Long telomeres protect against age-dependent cardiac disease caused by NOTCH1 haploinsufficiency

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Diseases caused by gene haploinsufficiency in humans commonly lack a phenotype in mice that are heterozygous for the orthologous factor, impeding the study of complex phenotypes and critically limiting the discovery of therapeutics. Laboratory mice have longer telomeres relative to humans, potentially protecting against age-related disease caused by haploinsufficiency. Here, we demonstrate that telomere shortening in NOTCH1-haploinsufficient mice is sufficient to elicit age-dependent cardiovascular disease involving premature calcification of the aortic valve, a phenotype that closely mimics human disease caused by NOTCH1 haploinsufficiency. Furthermore, progressive telomere shortening correlated with severity of disease, causing cardiac valve and septal disease in the neonate that was similar to the range of valve disease observed within human families. Genes that were dysregulated due to NOTCH1 haploinsufficiency in mice with shortened telomeres were concordant with proosteoblast and proinflammatory gene network alterations in human NOTCH1 heterozygous endothelial cells. These dysregulated genes were enriched for telomere-contacting promoters, suggesting a potential mechanism for telomere-dependent regulation of homeostatic gene expression. These findings reveal a critical role for telomere length in a mouse model of age-dependent human disease and provide an in vivo model in which to test therapeutic candidates targeting the progression of aortic valve disease.

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Long telomeres protect against age-dependent cardiac disease caused by NOTCH1 haploinsufficiency

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Introduction

Use of animal models to investigate age-dependent diseases in humans typically requires long-term aging. Furthermore, human diseases caused by genetic haploinsufficiency often fail to be modeled at any age in mice. Telomere shortening is thought to contribute to age-related diseases in humans, but this has yet to be demonstrated experimentally in an in vivo model. Many human diseases of homozygosity are recapitulated in mice, though in some cases, telomere shortening in mice homozygous for human disease mutations has revealed disease-related phenotypes (1, 2). It has yet to be demonstrated whether long telomeres in mice are protective against diseases of haploinsufficiency, which are frequently associated with age-related decline. Telomeres in lymphocytes of patients with calcific aortic valve disease (CAVD) are shortened, but whether telomere shortening is a causative factor is unknown (3). The incidence of CAVD increases with age, resulting in over 100,000 valve replacements for aortic valve stenosis (AS) (narrowing) annually in the United States. Fifty percent of individuals with CAVD also have coronary, aortic, or carotid artery calcification and therefore are at increased risk for myocardial infarctions and strokes.

Although CAVD often occurs in those with typical 3-leaflet aortic valves (AVs), a congenital anomaly of the AV in which the valve develops into 2, rather than 3, distinct leaflets (bicuspid aortic valve [BAV]) markedly raises the risk for CAVD. Approximately one-third of individuals with BAV, which occurs in 1%–2% of the population, will develop CAVD that typically has an earlier onset than usual. Congenital defects of the AV have high phenotypic variability, ranging from asymptomatic disease that becomes apparent later in life upon calcification to neonatal presentation with a thickened AV requiring immediate intervention. The most severe form of AS in the fetus can cause hypoplastic left heart syndrome, and all forms of AS are thought to be genetically linked. Humans with heterozygous NOTCH1 (hN1) mutations, the only known genetic cause of AV disease, exhibit the full spectrum of this phenotypic variability (4–7). However, mice heterozygous for N1 have normal AVs (8), limiting further investigation in vivo.

Results and Discussion

To examine the role of telomere length in valve disease, we generated successive generations of N1 mice lacking telomerase activity by crossing C57BL/6 mice heterozygous for N1 to those lacking the telomerase RNA component Terc (mTR) (9), as described in Supplemental Figure 1A (supplemental material available online with this article; https://doi.org/10.1172/JCI90338S1). Analyzing mice lacking telomerase activity over successive generations (mTR−/− generation 1–3, referred to as mTR<sup>0/−</sup>-mTR<sup>0/−</sup>) allowed the study of N1−/− mice with progressively shorter telomere lengths to determine the dose-dependent effect of telomere shortening on valve disease.

Diseases caused by gene haploinsufficiency in humans commonly lack a phenotype in mice that are heterozygous for the orthologous factor, impeding the study of complex phenotypes and critically limiting the discovery of therapeutics. Laboratory mice have longer telomeres relative to humans, potentially protecting against age-related disease caused by haploinsufficiency. Here, we demonstrate that telomere shortening in NOTCH1-haploinsufficient mice is sufficient to elicit age-dependent cardiovascular disease involving premature calcification of the aortic valve, a phenotype that closely mimics human disease caused by NOTCH1 haploinsufficiency. Furthermore, progressive telomere shortening correlated with severity of disease, causing cardiac valve and septal disease in the neonate that was similar to the range of valve disease observed within human families. Genes that were dysregulated due to NOTCH1 haploinsufficiency in mice with shortened telomeres were concordant with proosteoblast and proinflammatory gene network alterations in human NOTCH1 heterozygous endothelial cells. These dysregulated genes were enriched for telomere-contacting promoters, suggesting a potential mechanism for telomere-dependent regulation of homeostatic gene expression. These findings reveal a critical role for telomere length in a mouse model of age-dependent human disease and provide an in vivo model in which to test therapeutic candidates targeting the progression of aortic valve disease.

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Histologic examination of \( N^1WT \) mice at 2 months of age did not reveal any evidence of calcification, marked by Alizarin staining (Figure 1C, \( n = 16 \)). In contrast, 100% of AVs examined from \( N^1+/− \) mice with AS by echocardiography were positive for Alizarin staining (Figure 1C, \( n = 4 \)). The calcification was extensive, in many cases encompassing an entire leaflet of the AV and the aortic root and in some cases extending to the aortic vasculature (Figure 1D). Calcific regions showed coincident expression of the osteoblast transcriptional regulator RUNX2 (10), indicating an osteogenic switch (Figure 1E). 30% of \( N^1+/− \) mice with normal echocardiography showed calcification by Alizarin staining by 2 months of age, indicating variable disease progression (Figure 1C, \( n = 17 \)). Overall, 43% of \( N^1+/− \) mice had AV calcification, with 44% of those having progressed to AS by echocardiography (\( n = 21 \)).

A subset of \( N^1+/− \) mice also showed increased pulmonary valve (PV) peak velocity and peak pressure gradient of blood flow across the valve, indicative of PV stenosis (PS), compared with \( N^1WT \) mice (Supplemental Figure 2, A and B). Mice with PS also had right ventricular (RV) hypertrophy secondary to the PV narrowing (Supplemental Figure 2, C and D). 100% of mice with...
PS by echocardiography showed thickened PV leaflets histologically, but lacked calcification (Supplemental Figure 2E, n = 4). N1+/− mTRG2 mice that did not have PS by echocardiography had leaflets of intermediate thickness, with a total 33% of all N1+/− mTRG2 mice exhibiting PV thickness more than 2 SD greater than that seen in N1WT mTRG2 mice (Supplemental Figure 2F, n = 24). Interestingly, heterozygous mutations in N1 or its ligand JAG1 can also cause human PS or mitral valve stenosis, likely reflective of N1’s regulation of endocardial cushions that contribute to cardiac valve development (4, 7, 11, 12). In total, 31% of N1+/− mTRG2 mice exhibited an echocardiographic defect in either the semilunar (aortic or pulmonary) valves (only 1 mouse exhibited defects in both valves), while 55% exhibited a defect by histology at 2 months of age.

Although adult N1+/− mTRG2 mice developed valve disease, neonatal N1+/− mTRG2 mice showed no defects by echocardiography, indicating that the valve disease progresses in an age-dependent fashion (P < 0.05 by Barnard’s test, neonatal, n = 11; adult, n = 26). While AV defects were observed in the first generation, the severity of AV defects increased between the first and second generations of mice lacking mTR. By the third generation, we observed approximately 70% lethality by weaning age (Figure 2A and Supplemental Figure 3A), and 75% of N1+/− mTRG3 animals at E18.5 had thickened AV leaflets and/or septal defects, explaining the early lethality (Figure 2, B and C, and Supplemental Figure 3B). Because a spectrum of AV disease, ranging from adult onset CAVD to newborn AS, is often observed within families, it is intriguing to consider telomere length as a contributor to phenotypic variability.

Since N1 is thought to stabilize endothelial cells (ECs), preventing their proliferation (13), we investigated whether increased proliferation in the valves of N1-haploinsufficient mice might result in further telomere shortening. Phospho–histone H3 (pH3) immunostaining revealed increased proliferation specifically in the valve affected by stenosis in N1-haploinsufficient mice, with no difference in apoptosis by TUNEL staining (Figure 2D and Supplemental Figure 3 C–F). N1+/− mTRG2 mice with AS had increased proliferation in the PV compared with N1WT mTRG2 mice and N1+/− mTRG2 mice with PS (Figure 2D). Conversely, mice with PS had increased proliferation in the PV compared with N1WT mTRG2 mice and N1+/− mTRG2 mice with AS (Supplemental Figure 2F). Correspondingly, N1+/− mTRG2 mice with AS showed significantly reduced telomeres compared to N1+/− mTRG2 mice with PS.
specifically in their AVs compared with $N_1^{WT}$ $mTR^{G2}$ mice or $N_1^{-/}$$mTR^{G2}$ mice without AS (Figure 2E and Supplemental Figure 3, G and H). $N_1^{WT}$ $mTR^{G2}$ mice with PS also had reduced telomeres specifically in their PVs compared with $N_1^{WT}$ $mTR^{WT}$ mice or $N_1^{-/}$$mTR^{G2}$ mice without PS (Supplemental Figure 3, G and H). Thus, increased proliferation in diseased valves correlated with further telomere shortening in those valves, with telomere shortening below a quantitative FISH (Q-FISH) signal threshold of approximately 30 units segregating with the diseased valve.

To determine the genes dysregulated due to telomere shortening and how N1 haploinsufficiency differentially affected AVs in the context of the baseline telomere length, we performed RNA sequencing on dissected AVs from WT, $N_1^{WT}$, $mTR^{G2}$, and $N_1^{-/}$$mTR^{G2}$ mice. Telomere shortening alone ($N_1^{WT}$ $mTR^{G2}$) resulted in dysregulation of genes enriched in gene ontology terms related to GPCRs and β receptor response (Figure 3A and Supplemental Tables 1 and 2). GPCRs are one of the few known classes of proteins that serve as direct mechanosensors of hemodynamic shear stress in ECs (14). Given that laminar shear stress protects against atherosclerosis and CAVD (15), age-dependent telomere shortening may place AVs at greater risk by disrupting protective shear-induced GPCR signaling. Shear-induced N1 signaling also activates anticalcific gene networks in ECs (16–18), raising the possibility that N1 haploinsufficiency and dysregulated GPCR signaling in telomere-shortened AVs may contribute to the accelerated age-dependent calcification in $N_1^{-/}$ individuals.

Indeed, N1 haploinsufficiency in mice with long telomeres ($N_1^{WT}$ $mTR^{WT}$) downregulated genes involved in cell adhesion, including multiple integrins and actin-binding genes, including Parvb, which has been previously linked to coronary atherosclerosis (19) (Figure 3B). N1-haploinsufficient mice with shortened telomeres ($N_1^{-/}$ $mTR^{G2}$) also upregulated Tcfl2, which promotes migration of profibrotic cells to atherosclerotic lesions in vivo (20), and Ngr1, which may contribute to proliferation within thickened
valve leaflets (21) (Figure 3B). More broadly, gene dysregulation in $N1^{+/−}$ mTRG2 mice had significant overlap with genes perturbed in human $N1^{+/−}$ ECs (16–17), in which telomeres are naturally shorter, including those involved in prosteogenic and proinflammatory pathways (Figure 3C and Supplemental Figure 3I). For example, $N1^{+/−}$ mTRG2 AVs downregulated osteoclast factors such as Car2 (22), important for bone resorption, compared with $N1^{WT}$ mTRG2 AVs. Additionally, like human $N1^{−/−}$ ECs (16), $N1^{−/−}$ mTRG2 AVs upregulated both Apelin and its receptor, which are therapeutic targets for atherosclerosis and upregulated in human coronary atherosclerotic plaques as well as calcified human AVs (23) (Figure 3, D and E, and Supplemental Figure 3J).

3D chromatin analysis suggests telomeres loop back to promoters in the genome and thereby affect gene expression (24). We examined the reported sites of telomere contacts genome-wide in human umbilical vein ECs (25) and found that genes dysregulated in telomere-shortened mTRG2 valves were enriched for telomere-contacting promoters compared with all genes (Figure 3F). Furthermore, genes dysregulated in $N1^{+/−}$ mTRG2 valves, which have even further reduced telomere length compared with $N1^{WT}$ mTRG2 valves, were even more enriched for telomere-contacting promoters. Thus, genes dysregulated with decreasing telomere length were increasingly likely to be regulated by promoters associated with telomeres within the 3D chromatin structure in the nucleus, suggesting that disruption of this looping with telomere shortening may result in gene dysregulation responsible for age-related CAVD.

This study reveals that telomere shortening erodes the tolerance to haploinsufficiency in age-related disease and that shortening of long telomeres protective in mice can accurately recapitulate a human disease of haploinsufficiency. It is remarkable that despite the broad function of N1 in a myriad of tissues, telomere shortening in N1 heterozygous mice resulted only in cardiovascular disease, precisely recapitulating the clinical phenotype observed in humans with N1 haploinsufficiency. Reduced dosage of N1 led to upregulation of cell cycle genes and increased cell proliferation in the valve, which may shorten telomeres over time past a critical threshold (Figure 3G). Calcification of the aortic vasculature, commonly observed in humans with CAVD in an age-dependent manner, was also elicited by telomere shortening. It is interesting that progressive shortening of telomeres not only increased severity of disease, but also mimicked the range of AV disease seen in humans, including neonatal onset. The mechanism by which telomere length affects aging or disease more broadly remains unclear, but the enrichment in genes contacted by telomere looping among those dysregulated in mouse $N1^{+/−}$ AVs with shortened telomeres suggests a potential mechanism for telomere-dependent regulation of homeostatic gene expression.

While we focused on heterozygosity of N1, the majority of human diseases of haploinsufficiency lack a phenotype in mice heterozygous for the orthologous factor, similar to N1. In contrast, many human diseases of homozygosity are recapitulated in mice, although in the case of Duchenne muscular dystrophy, the mouse phenotype caused by homozygous mutations in Mdx was only fully manifested upon telomere shortening (1, 2). It will be interesting to determine whether telomere shortening represents a more general approach to modeling human disease in mice, particularly those diseases caused by dose-sensitive mutations. The model described here provides a unique opportunity for dissecting the mechanisms by which telomeres affect age-dependent disease and specifically provides an in vivo system for testing novel therapeutics for AV disease in adults and neonates.

**Methods**

Experimental details are in Supplemental Methods. All original RNA sequencing data were deposited in the NCBI’s Gene Expression Omnibus (GEO GSE83963).

**Study approval.** Protocols were reviewed and approved by the UCSF Institutional Animal Care and Use Committee.

**Author contributions**

CVT designed and performed experiments and bioinformatic analyses. FM designed, performed, analyzed, and interpreted telomere Q-FISH experiments and edited the manuscript. YH performed echocardiography. SSR and YH performed E18.5 dissections. CVT, YH, SSR, and LL performed perfusion fixation, mouse breeding, and genotyping. HMB designed experiments and analyses, supervised telomere Q-FISH experiments and interpretation, and edited the manuscript. DS designed experiments and analyses and supervised the work. CVT and DS wrote the manuscript.

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