SUPPLEMENTARY DATA

Supplementary Methods S1

Telomere-assisted chromosome fragmentation (TACF) in the DT40-CRE background: both ends of the linear HAC are capped with an identical (sub-) telomeric cassette, derived from the linearization plasmid pSP73-TEL08-BLAS-loxP

To identify the (sub-) telomeric ends present at each end of the linear HAC, the latter was shuttled from the BALB/c-3-19-19 donor cell line to the chicken DT40-CRE cell line, proficient in TACF, by MMCT. A linear targeting plasmid, pBS-TEL-aSAT-HYG, was expected to replace either of the Mb-sized chromosomal arms of the L-HAC via homologous recombination at the alphoid-20 sequences (Supplementary Figure S1A-C). 25 Hygromycin resistant DT40-CRE clones were further analyzed by PFGE with a probe for the alphoid-20 sequences. Three DT40-clones carried a HAC that was reduced in size (3.5 – 4.5 Mb) when compared to the original L-HAC whose length exceeds 6 Mb (data not shown), and 2 of them were selected for further analysis. To analyze the structure of both HACs in the absence of potential background random integrations of the targeting construct, the size reduced L-HACs were transferred into *hprt* $^{-/-}$ CHL cells by MMCT (Supplementary Figure S1A-C). FISH analysis with alphoid-20 sequences and chicken Cot-1 DNA, showed no detectable insertion of chicken sequences in the two CHL-clones selected for analysis (CHL-3-19-19-57-11 and CHL-3-19-19-79-30) (data not shown). STS analysis of the 1p22.1-21.3 region present on the original L-HAC was performed to verify that both size-reduced HACs carry the opposite arms of the original linear HAC. Markers D1S2719 and D1S1698 were found to be present on the 4 Mb-sized HAC in clone CHL-3-19-19-57-11 and could not be detected in the 4.6 Mb HAC of clone CHL-3-19-19-79-30 while marker D1S236 showed the opposite PCR amplification pattern. As such the hypothesis that both CHL clones contained size-reduced L-HACs carrying opposite chromosomal arms was confirmed (Supplementary Figure S1A-C). These findings were corroborated by FISH analysis with PACs RP4-573H3 and RP5-837O21 as probes. PAC RP4-573H3 localizes between D1S2719 and D1S1698 and is detected on the HAC only in clone CHL-3-19-19-57-11, while the signal for RP5-837O21, which contains the *GLCLR* gene, is restricted to the HAC present in clone CHL-3-19-19-79-30 (Supplementary Figure S1A-C). As a control, both PACs were found to be present on the original HAC on opposite chromosome arms (Supplementary Figure S1A). As the size-reduced HACs in clones CHL-3-19-19-57-11 and CHL-3-19-19-79-30 lack opposite chromosomal arms the presence of the NEO-loxP-BLAS DNA cassette was tested. By PCR with the NEOf and BLASf primers (Supplementary Figure 1D) and Southern blot analysis of EcoRI-digested DNA with a neomycin probe (data not shown), it was shown that an intact subtelomeric NEO-loxP-BLAS DNA cassette was present in both CHL clones. Probing XbaI or StuI-digested genomic DNA of both CHL clones for hygromycin sequences enabled the detection of the expected restriction fragments of respectively 4.9 kb and 1.7 kb on a Southern blot (Supplementary Figure 1E), suggestive of the presence of an intact alphoid-20-hygromycin subtelomeric DNA cassette on the opposite end of both size-reduced HACs. Together these data confirm that the original linear HAC was capped with identical NEO-loxP-BLAS derived telomeres and confirmed that the linear HAC contained two inverted NEO-loxP-3'*HPRT1* cassettes.

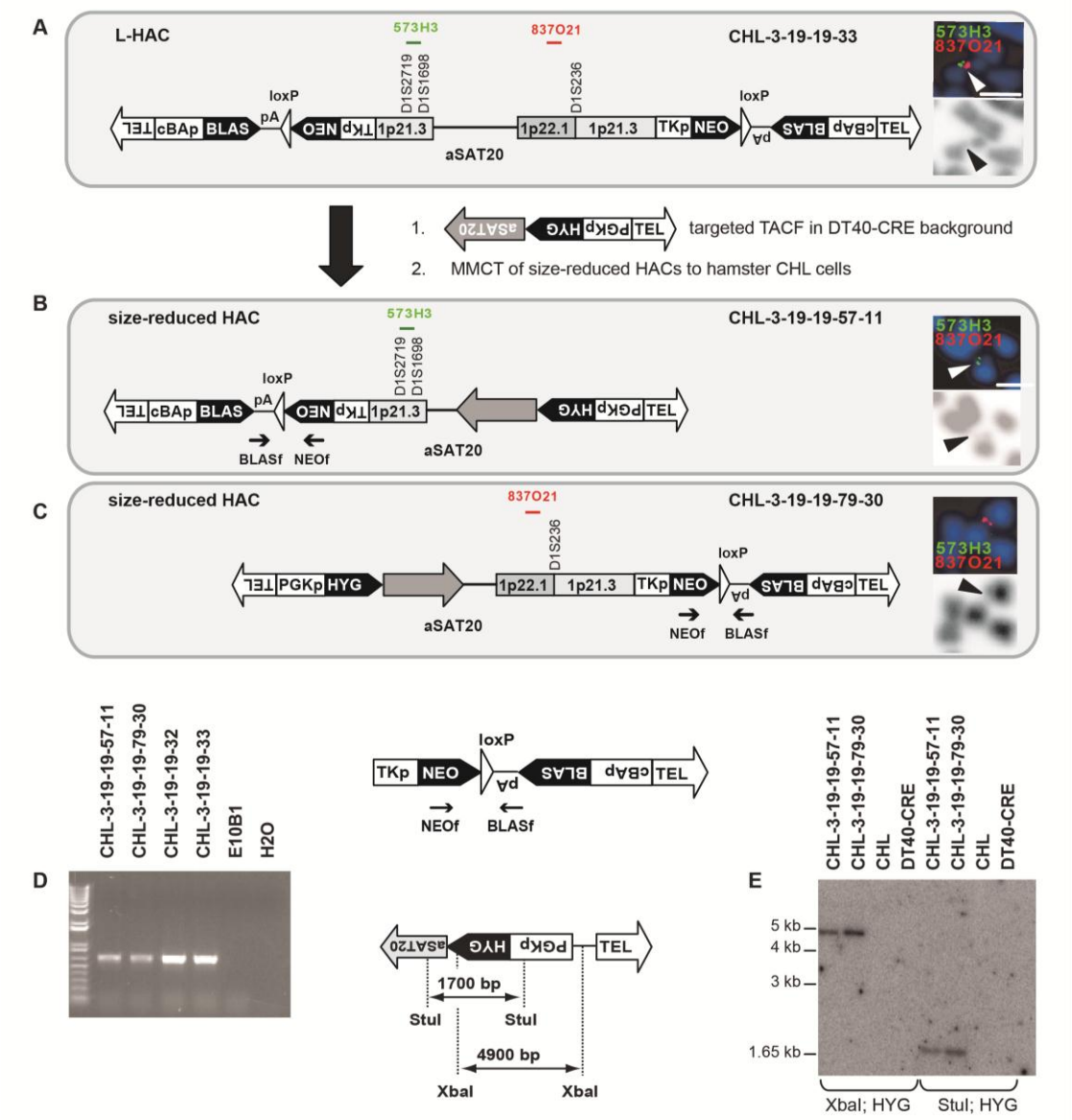
Plasmid pBS-TEL-aSAT-HYG

Plasmid pBS-TEL-aSAT-HYG was created by cloning PCR-amplified HAC alphoid DNA into the pGEM-T easy vector (Promega, Madison, Wisconsin). Subsequently, a 1783 bp BamHI/EcoRV fragment from plasmid pBS-Hyg/SV40-5'*HPRT*/loxP (61) carrying a hygromycin resistance gene driven by a PGK promoter was blunted and inserted into the unique, blunted SpeI site of the pGEM-Teasy vector. The 'aSAT-HYG-PGK' DNA cassette was isolated from the pGEM-T easy vector by NotI digestion and ligated into the unique NotI site of a modified pTel1.6 plasmid. In the original pTel1.6 plasmid (a gift of Dr. C. Farr, University of Cambridge, Cambridge, UK), the NotI site at the 5' end of the 1.6 kb (TTAGGG)n repeat was inactivated by partial NotI digestion, blunting and re-ligation; leaving the NotI site at the 3' end of the (TTAGGG)n repeat intact. Subsequently, the NotI site at the 5' end of the PGK promoter was inactivated in a similar manner resulting in plasmid pBS-TEL-aSAT-HYG. Plasmid pOG231 carrying a CRE recombinase expression construct was obtained from O'Gorman et al. (43).

Reference

43. O'Gorman, S. (2005). Second branchial arch lineages of the middle ear of wild-type and Hoxa2 mutant mice. *Dev. Dyn.* **234**,124-131.

Supplementary Figures



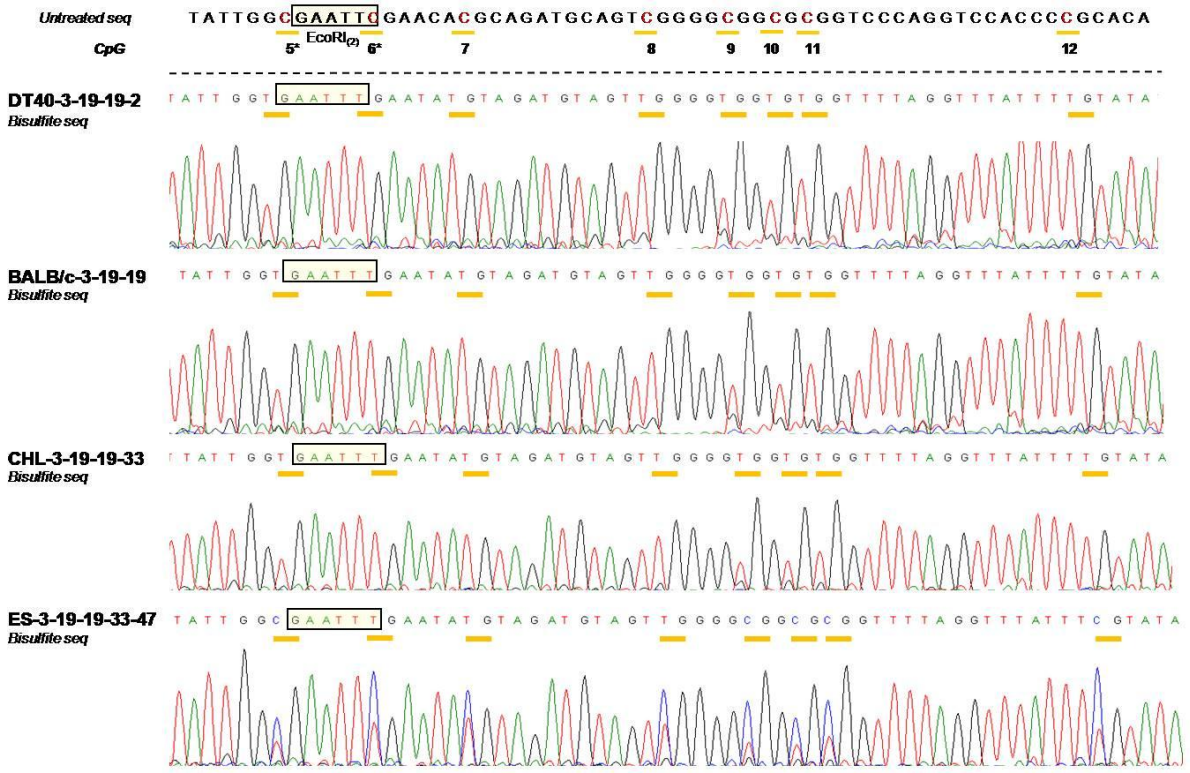
Supplementary Figure S1

Supplementary Figure S1. Characterization of the subtelomeric ends of the linear HAC. (A-C) Structural characterization of the subtelomeres at the L-HAC via TACF in the DT40-CRE background. The targeting plasmid pBS-TEL-aSAT-HYG was transfected into the DT40 cells to replace either of the chromosomal arms by HR at the aSAT20 sequences. The obtained size-reduced L-HACs were transferred to the CHL background by MMCT for structural characterization. Two CHL clones carrying size-reduced HACs that have lost opposite chromosomal arms (CHL-3-19-19-57-11 and CHL-3-19-19-79-30) were selected. FISH analyses of the HACs in the CHL background are indicated on the right. Markers D1S2719, D1S1698 and D1S236 are displayed vertically at their respective location and the PAC FISH-probes (RP4-573H3 and RP5-837O21) are indicated in green and red, respectively. (D) PCR analysis with primers NEOf and BLASf confirmed that both L-HACs contain the NEO-loxP-BLAS DNA cassette. (E) Southern blot analysis of genomic DNA digested with StuI or XbaI probed for the hygromycin sequences. Expected fragment lengths are indicated. DNA from *hprt* $^{-/-}$ CHL cells and DT40-CRE cells was used as negative controls.

	C-HAC	L-HAC	
genes / markers	CHL background E10B1	CHL-3-19-32	CHL-3-19-33
RPL5	-	-	-
MTF2	-	-	-
DR1	+	+	+
BCAR3	+	+	+
GLCLR	+	+	+
ABCA4	+	+	+
D1S236	+	+	+
ABCD3	+	+	+
F3	+	+	+
CNN3	+	+	+
D1S2819	+	+	+
D1S1556	+	+	+
D1S2664	+	+	+
D1S3161	+	+	+
D1S2102	+	+	+
D1S2719	+	+	+
D1S1698	+	+	+
PTBP2	+	+	+
DPYD	+	+	+

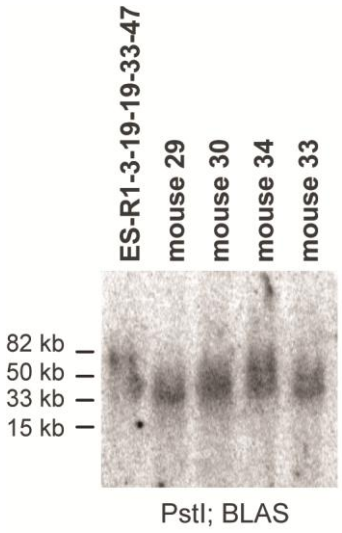
Supplementary Figure S2

Supplementary Figure S2. List of the genes and markers of the 1p22.1 and 1p21.3 region of the HAC that were tested by STS-PCR analysis. '+': fragment obtained by PCR, '-': absent from the HAC according to the PCR results.



Supplementary Figure S3

Supplementary Figure S3. Bisulfite sequencing of the L-HAC subtelomeric region in the DT40, BALB/c, CHL and ES-R1 cells. The chromatograms display the sequences of the subtelomeric region (CpG 5*-12) of the L-HAC upon bisulfite treatment and sequencing. The location of the CpGs is marked by yellow bars. RE site $EcoRI_{(2)}$ is framed in black. The untreated sequence is indicated on top and has all C's that are followed by a G nucleotide highlighted in red. Cytosines are present only in the chromatogram of the ES cells and never in the chromatograms for the DT40, BALB/c and CHL cells.



Supplementary Figure S4

Supplementary Figure S4. TRF analysis of the artificial telomeres in mouse lung tissue. Genomic DNA of ES clone ES-R1 3-19-19-33-47 and from the lung tissue of 4 littermates was digested with PstI and probed for blasticidin. The L-HACs present in the four littermates have artificial telomeres of similar length ranging from 30 -70 kb. Clone ES-R1 3-19-19-33-47 served as positive control.

Supplementary Tables

Supplementary Table S1. List of qPCR and PCR primers.

application	*gene/STS/PAC*	*Primer*	*sequence*
qPCR genomic	NEO	NEOf	5'-GCTGTGCTCGACGTTGTCA
		NEOr	5'-CGGCACTTCGCCCAATAG
	PAC 837O21	837O21f	5'-TCCCTGTTTAGAGTGCGAATC
		837O21r	5'-GGAGACCACAAGAGGGTT
	Rer1	*Rer1f*	5'-TTCACACACGGGAAGAGGAGATA
		Rer1r	5'-GTCCAGCTTCTAACTAGCAAATG
qPCR cDNA	NEO	NEOf	5'-GCTGTGCTCGACGTTGTCA
		NEOr	5'-CGGCACTTCGCCCAATAG
	Hprt	*Hprt*f	5'-GCTTTCCCTGGTTAAGCAGTACA
		*Hprt*r	5'-GAGAGGTCCTTTTCACCAGCAA
	Actin	*Actin*f	5'-ACCCACACTGTGCCCATCTAC
		*Actin*r	5'-AGCCAAGTCCAGACGCAGG
	Ubc	*Ubc*f	5'-AGGTCAAACAGGAAGACAGACGTA
		*Ubc*r	5'-TCACACCCAAGAACAAGCACA
PCR (on CV)	NEO/BLAS	NEOf	5'- AGCACGTACTCGGATGGAAG
		BLASf	5'-CCCCCTGAACCTGAAACATA
	HPRT1	HPRT1f	5'-TGGCGTCGTGATTAGTGATG
		HPRT1r	5'- GCTGAACAAGTACCAAACATGTAAA
1p21.3-22.1 STSs	D1S236	D1S236f	5'-AAACCACCTACCAATGTCTGTC
		D1S236r	5'-GAAGCTGTCGTTATGGGGT
	D1S2664	D1S2664f	5'-CAGCCCACAGAATAACACTG
		D1S2664r	5'-TTCATGCTATGATTTTCCGC
	D1S2719	D1S2719f	5'- CCCTTAACCACTTCTGGATT
		D1S2719r	5'-CATGCTTTTGTTGGCTTATTAC
	D1S1698	D1S1698f	5'- GTCTTTGTCAATATGTAGATTGGGG
		D1S1698r	5'-TCTGTTCCCTGACACTGCTG

Supplementary Table S2. Germline transmission of the L-HAC upon breeding 6 HAC^{+}-F2 males with wild-type NMRI females.

female	*F2 male*	*L-HAC^{+} pups per litter*						*total number*	*% L-HAC^{+} pups per parent*
	F2-5	0/13	4/14	1/14	1/16	4/18		10/75	13.33
	F2-28	9/16	4/14	3/18				16/48	33.33
NMRI	*F2-34*	4/14	8/15	6/17	5/17	7/15		30/78	38.46
	F2-41	2/3	2/13	8/14	10/14	4/13	5/10	31/67	46.27
	F2-45	5/12	6/16	7/15	3/13	5/15		26/71	36.62
	F2-55	3/14	4/16	3/8	11/17			27/70	38.57
							total % L-HAC^{+} pups		34.23