PHF10 Is Required for Cell Proliferation in Normal and SV40-Immortalized Human Fibroblast Cells

S.S. Banga\textsuperscript{a, b} L. Peng\textsuperscript{b} T. Dasgupta\textsuperscript{b} V. Palejwala\textsuperscript{a} H.L. Ozer\textsuperscript{a, b}

\textsuperscript{a}Department of Microbiology and Molecular Genetics, \textsuperscript{b}New Jersey Medical School-University Hospital Cancer Center, University of Medicine and Dentistry of New Jersey, Newark, N.J., USA

Abstract

Normal human diploid fibroblasts have limited life span in culture and undergo replicative senescence after 50–60 population doublings. On the contrary, cancer cells typically divide indefinitely and are immortal. Expression of SV40 large T and small t antigens in human fibroblasts transiently extends their life span by 20–30 population doublings and facilitates immortalization. We have identified a rearrangement in chromosome 6 shared by SV40-transformed human fibroblasts. Rearrangements involving chromosome 6 are among the most frequent in human carcinogenesis. In this paper, we extend analysis of the 6q26–q27 region, a putative site for a growth suppressor gene designated \textit{SEN6} involved in immortalization of SV40-transformed cells. Detailed molecular characterization of the rearranged chromosomes (6q*, normal appearing; and 6q\textsuperscript{t}, translocated) in the SV40-immortalized cell line HALneo by isolating each of these 2 chromosomes in mouse/HAL somatic cell hybrids is presented. Analysis of these mouse/HAL somatic cell hybrids with polymorphic and nonpolymorphic markers revealed that the 6q* has undergone a chromosomal break in the \textit{MLLT4} gene (alias \textit{AF6}). This result in conjunction with previous published observations leads us to conclude that \textit{SEN6} lies between \textit{MLLT4} and \textit{TBP} at chromosomal region 6q27. Examination of different genes (\textit{MLLT4}, \textit{DLL1}, \textit{FAM120B}, \textit{PHF10}) located within this interval that are expressed in HS74 normal fibroblast cells reveals that overexpression of epitope-tagged truncated \textit{PHF10} cDNAs resulted in reduced cell proliferation in multiple cell lines. Paradoxically, down-regulation of \textit{PHF10} by RNAi also resulted in loss of cell proliferation in normal fibroblast cells, indicating \textit{PHF10} function is required for cell growth. Taken together, these observations suggest that decreased cell proliferation with epitope-tagged truncated \textit{PHF10} proteins may be due to dominant negative effects or due to unregulated expression of these mutant proteins. Hence we conclude that \textit{PHF10} is not \textit{SEN6} but is required for cell growth.

The development of human cancer is a complex and multistep process. SV40-mediated immortalization has long been utilized as a model system for human carcinogenesis which commonly involves inactivation of pRb and p53 tumor suppressor pathways, telomere stabilization and other genetic alterations. Normal human diploid fibroblast cells have a limited life span in culture [Hay-
flick, 1965). These cells normally undergo 50–60 cell divisions, after which they stop dividing and enter a state of ‘replicative senescence’, with arrest within the G1 stage of the cell cycle [Stein and Dulic, 1995; Smith and Pereira-Smith, 1996]. This growth arrest has been attributed to the shortening and/or dysfunction of telomeres [Blackburn, 2000; Rodier et al., 2005; Stewart and Weinberg, 2006] resulting in activation of pRb and p53 tumor and growth suppressor pathways through up-regulation of p16 and p21 [Shay et al., 1991; Alcorta et al., 1996]. However, cells transformed by SV40 DNA tumor virus or its large T antigen (Tag) and small t antigen genes will replicate for an additional 20–30 generations. This extension of life span is achieved by the inactivation of the tumor suppressor proteins, p53 and pRb, through binding to the Tag protein [Bryan and Reddel, 1994]. Inactivation of p53 releases cell cycle checkpoints [Schwartz and Rotter, 1998; Vaziri and Benchimol, 1999], while inactivation of pRb leads to activation of the E2F family of transcription factors that are required for cell cycle progression [Nevis, 1992; Weinberg, 1995] inter alia. Inactivation of p53 and further shortening of the telomeres caused by the extended life span leads to genetic instability that contributes to extensive genomic rearrangements and thus ultimately to massive cell death (due to apoptosis) termed crisis [Macera-Bloch et al., 2002]. However, a rare cell survives or bypasses crisis and continues to grow indefinitely to massive cell death (due to apoptosis) termed crisis [Macera-Bloch et al., 2002].

Unexpectedly, cytogenetic analysis of 2 immortal cell lines AR5 and HALneo that originated from the same primary SV40 transformant (SVtSA/HF-A) revealed that they are of independent origin. AR5 is hemizygous and therefore has retained only one chromosome 6, whereas HALneo has retained the other homolog of chromosome 6. In addition, FISH analysis revealed that HALneo appears to have retained a fragment of chromosome 6 that is derived from the 6q26–q27 region and is translocated to another unknown chromosome [Banga et al., 1997]. Further analysis of each of these 2 chromosomes of HALneo in mouse/HAL cell hybrids, however, demonstrated that the apparently intact chromosome 6 has a terminal deletion whereas the chromosome 6 fragment is derived from the region of chromosome 6 that is retained in AR5 [Banga et al., 1997]. Therefore, both these immortal sublines (AR5 and HALneo) in fact share the same region of 6q26–qter. In this report, we describe detailed molecular characterization of the normal appearing chromosome 6 (designated 6q*) and the fragment of chromosome 6 (designated 6q') present in HALneo isolated in mouse/HAL cell hybrids using polymorphic and non-polymorphic markers. Analysis of these hybrids not only revealed integrity of these chromosomes with respect to their origin but also exposed a genetic rearrangement that would otherwise have gone undetected. Using this approach we were able to determine that chromosome 6q* has undergone one breakpoint in the MLLT4 gene which is located at 6q27. Based upon these and previously published results, we have redefined the location of SEN6 to 6q27 between MLLT4 and TBP. Since several of the human tumors and SV40-transformed cells share loss of heterozygosity (LOH) in 6q26–qter [Saito et al., 1992; Foulkes et al., 1993; Banga et al., 1997; Tibiletti et al., 1998; Letessier et al., 2007], inactivation of SEN6 may be responsible for immortalization of these tumors as well. Overexpression studies involving different genes in this interval revealed epitope-tagged PHF10 cDNAs (a plant homeodomain-containing gene of unknown function in humans) resulted in growth suppression in multiple human cell lines. On the contrary, depletion of PHF10 in normal human fibroblast cells resulted in loss of cell proliferation by RNA interference (RNAi). Taken together, these observations suggest that decreased cell proliferation with epitope-tagged truncated PHF10 proteins may be due to dominant negative ef-
fected or due to unregulated expression of these mutant proteins. Hence we conclude that PHF10 is not SEN6 but rather is required for cell growth.

Materials and Methods

Cell Lines and Culture Conditions
SCSV3hygro is an SV40-immortalized mouse cell line that is deficient in double-strand break repair [Banga et al., 1994] and has been stably transfected with a selectable marker that provides resistance to hygromycin (pCMVHygS). HALneo is a human fibroblast cell line that is immortalized with a temperature-sensitive SV40 T antigen [Hubbard-Smith et al., 1992] and has been stably transfected with a selectable marker neo (pRSVneo) conferring resistance to G418. SCSV3hygro and HALneo cells were grown at 7.5% CO2 in medium supplemented with 10% fetal calf serum, penicillin and streptomycin. SCSV3hygro cells were maintained in medium containing 200 μg/ml of hygromycin at 37°C and HALneo cells were maintained in medium containing 150 μg of G418 at 35°C. HSF43 is a human foreskin fibroblast cell line and CT10-2A is an immortal cell line derived from HSF43 by SV40 transformation [Ray and Kraemer, 1992]. HSF43, the fetal human bone marrow stromal cell line, which was used as the parent of the SV40-transformed cells generated in this laboratory, has been maintained as previously described [Small et al., 1982]. Other non-immortal and immortal SV40-transformed human cell lines including HALneo were maintained as previously described [Neufeld et al., 1987; Banga et al., 1997]. Non-immortal cell lines which were used to generate immortal derivatives were also termed preimmortal cell lines. The SV40-transformed immortal cl39T-Tet-On cell line stably expressing rtTA (reverse transactivator) was generated by transfection of pTet-On plasmid (Clontech). The cl39T-Tet-On cell line was isolated and maintained under tetracycline repressed conditions.

Fluorescence in situ Hybridization
Metaphase preparations were hybridized with whole-chromosome-6-specific painting probe (SpectrumGreen) according to the instructions provided by the supplier (Vysis Inc.). Chromosomes were stained with propidium iodide. Fluorescent signals were detected by Olympus Fluorescent microscope and photographs were taken with a B20 camera using 400 ASA Kodak film.

Mouse/HAL Somatic Cell Hybrids
To obtain mouse/HAL somatic cell hybrids between SCSV3hygro and HALneo cells, 1.5 × 10⁶ cells of SCSV3hygro and 2 × 10⁶ cells of HALneo were grown together without selection in a 10-cm petri dish for 14 h at 35°C. The cells were fused using polyethylene glycol (PEG, BMB) for 2 min at 37°C followed by extensive washes with serum-free medium. Cells were then grown in non-selective medium for 22 h at 35°C in a 7.5% CO2 humidified chamber. After trypsinization, cells were subcultured into a 10-cm dish in culture medium containing 200 μg/ml of hygromycin and 400 μg/ml of G418 to select hybrid cells. Cells were then incubated for 10–12 days at 37°C. Discrete clones were picked and plated at low density in a 10-cm dish. Subclones were picked from 10-cm petri dishes and replated in 24-well plates individually. When cells in a well reached 50–90% confluency, 80–90% of cells were harvested from each well into 1.5-ml Eppendorf tubes for rapid DNA isolation. Remaining cells in each well were re-fed with selection medium to continue culture for additional isolation of DNA and for long-term storage.

Rapid Isolation of DNA from Somatic Cell Hybrids
Cells from each hybrid were washed twice with PBS and then resuspended in 50 μl of buffer containing 10 mM Tris Cl pH 8.3, 1 mM EDTA pH 8.0, 25 mM NaCl and 200 μg/ml proteinase K. After brief vortexing, cells were then incubated at 37°C for 20–30 min. The Eppendorf tubes containing DNA were incubated at 95°C for 8–10 min to denature proteinase K as well as DNA and cooled immediately on ice. The DNA samples were kept at −20°C until further analysis. Using this method one can isolate DNA for PCR analysis from a number of clones at a time. Once the hybrids containing a single copy of human 6q were identified, more DNA was isolated by following standard procedures.

Primers and Sequencing
Primers used in the study were either synthesized at the Molecular Resource Facility (New Jersey Medical School) or purchased from Research Genetics Inc. Primer sequence data for many genes, expressed sequence tag (EST), sequence tag site (STS) and markers that are not provided in the manuscript can be obtained from the authors. To sequence the cDNA of MLLT4 in different immortal cell lines, we performed RT-PCR using total RNA of each cell line with primer pairs that were generated from the sequence of the MLLT4 cDNA clone [Prasad et al., 1993] provided by R. Dalla-Favera (New York). Additional exons present in longer splice variants were directly sequenced from respective genomic DNAs by designing primers from the sequence information available in the public databases.

Polymerase Chain Reaction
2 μl of DNA was taken from each mouse/HAL hybrid for PCR amplification in a reaction volume of 25 μl. A standard PCR reaction contained 1× Taq buffer (BMB), 200 μM dNTPs, 50 pm of each primer and 1 U of Taq polymerase (BMB). PCR conditions were as follows: 3 min at 94°C for 1 cycle, 1 min at 94°C, 1 min at 55–57°C (depending upon primer used), 1 min at 72°C for 35 cycles and 6 min at 72°C for 1 cycle. One tenth of the amplified products were separated in 4% agarose gels containing ethidium bromide to visualize amplified products.

RT-PCR and Real-Time Quantitative RT-PCR
Expression of each gene or EST from the 6q27 region was detected by RT-PCR using total RNA from a normal HS74 and a preimmortal SV40-transformed cell line (RT-PCR Kit, Invitrogen). Each experiment was repeated at least twice. For QRT-PCR of gene expression analysis of different transcripts of MLLT4, serial dilutions of input RNA (100–1.56 ng) were analyzed using the ABI PRISM 7500HT sequence detector system, primers, probes, and the TaqMan One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems). Three distinct primer pairs were used to evaluate different MLLT4 splice variants (Applied Biosystems). The reaction mixture was held at 48°C for 30 min for reverse transcription of RNA into cDNA. This was followed by incubation at 95°C for 10 min to activate the Taq polymerase. PCR amplification of cDNA was performed for 40 cycles using the following cycling conditions: denaturing for 15 s at 95°C, and annealing
and extending for 60 s at 60°C. All samples were tested in triplicate, and average values were used for quantitation. Analysis was performed using SDS v2.1 software (Applied Biosystems) according to the manufacturer’s instructions. GAPDH was used to normalize RNA loading. Relative Standard Curve Method was used for quantitation of gene expression.

**Plasmids and cDNA Cloning**

cDNA of DLL1 (kindly provided by S. Atravanis-Tsakonas, Charleston, Mass.) was tagged with an HA epitope at the 3’ end by PCR and then cloned into tetracycline regulatable vector pTRE-Tight-PGK-Puro carrying the PAC gene for puromycin resistance. pTRE-Tight-PGK-Puro vector was constructed by cloning PGK-Puro into pTRE-Tight (Clontech). pTRE-Tight-PGK-Puro vector was constructed by cloning PGK-Puro into pTRE-Tight-PGK-Puro carrying the pTRE-Tight-PGK-Puro vector to generate pTRE-Tight ([H]A)BamH1(FLAG)·PGK-Puro. A longer version of pTRE (pTRE10) cDNA (ORF 99-1454 nt) lacking exon 1 of the revised version NM_018288.3 by NCBI (fig. 1) was cloned from normal HS74 cells by RT-PCR using the primer pair carrying BamH1 (underlined) sites (5’ CTGGAATTCATG-GGCTCAGGAGATAGTTC 3’) and 3’ CTGATCCCGGCTTGGCTTTTTCC 3’). This additional exon 2 adds 123 nt (41 aa) to the pTRE10 variant 2 ORF. The isolation of pTRE10 cDNA was based on ORF analysis of NM_133325.1 sequence and existence of a similar size ORF in Pan troglodytes (XP_518861.2). To tag pTRE10 with HA and FLAG epitopes, its ORF was amplified using appropriate primers (see fig. 1, black arrows) and cloned in frame into pTRE-Tight · ([H]A)BamH1(FLAG)·PGK-Puro vector at the BamH1 site. Each cDNA was sequenced to confirm its integrity. To clone ([H]A)PHF10(FLAG) and PHF10(FLAG)·PGK-Puro plasmids, respectively, by digesting with EcoRI and ClaI, and then each HA- and/or FLAG-tagged cDNA was cloned into pLPCX under the control of CMV promoter. cDNA of MLLT4 was provided by R. Dalla-Favera (New York).

![Fig. 1. PHF10 gene, its mRNA species and respective protein isoforms.](#)

**Fig. 1.** PHF10 gene, its mRNA species and respective protein isoforms. A PHF10 is comprised of 12 exons with a coding potential of 498 aa. Exons and introns are not drawn to scale. B This mRNA lacks exons 1 and 2, and codes for a 410-aa protein (PHF10x) with 2 zinc finger domains (blue rectangles). C This mRNA lacks exons 1 and 2, and codes for a 408-aa protein (PHF10b) due to alternative splicing (represented by a line within the mRNA and by a small blank rectangle in the protein). D Analysis of the published sequence of PHF10 by us revealed an ORF that included exon 2. This resulted in the addition of 41 aa at the 5’ end to the published isoforms shown in B or C, and thus increased their coding capacity to 451 or 449 aa. A cDNA corresponding to a 451-aa protein (PHF10p) was cloned by RT-PCR using primers originating at 5’ and 2, and codes for a 408-aa protein (PHF10b) with 2 zinc finger domains (blue rectangles).
Western Blot Analysis

Total protein was isolated from different normal and SV40-transformed cell lines in RIPA buffer (10 mM Tris, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 mM sodium orthovanadate, 1 mM phenylmethyl-sulfonyl fluoride, 40 μg/ml aprotinin). After quantitation, 30 μg protein of the cell extracts were electrophoresed in a 7.5% denaturing polyacrylamide gel and blotted onto a membrane. The membrane was then probed with anti-MLLT4 monoclonal antibody (BD Biosciences). After washing, the blot was exposed to horseradish peroxidase-conjugated secondary antibody and protein visualized using the chemiluminescence detection system (PerkinElmer TM Life Sciences). Anti-α-actinin antibody (Sigma-Aldrich) was used as a control for sample loading.

Northern Blot Analysis

Total RNA from different cell lines was isolated using TRIzol (Invitrogen). PolyA+ RNA was isolated using Oligotex mRNA Midi kit (Qiagen). 3 μg of PolyA+ RNA was fractionated on 0.8% agarose-RNA borate formaldehyde gels. After blotting, membranes were hybridized with 32P-labeled probes (whole cDNA or fragments derived from respective genes) overnight using PerfectHyb Plus hybridization buffer (Sigma). After further washing, membranes were exposed to X-ray films. An RNA ladder was used as marker (Invitrogen).

Retroviral Production

10 μg of different retroviral vectors were transfected by the calcium phosphate transfection method in Phoenix packaging cells (gift from G. Nolan) plated 20–24 h before in 100-mm dishes. Briefly, after 12 h of transfection, cells were fed with fresh medium, and different retroviruses were harvested after 36 h. After filtration with 0.45-μm filter, aliquots of 1.5 ml were made and stored frozen at –80°C. A frozen-thawed aliquot of each retrovirus (supplemented with 8 μg/ml polybrene, Sigma) was titered on NIH3T3 cells to determine virus infectious units. Viruses that gave reproducibly similar numbers of puromycin-resistant colonies on NIH3T3 cells were used in growth studies on the normal, telomerase-immortalized and SV40-transformed human cells.

Growth Assays

Different normal, telomerase- and SV40-immortalized fibroblast cells were plated at 2 × 105 cells per 60 mm dish. After 12 h, aliquots corresponding to different retroviruses containing PHF10α (HA) and (HA)PHF10β (FLAG) cDNAs or empty vector were thawed, supplemented with 8 μg/ml of polybrene, and then used for transduction. After 48 h, cells were selected with fresh medium containing 0.3–0.7 μg/ml puromycin. Cells were fixed, stained with crystal violet and colonies counted. Each experiment was repeated 3 times.

RNAi

To down-regulate the expression of PHF10 in normal cells, different shRNAs (shRNA#1: 5′ CAGCATTGCGCAGTGATGA 3′; shRNA#2: 5′ CAGCCAAAGATGTGAGTA 3′; shRNA#3: 5′ CAGAAAGTTGAAGCCAGTA 3′, and shRNA#4: 5′ AGGTCAGGT CCTTACCCTG 3′) were synthesized along with their complementary strands, annealed and then cloned into a retroviral vector, pSIREN-RetroQ (Clontech). The control shRNA (5′ CATCAGTGCGTAGTGGTGTA 3′) was synthesized by making 4 nucleotide substitutions in the shRNA#1 as shown by bold letters. Only RNAi sequences used to generate shRNAs are shown. Retroviruses encoding these shRNAs were generated as described above.

Results and Discussion

Generation and Identification of Mouse/HAL Somatic Cell Hybrids Containing Chromosome 6 or a Fragment of Chromosome 6

To separate human chromosome 6q* from the 6q′ fragment present in HALneo [see fig. 2, Banga et al., 1997], we generated mouse/HAL cell hybrids as described in Materials and Methods. Using this strategy, we iso-
lated 130 hybrid clones. To identify mouse/HAL hybrids containing either chromosome 6q* or 6q\textsuperscript{t}, these clones were screened by PCR using a polymorphic marker D6S264 (derived from 6q26) which identifies 2 different alleles in HS74 and HALneo. Nine hybrids retain the upper allele, whereas 3 hybrids retain the lower allele that is preserved in AR5. To specifically identify hybrids containing chromosome 6q* and which contain a 6q\textsuperscript{t}, we performed PCR with another polymorphic marker, D6S300 (derived from 6q14), that amplifies only 1 (upper) allele in HALneo and thus distinguishes chromosome 6q* from the 6q\textsuperscript{t} (see fig. 3B). Analysis of the mouse/human hybrids with this marker revealed that 9 of the hybrids (data for 5 of those hybrids are shown in fig. 3B) amplified the same upper allele that is present in the HALneo. The remaining 3 hybrids did not show any amplification with this marker indicating these contain chromosome 6q\textsuperscript{t}; data for 2 of those hybrids are presented in figure 3B. Taken together, these results indicated that hybrids that amplified upper alleles with D6S264 and D6S300 markers carry chromosome 6q* (hereon referred to as Type I hybrids) and the hybrids which amplified the lower allele (D6S264 marker but not with D6S300 marker amplified) have chromosome 6q\textsuperscript{t} (hereon referred to as Type II hybrids). These results also revealed that chromosome 6q\textsuperscript{t} is derived from the chromosome that is retained in AR5, since both amplified the same allele.

**Characterization of the Type I and Type II Somatic Cell Hybrids**

Different polymorphic markers, non-polymorphic markers, expressed sequence tags, sequence tag sites or genes derived from the 6q25–q27 regions were used to characterize 6q* and 6q\textsuperscript{t} chromosomes by PCR with the intent to identify homozygous deletions or other chromosomal rearrangements that may define the location of SEN6. Summary of these results is presented in figure 4. Analysis with the polymorphic markers confirmed that the 6q\textsuperscript{t} chromosome is derived from the chromosome re-
tained in AR5 whereas the 6q* chromosome has undergone a terminal deletion, consistent with our earlier published work [Banga et al., 1997]. Overall, our data are consistent with published mapping and sequencing data, suggesting no further large rearrangements or deletions have occurred in 6q* and 6q1 chromosomes in the 6q25–q27 region.

Identification of a Chromosomal Break in MLLT4 in the 6q* Chromosome

Our FISH experiments involving 4,839 kb MLLT4 cDNA (NM_005936.2) and a 2.3-kb genomic fragment from the 5’ end (gift from R. Hauptschein and R. Dalla-Favera, New York) as probes on the HALneo cells showed a strong hybridization signal on the 6q1 chromosome as compared to the 6q* chromosome (data not shown). This observation suggested that 6q* might have undergone a chromosomal break in MLLT4. To further investigate this observation, we amplified Type I and Type II hybrid DNAs with 2 primer pairs from the 5’/H11541 end (from 1 to 90 nt and from 1 to 105 nt) as well as with 2 primer pairs from the 3’/H11541 end that amplify exon 28 (from 3,834 to 4,203 nt and from 4,077 to 4,839 nt) of the MLLT4 cDNA (NM_005936.2). Data for one primer pair from the 5’/H11541 end and one from the 3’/H11541 end of MLLT4 resulted in the amplification of the expected 763-bp band (from 4,077 to 4,839 nt) of the MLLT4 cDNA (NM_005936.2). Data for one primer pair from the 5’ end and one from the 3’ end is shown in figure 5. Indeed, both types of hybrids amplified the 105-bp fragment (from 1 to 105 nt) with the 5’ end primer pair, indicating no loss of these sequences occurred from the 5’ end of MLLT4. On the other hand, amplification of DNAs of both types of hybrids using primers from the 3’ end of MLLT4 resulted in the amplification of the expected 763-bp band (from 4,077 to 4,839 nt) only in a Type II hybrid whereas no amplification was observed in 3 Type I hybrids (see fig. 5) examined. These observations clearly...
demonstrate that MLLT4 has undergone a chromosomal break in the 6q* chromosome retained in HALneo immortal cell line. Further localization of the break point was not undertaken because of the large size of MLLT4 (~140 kb). The data presented here also suggest that SEN6 is located at or distal to MLLT4 between MLLT4–TBP on 6q27. Alternatively, MLLT4 itself could be a candidate gene for SEN6.

**Expression Analysis of Genes at 6q27 within the Deleted Region, MLLT4–TBP**

Sequencing of the human genome has identified a number of genes within the deleted region (http://genome.ucsc.edu). To gain insight whether these genes present within the candidate region are expressed in our immortal or normal cell lines, we performed RT-PCR using total RNAs. Data presented in table 1 show the expression pattern of different genes. Interestingly, we could not detect the expression of 5 genes (C6orf54, FRMD1, DACT2, SMOC2 and TCTE3) in the parental cell line HS74 or in a preimmortal SV40-transformed cell line by RT-PCR. Our repeated efforts to amplify the above mentioned genes failed to reveal any gene-specific expression, indicating that either these genes are not expressed at all or are expressed at levels below the detection threshold. These observations suggest that these genes are not likely candidates for SEN6, because they are not expressed in the parental cell lines. On the other hand, we are able to detect the expression of MLLT4, KIF25, PHF10, DLL1, THBS2, FAM120B, and WDR27, and they are, therefore, more likely candidate genes for SEN6.

![Fig. 5. Identification of a chromosomal break in MLLT4. PCR amplification of different DNAs with either primer from the 5’ end (5’ATGTGCGCGGCGCGCGCGT3’ and 5’CCAAATCCCCGCTGGGCTGG3’) or with primer from the 3’ end (5’TGCCGGGTATTTTCGATGGAATG3’ and 5’CTATCCCCATGGGAAACACGC3’) which amplified 112- and 763-bp fragments, respectively. Italicized nucleotides in one of the first primer pairs do not match with the first exon and the underlined nucleotide is next to the end of the first exon with the result that this primer pair amplifies the entire first exon (105 bp) but the length of the amplified fragment is 112 bp. HSF43 and HS74 are 2 normal human diploid fibroblast cell lines. HALneo and AR5 are immortal cell lines. Type I and Type II mouse/HAL hybrids carry 6q* and 6q† chromosomes, respectively.](image)
Identification of Growth Suppressor at 6q27

We analyzed 4 genes, MLLT4, DLL1, FAM120B and PHF10, as candidate genes for SEN6.

1. Analysis of MLLT4 in SV40-immortalized cells. The identification of a chromosomal break in MLLT4 in HAL-neo makes this gene a strong candidate for SEN6. Interestingly, this gene has been disrupted in acute myeloid leukemia with t(6;11)(q27;q23) [Prasad et al., 1993; Tanabe et al., 1996]. MLLT4 encodes a multidomain protein with 2 RAS-binding domains, a forkhead-associated domain, a dilute domain, 2 proline-rich domains, one PSD-95/Dlg-1/ZO-1 domain (PDZ) and an F-actin-binding domain [Kuriyama et al., 1996; Saito et al., 1998; Schneider et al., 1999]. MLLT4 expresses 3 transcripts of 11.2, 7.3 and 5.5 kb which encode 3 proteins (MLLT4 type 2 – 1,816 aa, MLLT4 type 3 – 1,743 aa and MLLT4 type 1 – 1,611 aa) that differ at their 3’ terminal regions [Saito et al., 1998; Lorger and Moelling, 2006]. Afadin, a mouse homolog of the human MLLT4 protein, shows 2 splice variants: a short variant (s-afadin) and a long variant (l-afadin) which correspond to MLLT4 type 1 and MLLT4 type 3 proteins. The shortest form of MLLT4 protein (MLLT4 type 1) lacks the actin-binding domain [Saito et al., 1998; Lorger and Moelling, 2006] and is localized at the cell membrane as well as in the nucleus [Mandai et al., 1997; Buchert et al., 1999, 2007], whereas the long isoform of MLLT4 protein (MLLT4 type 3) has an actin-binding domain and is primarily localized to the adherens junctions in epithelial cells [Buchert et al., 1999, 2007]. MLLT4 type 2 protein is not well studied. In addition to its interaction with many proteins including Ras, MLLT4 also interacts with Bcr, a fusion partner of Abl in Philadelphia chromosome, t(9;22), which is found in 95% of patients with chronic myelogenous leukemia [Faderl et al., 1999]. These observations suggest that MLLT4 plays an important role in cell signaling at sites of cell-cell contact to maintain cells in a nonproliferating state [Radziwill et al., 2003]. Loss of expression of MLLT4 protein has recently been reported in breast cancer, further suggesting its role as a tumor suppressor [Letessier et al., 2007].

These features of MLLT4/afadin make it likely to be a growth suppressor, and therefore we investigated this gene in more detail.

a. Expression analysis of MLLT4 in normal, preimmortal and SV40-immortalized cell lines. Since the MLLT4 gene produces multiple alternatively spliced transcripts [Saito et al., 1998], we did QRT-PCR using primers and TaqMan probes (Applied Biosystems) that specifically identifies each of the 3 transcripts. All are expressed in the fibroblast cell lines. Data for MLLT4 type 2 is not available.

Table 1. Expression analysis of different genes from the 6q27 region (between MLLT4 and TBP) in normal (HS74) and preimmortal (SV.RNS/HF) cell lines

| Genes/ESTs | Fragment size | Starting nucleotide position of the forward primer | Forward primer sequence (5’ → 3’) | Reverse primer sequence (5’ → 3’) | HS74 RNA | Preimmortal (SV.RNS/HF) RNA |
|------------|--------------|---------------------------------------------------|----------------------------------|-----------------------------------|--------|---------------------------|
| MLLT4      | 723          | 168,095,029                                       | tgcgggtatgtcgtggaatg             | ctaccccatggaaacacgc               | +      | +                         |
| C6orf54    | 171          | 168,137,343                                       | cagagtgcggagacctgtag             | gagaagctggtaagagggtgt            | –      | –                         |
| KIF25      | 240          | 168,182,158                                       | gaccttctgcaaaagacag              | gcggagctgtggtggag                | +      | +                         |
| FRMD1      | 286          | 168,207,245                                       | gactacatctccggcacat              | ttcgacctttccacga                 | –      | –                         |
| DACT2      | 109          | 168,451,285                                       | ctacagcccttcacaagacg             | tcgtgtcgctgactcagc               | –      | –                         |
| SMOC2      | 180          | 168,585,308                                       | tgcagacccccggacaaggg             | ccacagaagctgggaagaac             | –      | –                         |
| THBS2      | 252          | 169,367,375                                       | cgtgcaaatgacctttggt              | caaatataccccgctccatc             | +      | +                         |
| WDR27      | 279          | 169,804,358                                       | ctgagactgctcgcctgtga             | gactccacacagccaatc               | +      | +                         |
| PHF10      | 244          | 169,852,305                                       | aagtcgacactccaaagaa             | caagtccaggtgaggtctt              | +      | +                         |
| TCTE3      | 254          | 169,893,470                                       | acagcgagtagggaagagc             | ttaatttgcaggggacct              | +      | +                         |
| DLL1       | 232          | 170,436,652                                       | tccgatcttcagcggtgctc             | Gacgtcctcctgctgcttac             | +      | +                         |
| FAM120B    | 199          | 170,474,109                                       | cgctcagaagaagctacc              | ccaacgagctcagagcgg               | +      | +                         |

Expression of the above genes/ESTs was detected by RT-PCR using total RNA. Each RT-PCR experiment was repeated at least twice. In some cases another set of primers were also used to confirm the absence of expression. One tenth of the RT-PCR products were electrophoresed in 4% agarose gels containing ethidium bromide to visualize amplified products.

– indicates no amplification of the expected DNA fragment, whereas + indicates amplification of the expected gene fragment.
shown because it does not vary among different cell lines. Data shown in figure 6 for MLLT4 type 1 and MLLT4 type 3 indicate that expression of the transcripts is quite varied among normal (HS74), preimmortal (SVtsA/HF-A, SV.RNS/HF) and immortal (AR5, HALneo, SV.RNS/HF-1, cl39T, SVtsA/HF-CH2) cell lines. However, expression of the 2 transcripts within each cell line is similar. Notably, the MLLT4 type 1 and MLLT4 type 3 transcripts in HALneo are reduced as compared to other cell lines, including the normal (HS74) and its preimmortal (SVtsA/HF-A) parental cell line. Reduced expression of the corresponding MLLT4 type 1 and MLLT4 type 3 proteins in HALneo is further observed by Western blot analysis (fig. 7). Taken together, reduced expression of MLLT4 in HALneo indicated that the gene might be mutated in other SV40-immortalized cell lines.

b. Mutation analysis of MLLT4 in SV40-transformed cell lines. To determine whether MLLT4 is mutated in SV40-immortalized cells (AR5, HALneo, SV.RNS/HF-1, SVtsA/HF-CH2 and cl39T), we sequenced the entire cDNAs of the 3 transcripts [Saito et al., 1998] by a combination of RT-PCR and genomic sequencing. Although sequence analysis confirmed the presence of published polymorphisms, it did not reveal any inactivating mutations in the coding region of any of the 5 immortal cell lines analyzed (data not shown). Most important, there were no inactivating mutations in AR5 and HALneo, which would be expected to share a mutation since they are both derived from the parental preimmortal SVtsA/HF-A and retain the same intact allelic copy of the MLLT4-coding region. This result suggested that inactivation of MLLT4 is not responsible for the immortalization of SV40-transformed human fibroblasts. This observation is consistent with results reported by Minaguchi et al. [1999], who did not find any mutations in the MLLT4 gene in various ovarian carcinomas showing LOH in 6q27. It is recognized that epigenetic changes or alterations other than mutation in the coding region could be responsible in HALneo, but the absence of reduced expression in other immortal cell lines makes it less likely to be the shared SEN6 mechanism.

2. Analysis of DLL1 and FAM120B (BC012177, KIAA1838) as growth suppressors. We then investigated the role of DLL1 a ligand to NOTCH receptor and BC012177 (a putative transmembrane encoding protein of unknown function) [Holden and Raymond, 2003] as growth suppressors by cloning their respective cDNAs in pTRE-Tight-PGK.Puro vector and by stably expressing them in cl39T-Tet-On cells (stably expressing rtTA, reverse tetracycline transactivator). Clones were selected by puromycin selection in the absence of doxycycline and were then subjected to growth inhibition studies in the presence as well as absence of doxycycline. Both genes did not show any effect on overall growth of the cells (data not shown).
Moreover, Northern blot and RT-PCR analyses also did not show any change in gene expression for both these genes in a number of SV40-immortalized cell lines tested (data not shown). These observations eliminated DLL1 and FAM120B as putative candidate genes for SEN6.

3. Analysis of PHF10 as a growth suppressor. PHF10 belongs to a family of plant homeodomain (PHD) containing proteins which play a role in transcription regulation via chromatin remodeling [Aasland et al., 1995]. The NCBI database initially described that PHF10 had 2 known splice variants which encode 410-aa (PHF10α, NP_060758.1) or 408-aa (PHF10β, NP_579866.1) proteins based upon the model of 2 untranslated exons 1 and 2 (see fig. 1B and C). During the course of this study, the NCBI database has revised its interpretation to include exons 1 and 2 as part of the ORF. PHF10 has a SAY (supporter of activation of yellow) domain and 2 PHD finger motifs. Although PHF10 is evolutionarily conserved among species, its function in humans is unknown. However, the Drosophila homolog of PHF10, SAYP, regulates transcription both in euchromatin as well as in heterochromatin, and is an essential gene [Shidlovskii et al., 2005]. Recently SAYP has been shown to be a novel signature subunit of the PBAP chromatin remodeling complex [Chalkley et al., 2008].

a. Expression of PHF10 in different SV40-immortalized cell lines. To determine whether PHF10 gene expression is affected in different preimmortal and immortal cell lines, we performed Northern blot analysis using a 500-bp fragment from the 5′ end of PHF10β cDNA as a probe. This analysis identified 4 different PHF10 transcripts (1.9, 2.5, 4.3 and 5.0 kb) that were expressed in all cell lines (fig. 8A). Although expression of these transcripts is quite variable among different immortal cell lines as compared to preimmortal SV.RNS/HF and normal HS74 cells, expression of all transcripts was reduced in AR5 and especially SV.RNS/HF-1 cell lines. Despite the fact that PolyA+ RNA of immortal SV.RNS/HF-1 is underloaded in figure 8A as compared to its preimmortal parent SV.RNS/HF, the results of QRT-PCR (data not shown) and Northern blot analysis (see fig. 8B) using additional RNA preparations confirmed that overall expression of PHF10 transcripts is reduced in SV.RNS/HF-1. In cl39T, larger transcripts (4.3 and 5.0 kb) were more abundantly expressed as compared to normal (HS74) or preimmortal (SV.RNS/HF) cells (fig. 8A). In contrast, expression of all 4 transcripts was higher in HALneo. Reduced expression of all of the PHF10 transcripts in AR5 and SV.RNS/HF-1 immortal cell lines might be due to mutation(s) in PHF10 or due to regulation by epigenetic mechanisms.

The 4 transcripts of PHF10 in normal and SV40 immortalized cell lines could arise due to alternate promoter usage, alternative splicing or site of polyadenylation. Molecular structure and identity of the 2.5-, 4.3- and 5.0-kb transcripts are unknown, although our repeated efforts to clone larger transcripts were unsuccessful by 5′
RACE. We believe that the 1.9-kb transcript most likely represents NM_018828.2 and NM_013325.1, 2 known isoforms of \textit{PHF10} (fig. 1). It is likely that the 4.3-kb transcript represents EST AJ420510 which has a long 3'/H11541 UTR. This EST lacks exons 7 and 10–12 present in other \textit{PHF10} cDNAs.

b. Analysis of mutations in the \textit{PHF10} gene. To determine whether reduced expression of \textit{PHF10} transcripts is due to mutations, we have amplified the presumptive coding region (222–1,454 nt) of \textit{PHF10} in 4 different SV40-immortalized cell lines (AR5, cl39T, SV.RNS/HF-1 and HALneo). Sequence analysis of at least 2 clones from each immortal cell line revealed no mutations within this region. Interestingly, both alternatively spliced forms of cDNA (\textit{PHF10}a and \textit{PHF10}b) were expressed in all these cell lines. Absence of mutations in multiple SV40-immortalized cell lines in \textit{PHF10} sequence suggests that epigenetic pathways could be playing a role in the regulation of \textit{PHF10} expression. It should be noted that we were unable to amplify 5’ exon 1 and promoter region of \textit{PHF10} because of its high GC-rich region, and therefore might have missed mutation(s) in that region.

c. Ectopic expression of \textit{PHF10}a and \textit{PHF10}b results in growth suppression of normal, telomerase-immortalized and SV40-immortalized cells. In initial experiments, we observed that doxycycline-regulated stable overexpression of \textit{PHF10}a(HA) cDNA resulted in the partial suppression of growth in cl39T-Tet-On immortal cells (fig. 9). These data suggested that \textit{PHF10} may have growth-suppressive properties. To eliminate the possibility that the modest effect we observed is due to clonal variation, and to extend this observation to other immortalized cell lines, we cloned \textit{PHF10}b(HA) and (HA)\textit{PHF10}b(FLAG) (see fig. 1C and D) in a retroviral vector pLPCX. The cloned cDNAs expressed corresponding proteins by...
Western blot analysis after transient transfection assays in 293T cells (data not shown). Four independently derived SV40-immortalized cell lines (cl39T, SV.RNS/HF-1, AR5, CT10–2A) were transduced with retroviruses carrying (HA)PHF10L (FLAG), PHF10 (HA) or empty vector. Overexpression of PHF10 (HA) and (HA)PHF10L (FLAG) clearly exhibited reduced growth of all 4 cell lines used irrespective of their origin as compared to empty vector (fig. 10, compare columns 3 and 4 with column 2). To further investigate whether this inhibition of cell proliferation is specifically restricted to SV40-immortalized cells, we transduced 2 independent normal fibroblast (HS74, HSF43) and 3 independently derived telomerase-immortalized human fibroblast cell lines (HS74-hTERT, HSF43-hTERT; IMR90-hTERT, lung fibroblast cell line immortalized with hTERT) with retroviruses carrying (HA)PHF10L (FLAG), PHF10 (HA) or empty vector. Interestingly, both cDNAs also affected growth of normal and telomerase-immortalized cell lines as compared to empty vector control indicating that growth inhibition by these PHF10 cDNAs is not limited to SV40-immortalized cells but instead is global in nature. Similar, inhibition of growth has been observed by the overexpression of PHD finger containing proteins such as members of the ING family of proteins (ING1–5) [Gunduz et al., 2008].

d. Down-regulation of PHF10 expression causes loss of cell proliferation in normal HS74 fibroblast cells. Since overexpression of PHF10 (HA) or (HA)PHF10L (FLAG) causes reduced cell proliferation, we hypothesized that depletion of PHF10 function by RNAi should be growth stimulatory or should extend the life span of normal fibroblasts. With that rationale, we cloned different PHF10 shRNAs into a retroviral vector (pSIREN-RetroQ, Clontech) and then cotransfected these with PHF10 (HA) cDNA to identify shRNAs that are effective in down-regulating PHF10 protein. Western blot analysis shows that shRNA#1 and shRNA#4 both significantly reduced ectopically expressed PHF10 (HA) protein as compared to control shRNA, shRNA#2 and shRNA#3 (fig. 11). All classes of endogenous PHF10 mRNA are reduced in transfected SV40-immortalized cl39T cells treated with shRNA#1 (data not shown). To determine the effect of down-regulation of PHF10 function by shRNA#1 and shRNA#4 on the extension of life span of normal HS74 cells, we transduced these cells serially, first with retroviruses carrying shRNA#1 and then with shRNA#4 within 24 h. Surprisingly, depletion of PHF10 negatively affected growth of HS74 cells as well (fig. 12). This result is contrary to the growth inhibition data obtained with the expression of PHF10 (HA) and (HA)PHF10L (FLAG) cDNAs (see fig. 10). Since a similar effect was seen with shRNA#1 and shRNA#4 in a cancer as well as in a normal telomerase-immortalized cell line [Banga, unpubl. observations], we conclude that PHF10 function is required for cell growth. Accordingly, the growth suppression observed with different epitope tagged PHF10 cDNAs is most likely to be due to the following reasons. (1) At the time of cloning, the available PHF10 cDNAs or ESTs represented complete or full-length PHF10 cDNAs.
NCBI has revised the full-length nature of PHF10 cDNA to include all 12 exons with a coding potential of 498 aa (see fig. 1). Therefore, it is now evident that we were examining the effect of overexpression of epitope-tagged truncated proteins in the cells, and therefore these proteins might be acting as dominant negative mutants. (2) This effect on cell proliferation is especially important for a protein whose function is essential for cell viability or survival. (3) This effect on growth suppression becomes more likely in the case of a protein that is a component of a multiprotein chromatin remodeling complex in humans [Banga, unpubl. observations] and in Drosophila [Chalkley et al., 2008]. It is also possible that growth inhibition could also be due to unregulated residual functioning of these overexpressed truncated PHF10 proteins during the cell cycle since they are driven by constitutively active CMV promoter. A similar effect on cell proliferation was previously observed when PW29, a nuclear protein involved in sister chromatid cohesion, was expressed as PW29–GFP fusion [Darwiche et al., 1999], whereas expression of H2B–GFP did not affect cell proliferation [Kanda et al., 1998]. Finally, it might be considered that marked alteration in the level of mutant PHF10 protein by overexpression or underexpression distorts a multiprotein complex dependent on PHF10 resulting in inhibition of cell proliferation or cell death.

Our overexpression studies involving epitope-tagged PHF10 also revealed that PHF10–derived growth inhibition in 4 different SV40-immortalized and 3 telomerase-immortalized normal fibroblast cell lines is independent of the presence of the catalytic subunit of telomerase, hTERT. For example, c39T immortal cells have long telomeres but do not express telomerase whereas AR5 (long telomeres) and SV.RNS/HF-1 (short telomeres) both express high levels of telomerase [Macera-Bloch, 2002]. In addition, all 4 cell lines used in this study were immortalized differently from each other. For example, CT10–2A was derived with wild-type SV40; c39T was immortalized with origin defective SV40 early region DNA; AR5 was obtained with origin defective SV40 encoding temperature-sensitive T antigen tsA58 DNA; and SV.RNS/HF-1 was obtained with origin defective SV40 DNA encoding a wild-type T antigen. These observations suggest that some other mechanism(s) of growth suppression independent of T antigen (and thereby independent of pRb and p53) is involved with PHF10.

In summary, we have further refined the location of the senescence-inducing gene defective in SV40-immortalized cell lines, the putative growth suppressor SEN6, to the 6q27 region between MLLT4 and TBP by characterizing Type I (6q*) and Type II (6q1) mouse/human hybrids with polymorphic and non-polymorphic markers. Generation of Type I and Type II hybrids made the 6q27 region more amenable to molecular analysis with different classes of DNA markers using agarose gels, and allowed us to identify the chromosomal break in MLLT4. This strategy should be applicable to analyze any chromosomal or chromosomal fragment from human cells of interest at the physical and expression level. Analysis of MLLT4, DLL1, FAM120B and PHF10 genes located in the deleted region and expressed in immortal, preimmortal and normal cells did not show features consistent with expectations for SEN6. In the case of epitope-tagged PHF10 cDNAs, growth suppression observed in normal, telomerase-immortalized and SV40-immortalized fibroblast cells could be due to a dominant negative effect or unregulated overexpression of PHF10 or PHF10 cDNAs, and hence PHF10 is also not a candidate gene for SEN6. This conclusion is consistent with the observation that PHF10 function is required for cell proliferation based upon RNAi studies.

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