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Prevalence and functional analysis of sequence variants in the ATR checkpoint mediator Claspin (CLSPN)

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Abstract

Mutational inactivation of genes controlling the DNA damage response contributes to cancer susceptibility within families and within the general population as well as to sporadic tumorigenesis. *Claspin* (*CLSPN*) encodes a recently recognized mediator protein essential for the ATR and CHK1-dependent checkpoint elicited by replicative stress or the presence of single-stranded DNA. Here we describe a study to determine whether mutational disruption of *CLSPN* contributes to cancer susceptibility and sporadic tumorigenesis. We resequenced *CLSPN* from the germline of selected cancer families with a history of breast cancer (n=25) or a multicancer phenotype (n=46) as well as from a panel of sporadic cancer cell-lines (n=52) derived from a variety of tumor types. Eight nonsynonymous variants, including a recurrent mutation, were identified from the germline of two cancer-prone individuals and five cancer cell-lines of breast, ovarian and hematopoietic origin. None of the variants was present within population controls. In contrast, mutations were rare within genes encoding the CLSPN-interacting protein ATR and its binding partner ATRIP. One variant of CLSPN, encoding the I783S missense mutation, was defective in its ability to mediate CHK1 phosphorylation following DNA damage and was unable to rescue sensitivity to replicative stress in CLSPN-depleted cells. Taken together, these observations raise the possibility that *CLSPN* may encode a component of the DNA damage response pathway that is targeted by mutations in human cancers, suggesting the need for larger population-based studies to investigate whether *CLSPN* variants contribute to cancer susceptibility.

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Keywords

Claspin; ATRIP; mutation; breast; cancer

INTRODUCTION

Disruption of the DNA damage response pathway plays a critical role in the development of both sporadic and inherited forms of breast cancer and other cancers (1). For example, somatic mutations within *p53*, a key effector of the cellular response to DNA damage are present in more than half of all sporadic tumors, while germline *p53* mutations are linked to Li-Fraumeni Syndrome (LFS), a multi-cancer phenotype that includes breast cancer (2,3). Germline mutations in *BRCA1* and *BRCA2*, genes that are also implicated in the cellular response to DNA damage, are responsible for a subset of familial breast cancer (4,5), while lower-penetrance alleles in the *CHEK2*, *PALB2*, and *BACH1/BRIP* DNA damage response genes are associated with a modest increase in the risk of developing this disease (6–10).

In mammalian cells, stalling of DNA replication forks, repair of UV-induced DNA damage, and exonuclease-mediated processing of double-strand DNA breaks all lead to the appearance of single-stranded DNA (ssDNA) and, consequently, to the induction of cell-cycle arrest via a complex signal transduction cascade (11). An early event in the DNA damage response to ssDNA is the recruitment of the ataxia telangiectasia and Rad3 related (ATR) protein, together with its binding partner ATRIP, to sites of DNA damage (12,13). Once activated, ATR can phosphorylate the CHK1 kinase on Ser³¹⁷ and Ser³⁴⁵ (14,15). Activated CHK1, in turn, phosphorylates the CDC25A and CDC25C phosphatases, leading to ubiquitin-mediated proteosomal degradation of CDC25A and nuclear export and inactivation of CDC25C (16,17). Consequently, CDKs remain in an inactive, phosphorylated state thus preventing cell cycle progression.

In *Xenopus*, the activation of Chk1 by ATR following replicative stress is dependent upon Claspin, a so-called mediator protein that, in a phosphorylated state, binds to Chk1 (18–20). In mammalian cells the orthologous protein, CLSPN, also mediates the activation of CHK1 following replicative stress or DNA damage (21,22). This activity is dependent upon phosphorylation of CLSPN at Thr916, a requirement for binding of CLSPN to CHK1 *in vivo* (23). Consistent with a role in the DNA damage response, siRNA-mediated downregulation of CLSPN in mammalian cells leads to an increase in premature chromatid condensation following HU-treatment, a reduction in the inhibition of DNA synthesis following UV exposure and a decrease in cell survival (21). Recent studies have shown that CLSPN specifically accumulates during the S phase of the cell cycle, and that its degradation in the G2 phase is essential for checkpoint recovery and associated entry into mitosis (24–27). Of note, Mrc1, the yeast homolog of CLSPN, is a component of normal DNA replication forks and has checkpoint-independent functions (28–32). Human CLSPN has recently been shown to facilitate the ubiquitination of PCNA following DNA damage, independently of ATR (33).

Here we describe the resequencing of *CLSPN*, a relatively poorly characterized component of the DNA damage response pathway, from the germline of familial cancer cases and within a panel of sporadic cancer cell-lines. We report prevalent variants within *CLSPN* as well as a single allelic variant within *ATRIP*. Functional analysis revealed that at least one variant of *CLSPN* is associated with hypersensitivity to replication-induced DNA damage both *in vitro* and *in vivo*.

MATERIALS AND METHODS

High-risk cancer cohorts

Our cohort was comprised of EBV-immortalized lymphoblastoid cell-lines established from probands with early-onset breast cancer and a family history of disease (n=25) (34), Li-Fraumeni Syndrome (LFS) (n=5), or variant-LFS (n=41). Breast cancer families were not linked to germline mutations in *BRCA1* or *BRCA2*. Likewise, multicancer families were not linked to germline mutations within *p53*. All clinical material was collected under appropriate Institutional Review Board-approved protocols. EBV-immortalized lymphoblastoid cell-lines were maintained in Iscoves modified Dulbecco's medium supplemented with 20% fetal bovine serum (FBS), L-glutamine (2mM), and penicillin (10U)-streptomycin (10μg) at 37°C in 5% CO₂.

Sporadic cancer cell-lines

Sporadic cancer cell-lines were obtained from ATCC and were comprised of the following: breast (MCF7ADR, MDA-MB-435, T47D, BT483, MDA-MB-436, MDA-MB-53, MDA-MB-468, MDA-MB-415, MDA-MB-231, MDA-MB-175, MDA-MB-157, HS157, HS467T, HS496T, HS578T, UACC893, BT549), ovarian (ES-2, IGROV-1, MDAH2774, OV1063, OVCAR3, OVCAR4, OVCAR5, OVCAR8, SKOV3, SW626), lung (NCIH460, NCI522, HOP92), CNS (SF295, SNB19, U251), hematopoietic ((CCRF-CEM (acute lymphoblastic leukemia), K562 (chronic myelogenous leukemia), MOLT4 (acute lymphoblastic leukemia), RPMI-8226 (multiple myeloma), SR (large-cell lymphoma)), colon (COLO205, HCT116, HCT15), renal (786-0, ACHN, CAKI-1, SN12C, U031), melanoma (LOXMVII, M14, SKMEL2, UACC62), and osteosarcoma (U2OS, SAOS2). Cell-lines were not authenticated after receipt. Cell-lines were grown in either DMEM or RPMI-1640 (hematopoietic cell-lines) supplemented with 10% FBS, L-glutamine (2mM), and penicillin (10U)-streptomycin (10μg) at 37°C in 5% CO₂.

Control populations

EBV-immortalized lymphoblastoid cell-lines were established from 200 anonymous blood donors with no previous diagnosis of cancer, the majority of whom were Caucasian, through the Massachusetts General Hospital blood bank. A second, multiethnic, control series consisted of DNAs extracted from 360 healthy individuals with approximately equal representation of African-American, Caucasian, Hawaiian, Japanese, and Latino ethnic subgroups (35).

Mutational Analysis

Genomic DNA was extracted from cell-lines using a standard phenol-chloroform procedure followed by ethanol precipitation and resuspension in TE buffer. All coding exons of *CLSPN* and *ATRIP* were amplified from genomic DNA using intronic primers. Primers and PCR conditions are available upon request. PCR amplicons were purified by exonuclease I (United States Biochemical, Cleveland, OH) and shrimp alkaline phosphatase (United States Biochemical, Cleveland, OH) treatment according to the manufacturer's recommendations. Purified amplicons were diluted and sequenced using the BigDye terminator kit version 1.1 in conjunction with an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Nucleotide sequences were analyzed for the presence of mutations by visual inspection and using Sequence Navigator and Factura softwares (Applied Biosystems, Foster City, CA) to mark and display heterozygous or homozygous positions. All non-synonymous sequence variants were confirmed from at least two independent genomic DNA amplifications. Denaturing HPLC (dHPLC) using the Wavemaker system (Transgenomic, Omaha, NE) was used to screen for variants within the coding sequence of *ATR*. Wavemaker software

(Transgenomic, Omaha, NE) and the Stanford prediction program (<http://insertion.stanford.edu/melt.html>) were used to determine the optimal annealing temperatures for mutational analysis by dHPLC. Amplicons exhibiting anomalies by heteroduplex analysis were sequenced to determine the underlying nucleotide change.

Genotyping of population controls

Non-synonymous sequence variants were genotyped within population controls by direct nucleotide sequencing (up to 200 Caucasian controls) as described above, or using a Sequenom MassArray system (up to 360 multiethnic controls). Primers and probes were designed for each variant using the SpectroDesign software and are available on request. Multiplex PCR was performed in 5- μ l volumes that contained 0.1 U of *Taq* polymerase (Amplitaq Gold, Applied Biosystems, Foster City, CA), 5 ng genomic DNA, 2.5 pmol of each primer, and 2.5 mol of dNTP. Thermocycling was at 95°C for 15 min, followed by 45 cycles of 95°C for 20 s, 56°C for 30 s, and 72°C for 30 s. Unincorporated dNTPs were deactivated using 0.3 U of shrimp alkaline phosphatase (Roche, Germany), followed by primer extension using 5.4 pmol of each primer extension probe, 50 μ M of the appropriate dNTP/ddNTP combination, and 0.5 units of Thermosequenase (Amersham Pharmacia, Piscataway, NJ). Reactions were cycled at 94°C for 2 min, followed by 40 cycles of 94°C for 5 s, 50°C for 5 s, and 72°C for 5 s. After the addition of a cation-exchange resin to remove residual salt from the reactions, ~7 nl of the purified primer-extension reaction was loaded onto a matrix pad (3-hydroxypicolinic acid) of a SpectroCHIP (Sequenom, San Diego, CA). SpectroCHIPS were analyzed using a Bruker Biflex III MALDI-TOF mass spectrometer (SpectroREADER, Sequenom, San Diego, CA) and the spectra processed using SpectroTYPER (Sequenom, San Diego, CA). Genotyping percentage exceeded 98% for each assay. Error rates as previously assessed by duplicate samples on this platform have been estimated at 0.3%.

Generation of CLSPN Expression Constructs

A C-terminal myc-tagged construct expressing full-length, wildtype *CLSPN* was generated by PCR and cloned into the pCDNA3.1/V5-His TOPO TA expression vector (Invitrogen, Carlsbad, CA). Site-directed mutagenesis was performed to generate mutant constructs expressing the naturally occurring Ile236Val, Ala1146Ser, Pro956Leu, Gly364Asp, Ile783Ser, or Arg1184Trp *CLSPN* variants. The integrity of all constructs was verified by nucleotide sequencing. The expression of each construct was confirmed by Western blot analysis using an anti-MYC antibody after transfection into U2OS cells. Constructs encoding the wildtype *CLSPN* and each of the *CLSPN* variants were subcloned into the pAd shuttle vector. Briefly, constructs in the pCDNA3.1/V5-His Topo vector were digested with BamHI and XhoI and cloned into the BglII/XhoI site of the pShuttle-CMV vector. Recombinant adenoviral plasmids were generated by homologous recombination in *E. coli* and adenovirus titers measured using the Adeno-X™ titer kit (BD Biosciences Clontech, Palo Alto, CA), according to the manufacturer's instructions.

Small Interfering RNA (siRNA) and DNA Transfections

Duplexes targeting *CLSPN*-3'UTR, or a control transcript, were obtained from Dharmacon, Inc (Lafayette, CO). The coding strand of the *CLSPN*-siRNA was AUUGCAGACAGAAAUCCAdTdT and the control-siRNA was UCCAGUGAAUCCUUGAGGUdTdT (21). SiRNA duplexes were transfected into U2OS cells using Oligofectamine (Invitrogen, Carlsbad, CA). Briefly, cells were grown to 30–40% confluency and transfected with siRNAs at 100 nM for 48h, after which time the culture medium was replaced and cells were allowed to recover at 37°C with 5% CO₂ for 24 h. The siRNA transfection was then repeated. In cotransfection experiments, U2OS cells were initially transfected with siRNA alone using Oligofectamine (Invitrogen, Carlsbad, CA).

After 24 h, cells were transfected with *CLSPN*-expressing constructs using Lipofectamine-2000 (Invitrogen, Carlsbad, CA). After a 24h recovery period, cells were mock-treated or UV-treated (10J/m²).

Immunoblotting

U2OS cells transfected with control or *CLSPN*-3'UTR siRNAs were treated with IR (10Gy), UV (10J/m²) or HU (2mM), or left untreated, 48h after the second siRNA transfection. Cells were harvested for Western blotting after 1h by lysis in NETN buffer consisting of 150mM NaCl, 1mM EDTA, 20mM Tris (pH 8), 0.5% Nonidet P-40, 1x protease inhibitor cocktail (Roche, Indianapolis, IN). Lysates were either untreated, or treated with λ phosphatase (400U) for 30 min at 30°C in the buffer provided (New England Biolabs, Ipswich, MA), and resolved on a 7.5% SDS/PAGE gel. For immunoblot analysis, proteins were transferred to PVDF membranes (Millipore, Bedford, MA), probed with the appropriate antibody, and visualized with the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer, Waltham, MA). Antibodies used were the anti-*CLSPN* (ab3720) antibody (Abcam, Cambridge, MA), the anti-CHK1 (G-4) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), the anti-phospho CHK1 (Ser345) antibody (Cell Signaling Technology, Danvers, MA), anti-Myc (9B11) antibodies (Cell Signaling Technology, Danvers, MA), and the β-tubulin antibody (Upstate, Lake Placid, NY).

Colony Formation Assay

U2OS cells co-transfected with control or *CLSPN*-3'UTR siRNA were plated at low density and treated with 2mM HU for 24 hr, at which time cells were washed and the culture medium replaced. Colonies were stained with Coomassie Blue and counted after two weeks.

RESULTS AND DISCUSSION

Germline and somatic sequence variants in *CLSPN*

We resequenced all coding exons of *CLSPN* from EBV-immortalized lymphoblastoid cell-lines established from 71 index cases with a family history of cancer. Germline variants of *CLSPN* were present among lymphoblastoid cell-lines from 1 of 25 (4%) probands from breast cancer families, and 1 of 46 (2%) probands from multicancer families (Table 1). We therefore extended our analysis to search for *CLSPN* mutations in a panel of sporadic cancer cell-lines representing diverse tumor types, including breast cancer. Non-synonymous sequence variants were uncovered in 5 of 52 (10%) sporadic cancer cell-lines representing eight distinct tumor types (Fig. 1). These were confined to cell-lines derived from tumors of the breast (2 of 17 lines), ovary (1 of 10 lines) and hematopoietic system (2 of 5 lines) (Table 1). All variants were missense mutations, none of which was present among a series of at least 140 control individuals (280 alleles), nor were they reported SNPs (Table 1). Among the six *CLSPN* variants found within sporadic cancer cell-lines, five occurred in the heterozygous state. An exception was the breast cancer cell-line MDA-MB-175, which was homozygous for a variant encoding the P956L substitution. A second cell-line, ES2, derived from an ovarian tumor, contained two independent missense mutations leading to the R1184W and I783S substitutions. Sequencing of cloned RT-PCR products generated from ES2 revealed that the substitutions were biallelic, occurring *in trans*. The R1184W variant was also present within a leukemia cell-line, MOLT4, but was absent amongst 184 healthy individuals, suggesting that this may represent a recurrent tumor-associated mutation. The *CLSPN* I236V, I783S, A1146S and R1184W variants each affected amino acid residues that are conserved between human and *Xenopus*, suggesting that they may be important for proper functional activity.

Sequence variant within *ATRIP*

Given our observation of *CLSPN* variants within a subset of sporadic cancer cell-lines, we extended our screen to search for somatic mutations within genes encoding proteins that have a direct functional interaction with *CLSPN* namely *ATR* and *ATRIP*. We previously reported the absence of mutations within *CHK1*, which encodes a *CLSPN*-interacting protein, within this same panel of cell-lines (6). Here, a single non-synonymous sequence variant was identified within *ATRIP* from the panel of 52 tumor-derived cell-lines. This consisted of a heterozygous missense mutation (c.2297C>T), predicted to encode an amino acid substitution *ATRIP*-T766M within the ovarian cancer cell-line OVCAR3. This affects a residue that is immediately proximal to the conserved PIKK interaction motif within *ATRIP*, although it does not itself display cross-species sequence conservation (36). The *ATRIP* variant was not detected within the germline of 241 healthy control individuals (482 alleles) indicating that it is not a common polymorphism within the general population.

Functional properties of *CLSPN* mutants

The relatively high frequency of sequence variants detected within *CLSPN*, compared with the frequency of *ATRIP* mutations within the same set of cell-lines, prompted us to examine the effect of the *CLSPN* variants on function. To evaluate the functional consequences of each *CLSPN* variant, we examined their ability to undergo phosphorylation in response to DNA damage, as well as their effect on DNA damage-induced phosphorylation of *CHK1*. We first confirmed that phosphorylation of *CHK1* at Serine 345 is reproducibly observed in U2OS cells (Fig. 2A), as well as in the breast cancer cell-lines MDA-MB-436, MDA-MB-453 and MDA-MB-468 (Fig.S1) in response to either UV or hydroxyurea (HU) exposure. Si-RNA-mediated knockdown of endogenous *CLSPN* in U2OS cells specifically suppressed this effect (Fig. 2A). The siRNA constructs were designed to target the 3' UTR of the native *CLSPN* transcript, making it possible to simultaneously express ectopic *CLSPN* constructs lacking the 3'UTR. Ectopic 3'UTR-truncated *CLSPN* was subject to physiological regulation, as demonstrated by its phosphorylation following HU exposure (Fig. 2B). We then engineered U2OS cells by knocking down endogenous *CLSPN*, while ectopically expressing variants identified in our mutation screen. We established two independent reconstitution assays in U2OS cells to investigate the possible functional defects of *CLSPN* mutants. In the first, we tested *CHK1* phosphorylation post-transfection of siControl and si*CLSPN* oligos following UV treatment. We chose to examine cells following UV treatment because this form of DNA damage gave the most robust induction of *CHK1* phosphorylation in siControl transfectants (Fig. 2A). In this reconstitution assay, wildtype *CLSPN* displayed a robust restoration of *CHK1* phosphorylation following UV treatment. Of six variants tested, one, *CLSPN*-I783S, reproducibly displayed a reduced complementation of endogenous *CLSPN* knockdown (Fig. 2C), as measured by *CHK1* phosphorylation, across three independent experiments. *CLSPN* has previously been implicated in a pathway that prevents premature chromatin condensation in response to HU induced DNA damage (21). Therefore, we established a second reconstitution assay in which we evaluated survival of U2OS cells post-transfection of siControl and si*CLSPN* oligos and following HU treatment. *CLSPN*-I783S reproducibly displayed a reduced complementation of endogenous *CLSPN* knockdown, as determined by cell viability, following treatment with HU (Fig. 2D). All other mutant constructs tested (I236V, A1146S, P956L, G364D, and R1184W) showed a level of complementation similar to the wild-type construct and therefore are currently of unknown functional significance (data not shown). Although we have not identified a cell-line with definitive homozygous inactivation of *CLSPN*, the ES2 ovarian cancer cell-line harbors two heterozygous mutations in *CLSPN*, occurring *in trans*, namely the I783S and R1184W mutations. This cell-line showed very low levels of *CHK1* phosphorylation following UV or HU treatment (Fig. S2). Taken together, these observations suggest that *CLSPN*-I783S encodes an attenuated allele,

potentially linked to tumorigenic properties. However, in the absence of assays that directly measure CLSPN activity, it remains possible that additional mutants identified in this study may encode proteins with attenuated activity beyond the level of detection by our assays. Similar effects have been noted for the CHEK2-I157T variant, which encodes an attenuated protein that is impaired in only certain aspects of the CHEK2-mediated DNA damage response (37–39). Alternatively, some *CLSPN* mutations uncovered within our screen may be passenger mutations rather than driver mutations in human tumorigenesis. As has been noted in other studies, robust functional assays are an absolute requirement to distinguishing between passenger and driver mutations (40).

In addition to the *CLSPN* variants observed in sporadic cancer cell-lines, we also detected germline variants of this gene within EBV-immortalized lymphoblastoid cell-lines established from probands from two cancer families. However, extended genetic analysis of matched tumor DNA for loss-of-heterozygosity, or of germline DNA from an affected relative for co-segregation, provided no evidence for an association of the variant alleles with disease susceptibility in these particular families (data not shown). This is consistent with recent analyses of breast cancer families which also found no evidence for linkage of *CLSPN* to breast cancer predisposition (41,42). Candidate gene analysis is complementary to genome wide association (GWA) studies, especially for rare variants in genes that are known to play a role in DNA damage pathways. As illustrated by a number of genes studied to date (7,8,10), multiple different variants, each of which is relatively rare in the population, may contribute toward cancer risk, without the ancestral founder effects that are best identified by GWA studies. As such, combining mutational analysis with detailed functional assays is particularly valuable in dissecting pathways that, in aggregate, contribute toward breast cancer susceptibility in the general population.

In conclusion, our findings have highlighted a potential role for mutational disruption of *CLSPN* in human tumorigenesis. Given that CLSPN is a multifunctional protein and that it modulates, but does not directly execute the phosphorylation of ATR substrates, it is perhaps not surprising that some functional defects of the CLSPN mutants have not been revealed by the specific *in vitro* functional assays that we used. As our understanding of the precise biochemical activity of CLSPN improves, additional assays may become available to shed further light on the functional impact of the germline and somatic mutations described here. In the meantime, resequencing of this gene among primary tumors of the breast, ovary and hematopoietic system may be required to more precisely determine the extent and role of *CLSPN* disruption in these malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Somatic and germline variants of *CLSPN*

A, Nucleotide sequence traces depicting missense mutations (arrows) within the breast cancer cell-line MDA-MB-175 (P956L), the leukemia cell-line MOLT4 (R1184W), and the ovarian cancer cell-line ES-2 (I783S). Wildtype sequences from control individuals are shown for comparison (upper panels). **B**, Schematic representation of the *CLSPN* protein, indicating the location of variants in relation to known functional domains. β TrCP degron, β TrCP recognition motif DSGxxS; NLS, potential nuclear localization signals; *CHK1-BD1*, -*BD2*, -*BD3*, CHK1 binding domains 1–3.

Figure 2. The *CLSPN*-I783S variant encodes a functionally defective protein

A, siRNA-mediated knockdown of endogenous *CLSPN* in U2OS cells is associated with reduced levels of phosphorylated CHK1. U2OS cells transfected with control (con) siRNA, or *CLSPN*-3'UTR-specific siRNA were untreated or treated with IR (10Gy), UV (10J/m²) or HU (2mM). Cells were harvested 1h post-treatment followed by immunoblotting to measure CLSPN, phospho-CHK1(Ser345), and total CHK1 protein levels. **B**, *In vivo* phosphorylation of CLSPN-I783S, following exogenous expression in U2OS cells, is not impaired. U2OS cells infected by adenoviral constructs expressing myc-tagged wildtype (WT) or mutant (I783S) CLSPN were either treated with HU (2mM), or left untreated. Western blots of λ-protein phosphatase (λ-ppase)-treated or -untreated lysates were probed with an anti-CLSPN antibody to measure total levels of CLSPN expression, or with an anti-MYC antibody to measure ectopic CLSPN levels. **C**, *In vivo* phosphorylation of CHK1 by CLSPN-I783S, is impaired. U2OS cells were cotransfected with CLSPN-3'UTR-specific siRNAs to knock down the endogenous protein, as well as with constructs exogenously expressing wildtype or I783S constructs lacking a 3'-UTR. Cells were divided into two plates and treated with 10J/M² UV, or left untreated. Cells were harvested 1hr post-treatment, and subjected to immunoblotting with antibodies against CLSPN, phospho-CHK1 (Ser345), or total CHK1. Protein levels were quantified by phosphor imaging and the amount of phosphorylated CHK1, relative to total CHK1, determined (*lower panel*). **D**, Wildtype CLSPN, but not CLSPN-I783S, partially rescues the HU-sensitivity of CLSPN-depleted U2OS cells. CLSPN-depleted U2OS cells were co-transfected with exogenous DNA constructs expressing wildtype CLSPN or CLSPN-I783S. Transfected cells were plated at low density and treated with 2 mM HU for 24h. After two weeks, the number of colonies was determined by counting. Experiments were performed in triplicate and representative results, indicating the number of cells surviving HU treatment relative to untreated cells, are shown.

Table 1*CLSPN* mutations among cancer families and sporadic cancer cell-lines

Sample	Phenotype	Nucleotide Change	Amino acid change	Frequency in population controls
<i>Germline of cancer families (n=71)</i>				
Case-1	Breast cancer	A>G, nt 706	I236V	0/546
Case-2	LFL	G>T, nt 3436	A1146S	0/184
<i>Sporadic cancer cell-lines (n=52)</i>				
MDA-MB-415	Breast cancer	G>A, nt 1091	G364D	0/551
MDA-MB-175	Breast cancer	C>T, nt 2867	P956L	0/551
ES-2	Ovarian cancer	C>T, nt 3550 T>G, nt 2348	R1184W I783S	0/184 0/140
MOLT4	Leukemia	C>T, nt 3550	R1184W	0/184
RPMI-8226	Multiple myeloma	A>G, nt 1361	H454R	0/165

Li-Fraumeni-like (LFL); Nucleotide (nt) and amino acid positions are based on Genbank Assession number GI:21735568