

Biochemical and Functional Characterization of the Klotho-VS Polymorphism Implicated in Aging and Disease Risk*

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Background: The mechanism by which polymorphisms in the anti-aging protein klotho lead to increased disease risk is unknown.

Results: *In vitro*, klotho-VS decreases homodimerization and increases heterodimerization with and activation of FGFR1c.

Conclusion: Altered dimerization explains klotho-VS association with increased disease risk.

Significance: Understanding how the VS variant leads to changes in klotho function will elucidate the role klotho plays in disease and lifespan.

Klotho (KL) is an age-regulating protein named after the Greek goddess who spins the thread of life. Mice deficient in KL are normal throughout development, but rapidly degenerate and display a variety of aging-associated abnormalities that eventually lead to decreased life expectancy. While multiple genetic association studies have identified KL polymorphisms linked with changes in disease risk, there is a paucity of concrete mechanistic data to explain how these amino acid substitutions alter KL protein function. The KLVS polymorphism is suggested to lead to changes in protein trafficking although the mechanism is unclear. Our studies have sought to further investigate the functional differences in the KLVS variant that result in increased risk of many age-related diseases. Our findings suggest that the F352V and C370S substitutions lead to alterations in processing as seen by differences in shedding and half-life. Their co-expression in KLVS results in a phenotype resembling wild-type, but despite this intragenic complementation there are still changes in homodimerization and interactions with FGFR1c. Taken together, these studies suggest that KLVS leads to altered homodimerization that indirectly leads to changes in processing and FGFR1c interactions. These findings help elucidate the functional differences that result from the VS polymorphism, which will help clarify how alterations in KL function can lead to human disease and affect cognition and lifespan.

α -Klotho (KL)² was first discovered in an attempt to overexpress a sodium proton exchange pump that led to the interrup-

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² The abbreviations used are: KL, α -klotho; ADAM, a disintegrin and metalloproteinase domain-containing protein; CSF, cerebrospinal fluid; FGF, fibroblast growth factor; IGF1, insulin growth factor 1; TNF- α , tumor necrosis factor alpha; TGF β , transforming growth factor beta; SNP, single nucleotide polymorphism; CVD, cardiovascular disease; V, F352V; S, C370S; FGFR, FGF receptor; KLWT, wild type KL; TCA, trichloroacetic acid; EV, pcDNA3.1, empty vector; mIgG, mouse IgG; GWAS, genome-wide association studies; pERK, phosphorylated ERK; tERK, total ERK.

tion of the KL promoter. The resulting mice mature normally until 3 weeks of age, at which time they develop a severe aging phenotype that includes osteoporosis, atherosclerosis, hypoglycemia, and cognitive decline (1). Our group has shown that KL levels are down-regulated in the brains of mammals as they age (2), likely due to the methylation of its promoter (3). Conversely, mice overexpressing KL have an increase in lifespan of 20 and 30% in female and male mice, respectively (4). These mice are more resistant to oxidative stress and are protected against renal injury in a model of glomerulonephritis (5, 6).

KL is a type 1 transmembrane protein expressed predominantly in the distal tubules of the kidney, choroid plexus, and pituitary glands (1). Most of the transmembrane form of the protein is localized extracellularly. It contains two weakly homologous domains, KL1 and KL2, that share homology with family I glycosidases. The transmembrane form can be shed from the cell surface by ADAMs 10 and 17 (7) and the shed form is detected in both serum and cerebrospinal fluid (CSF) (8). While its homologous family member β -klotho is reported to function solely as an FGF (fibroblast growth factor) co-receptor (9–11), the functions attributed to KL are more diverse. Most of our information about the function of KL comes from studies in mouse where KL inhibits insulin/IGF1, wnt, TNF- α , and TGF β signaling and serves as the obligate co-receptor to transduce FGF23 signaling. KL also functions to alter ion channel function through its sialidase activity (4, 12–19).

Beyond the mouse, human KL polymorphisms have been identified that reveal even slight changes in KL can impact both longevity and disease risk (14–20, 26, 39–49). Perhaps the most studied of the polymorphisms is KLVS. The KLVS variant consists of 6 single nucleotide polymorphisms (SNPs) that are always found together. Three SNPs lie in introns and do not alter splicing and another SNP at nucleotide 1155 causes no change in amino acid designation. However, two SNPs result in amino acid substitutions, namely F352V and C370S, which could alter protein function. Multiple studies show that humans heterozygous for the KLVS allele have an increased chance of survival over the age of 75 (20–22). One study also found a reduced representation of KLVS homozygotes after this age (22). Further characterization of the variant revealed

protective effects of heterozygosity after 81, despite reduced frequency of heterozygotes in the population before this age (21). In independent studies, the KLVS variant has also been linked to an increased risk of cardiovascular disease (CVD) and metabolic syndrome with a similar pattern of advantageous KLVS heterozygosity and increased disease risk associated with homozygosity (21, 23–25). This could be explained by the KLVS regulation of factors that independently increase risk of these diseases such as HDL-cholesterol and systolic blood pressure (21). There are also age-dependent increases in bone mineral density in both male and female heterozygotes, but whether there is a homozygote disadvantage could not be determined due to the low frequency of that genotype (26, 27). Contrary to the other effects, heterozygosity for KLVS increases the risk of BRCA1 associated breast and ovarian cancer and decreases the risk in homozygous patients from an Ashkenazi Jewish population, but not in a larger cohort of European ancestry (28, 29).

Despite many genetic association studies on the KLVS allele, there is limited information on the molecular mechanism behind the KLVS association with altered longevity and risk of disease. Given the increased risk of disease development and complex association with longevity, a thorough understanding of the mechanistic difference associated with KLVS is important in understanding the role of KL in humans. To understand how changes in KL could affect human longevity and disease risk, we generated plasmid constructs of transmembrane KL with individual (V for F352V, and S for C370S) substitutions and one containing the double substitution (VS, F352V/C370S) as observed in humans to examine how KL dimerization, half-life, shedding, and FGF co-receptor function are altered by the presence of the KLVS polymorphism.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, all chemicals were obtained from Sigma Aldrich.

Construction of Plasmids—Full-length KL, KL tagged at its C terminus with GFP or V5 in pcDNA3.1 plasmids were constructed as previously described (7). To construct polymorphic variants containing F352V, C370S, or both, site-directed mutagenesis was performed per the manufacturer's instructions (Agilent Technologies Inc., Santa Clara, CA) using the following sense and antisense primers (Integrated DNA Technologies): KL C370S: 5'-AGGTGGGTCCAAAGCTAAGAGCAAAAAGTC-3' and 5'-GACTTTTTTGTCTTAGCTT-TGGACCCACCT-3'; KL F352V: 5'-TCTCAGATTCAGTAA-CATCAGGCAGAATAGA-3' and 5'-TCTATTCTGCCTGAT-GTTACTGAATCTGAGA-3'.

Mouse FGF receptor (FGFR1c) plasmid with a V5 tag was a gift from Dr. Makoto Kuro-o, University of Texas Southwestern, Dallas, TX.

Cell Culture and Transfection—HEK 293 cells were maintained in DMEM (Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 5% penicillin-streptomycin (Mediatech Inc.) at 37 °C in 5% CO₂. Cells were transfected with a total of 0.5 μg of DNA per ml of medium. Total DNA was consistent across all experimental conditions with pcDNA3.1 empty vec-

tor (Invitrogen, Grand Island, NY) used as a control in experiments with multiple plasmid transfections. Cells were transfected using Attractene per manufacturer's instructions (Qiagen, Valencia, CA).

KL Shedding Assay—Cells were transfected with full-length wild type KL (KLWT) or KL containing either one or both of the mutations. Twenty-four hours after transfection, cells were washed with Dulbecco's phosphate-buffered saline (DPBS) (Mediatech Inc.) and incubated in serum-free DMEM for 24 h. Medium was collected, debris was removed by centrifugation, and bovine serum albumin (BSA) was added to adjust to a final protein concentration of 10 μg/ml. Cold (4 °C) trichloroacetic acid (TCA) was added to a final concentration of 25%. The mixture was incubated at -20 °C for 5 min and then at 4 °C overnight. After incubation, the samples were centrifuged at 16,000 × g, and the supernatant was removed. The resulting pellet was washed three times with ice-cold acetone with 5-min centrifugations. The pellet was dried at 98 °C for 5 min and then dissolved in 2× Laemmli sample buffer. Half of the TCA-precipitated protein resolubilized in Laemmli buffer was loaded onto 10% acrylamide SDS-PAGE gels. After the media collection, cells are washed twice with DPBS and lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5) containing complete protease inhibitor mixture (Roche, Mannheim GE). The cell lysate was centrifuged at 16,000 × g for 15 min, and the supernatant was collected for 10% acrylamide SDS-PAGE.

Half-life Assay—HEK 293 cells were transfected as described above. Twenty-four hours later, cells were washed twice with DPBS and incubated with a 1 mM solution of cell permeable NHS-SS-Biotin (Pierce) containing 0.1% DMSO for 30 min at room temperature. Afterward, biotinylation was quenched by washing the cells twice with serum-free DMEM. Cells were returned to DMEM containing serum and incubated at 37 °C for the indicated time points. At each time point, the medium was aspirated from the cells and cell lysates were collected as described above. After collection and centrifugation, the BCA Protein Assay (Pierce) was used to determine the protein concentration of the samples. Then 100 μg of protein from each sample were incubated at 4 °C with neutravidin beads (Thermo Scientific, Rockford IL) overnight to pull-down all biotinylated proteins. The bound protein was then eluted from the beads by incubating in 2× Laemmli sample buffer and separated on 10% acrylamide SDS-PAGE.

Blue Native-PAGE—Blue Native gel electrophoresis was performed to separate proteins without denaturation. The Invitrogen NativePAGE Novex 4–16% Bis-Tris Gel system was used in accordance to the manufacturer's protocol for the outlined experiments. Briefly, HEK 293 cells transfected as described above were solubilized in NativePAGE sample buffer containing 1% digitonin and complete protease inhibitor without EDTA (Roche) at 4 °C. After determining protein concentration, an equal amount of protein from each sample was prepared for separation by mixing with 5% Coomassie G-250 (Invitrogen, Grand Island, NY) to reach a final concentration of 1% Coomassie G-250. Pre-chilled anode and cathode buffers (dark blue and light blue) were used to run the gel. The dark blue cathode buffer was used until the dye front had moved

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through 1/3 of the gel and then was exchanged for the light blue cathode buffer until the completion of the run. After separation, the proteins were transferred onto an Immobilon 0.4 μM PVDF membrane (Millipore, Billerica MA). To fix the proteins, the resulting PVDF membranes were incubated in 8% acetic acid and then the membranes were destained with methanol. Concomitantly, the same samples were run in reducing conditions on 10% acrylamide SDS-PAGE gels to compare total expression of the transfected proteins. Afterward, both membranes were processed for Western blotting as described below.

Co-Immunoprecipitation—HEK 293 cells were co-transfected with combinations of KLWT and KLVS containing a V5 or GFP tag or with combinations of FGFR1c-V5 and EV, KLWT-GFP or KLVS-GFP. Other HEK 293 cells were transfected with V5 or GFP-tagged KLWT or KLVS alone. Twenty-four hours after transfection, cells were washed twice with ice-cold DPBS and collected in IP lysis buffer (1% Triton X-100, 0.01 M Tris-HCl, 0.01 M EDTA, 0.05 M NaCl, 0.05 M NaF, pH 7.2) containing protease inhibitors. After protein concentration was measured by BCA as described above, 100 μg of protein was loaded onto pre-washed V5-conjugated Sepharose beads (Sigma) and incubated overnight at 4 °C. An identical set of lysates containing equal protein were incubated with anti-GFP (Roche) for 1 h at 4 °C before adding pre-washed protein A/G beads (Pierce) and incubating overnight at 4 °C. Another set of samples for the experiments using FGFR1c-V5 were also prepared and incubated with mouse IgG (mIgG) for 1 h at 4 °C before adding pre-washed protein A/G beads (Pierce) and incubating overnight at 4 °C. After incubation, beads were washed and processed for SDS-PAGE as described below.

FGF23 Assay—HEK 293 cells were transfected with EV, KLWT or KLVS. Twenty-four hours after transfection, cells were incubated in serum-free medium for 2 h and then either bFGF (basic fibroblast growth factor) or FGF23 (R&D Systems, Minneapolis, MN) was added to the wells to reach a concentration of 100 ng/ml or 10 ng/ml, respectively. The cells were incubated for 15 min at 37 °C and then were immediately washed in DPBS and lysed in RIPA containing protease and phosphatase inhibitors (Roche, Mannheim GE). After lysis, samples were prepared for SDS-PAGE as described previously.

Western Blotting—Samples prepared as described were separated on 10% Tris-glycine gels. After SDS-PAGE, proteins were transferred onto nitrocellulose membranes (Millipore, Billerica MA). Membranes were blocked in 5% nonfat milk (Carnation, Wilkes-Barre PA) prior to incubation overnight in primary antibody dissolved in TBS-T containing 3% BSA. For detection of KL, the rat monoclonal antibody KM2076 (Cosmo Bio Co., Ltd., Japan) was used at a dilution of 1:2000. Mouse anti- β -tubulin (1:10,000) (Invitrogen, Grand Island, NY) was used as a loading control. FGFR1c-V5 was detected using a mouse monoclonal antibody against the V5 tag (1:5000) (Sigma) and phosphorylated ERK (1:10,000) and total ERK (1:1000) were detected using antibodies obtained from Sigma Aldrich and Cell Signaling Technologies, respectively. Appropriate HRP-conjugated secondary antibodies were obtained from KPL and were used at a dilution of 1:10,000. Detection was accomplished using Immobilon Chemiluminescent reagent

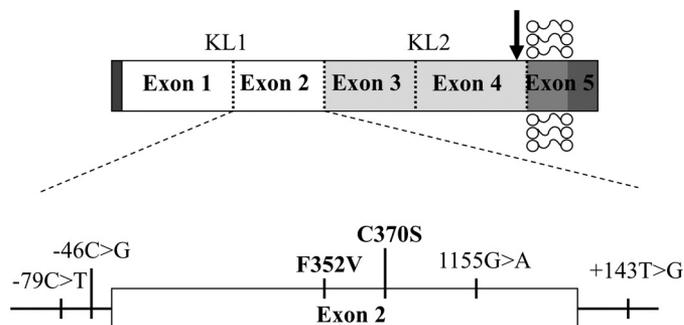


FIGURE 1. **Schematic representation of KL.** The KL protein is generated from 5 exons that make up the full-length protein. It is a type I transmembrane protein with a short intracellular region. Shown are the relative locations of the known domains (KL1 and KL2) as well as known cleavage site of ADAM 10/17 (arrow). Magnification of exon 2 depicts the approximate location of the six single nucleotide polymorphisms (SNP) that encode for the KLVS variant, including 3 SNPs not translated into protein located in the intron surrounding exon 2, two SNPs encoding for amino acid substitutions at phenylalanine 352 and cysteine 370 (in bold letters), and one SNP located at nucleotide 1155 in exon 2 that does not change the amino acid sequence. The figure was modified from Arking *et al.* (22).

(Millipore, Billerica MA). Quantitation of densitometry was performed using ImageJ software.

Statistical Analysis—Statistical analyses were performed with Graphpad Prism software version 5.0. Before further analysis, data sets were analyzed for significant outliers using the Grubbs Outlier Test. Data sets containing 3 or more groups were analyzed using 1-way ANOVA. Post-hoc Dunnett tests were performed when significance of $p < 0.05$ was achieved. Data sets containing only two groups were analyzed by Student's t -tests. Half-life data were analyzed by using the extra sum-of-squares F test to compare lines of best fit generated from a one-phase decay model. Data were considered significant when a global comparison of Y_0 , K , and plateau resulted in a rejection of the null hypothesis with a $p < 0.05$.

RESULTS

Shedding Is Altered in KL Variants—Expression of the VS polymorphism in a construct consisting of the KL1 portion of the extracellular domain revealed differential secretion (22). However, the effect of the two amino acid substitutions on shedding of full-length KL (Fig. 1) has not been investigated. Since the full-length 135 kDa transmembrane protein is detected in cells, and the 130-kDa shed protein including both the KL1 and KL2 domains is measured in both blood and CSF, these forms of the protein are likely to be the most physiologically relevant to investigate (8). To assess whether there are differences in the shedding of the transmembrane form, HEK 293 cells were transfected with transmembrane wild-type KL (WT), or KL constructs containing the V, S or VS variants. Shedding was measured by collecting conditioned serum-free medium and lysates from the cells over a 24-h period. To correct for differences in KL construct expression, shedding was expressed as a ratio of extracellular (medium) to intracellular (lysate) KL levels. Compared with WT, the V variant leads to a 4.6-fold decrease in the amount of KL shed into the extracellular space (Fig. 2), which is similar to the difference reported previously (22). Conversely, neither the S nor VS variant showed a significant difference in shedding from wild-type (Fig.

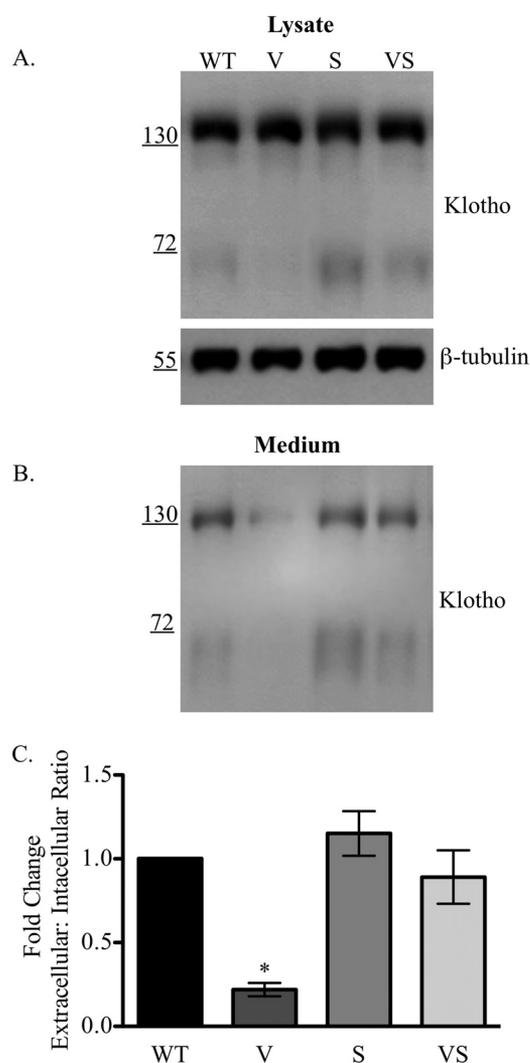


FIGURE 2. KL V dramatically decreases shedding. Shedding was examined by transfecting HEK 293 cells with wild-type (WT) KL or KL containing one or both of the amino acid substitutions in KLV (V, S, or VS). Shedding differences were measured by Western blot as change in the extracellular:intracellular KL protein ratio. *A*, representative blot showing KL expression and β -tubulin loading control in the lysate (intracellular), and KL levels in the (*B*) medium. *C*, quantification of band intensity was measured across multiple independent experiments. Graph represents the mean fold change of extracellular:intracellular KL (error bars are \pm S.E.; $n = 5$; * $p < 0.05$, ANOVA).

2). Although the V variant alone results in a drastic reduction in shedding, the presence of the S variant appears to correct the phenotype. This result suggests that there is intragenic complementation of the variants where co-expression of the S variant with the V variant corrects for the phenotype conferred by the V variant.

Half Life of KL Variants—Altered secretion could be the result of changes in turnover of KL protein. To investigate this possibility we assessed variant half-life using biotinylation to measure changes in protein degradation (30). Briefly, the proteins of HEK 293 cells transfected with KLWT or the variants were biotinylated, the reaction was quenched, and lysates were collected at time points from 30 min to 6 h (Fig. 3). Biotinylated proteins were isolated using neutravidin beads and changes in biotinylated KL were measured by Western blotting to assess the amount of KL remaining as compared with the amount seen

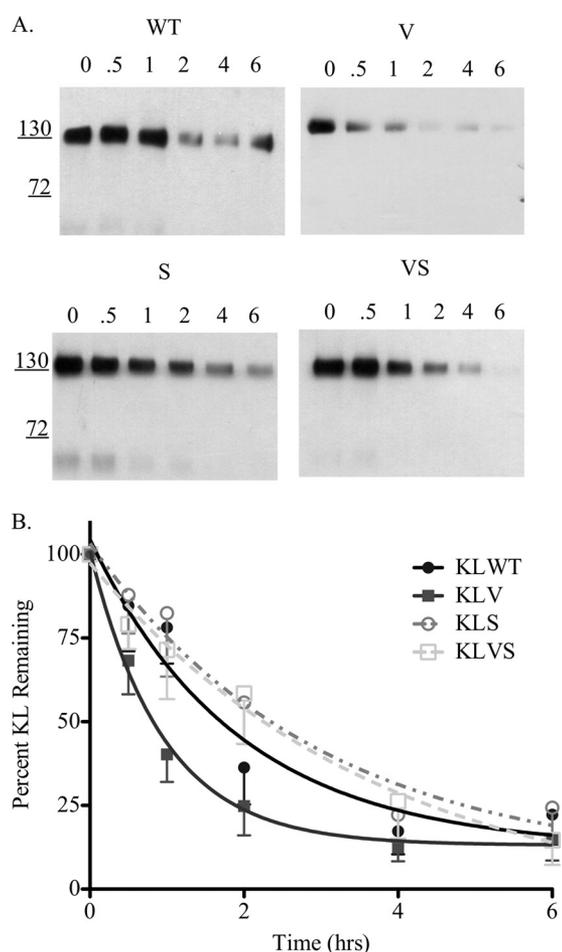


FIGURE 3. KL V decreases protein half-life. HEK 293 cells transfected with wild-type (WT) KL or KL containing one or two of the amino acid substitutions in KLV (V, S, or VS) were tested for changes in half-life of KL. Cells were biotinylated and half-life was assessed as amount of KL left 0.5, 1, 2, 4, or 6 h after biotinylation was quenched, and cells were placed back at 37 °C. *A*, representative Western blot of lysates pulled down with neutravidin beads after biotinylation and probed for presence of KL protein. *B*, graph depicts half-life of KL variants represented by percent KL remaining over time (h) (error bars are \pm S.E.; $n = 5$). Half-life values of variants measured by non-linear regression model of one-phase decay were as follows: KLWT- 1.33 h, KL V-0.66 h, KLS-2.03 h, and KLVS-2.53 h. The non-linear regression of KL V was found to significantly differ from KLWT by using the extra sum-of-squares F test ($n = 5$, $p < 0.05$).

at time 0. There was a decrease in the half-life of the V variant ($t_{1/2} = 0.6554$, $r^2 = .8245$), compared with WT ($t_{1/2} = 1.325$, $r^2 = 0.7065$, $p < 0.05$), but no differences in S ($t_{1/2} = 2.036$, $r^2 = 0.6021$) or VS ($t_{1/2} = 2.572$, $r^2 = .6862$) found by comparing non-linear regression models for one-phase decay through an extra sum-of-squares F test (Fig. 3). These observations coincide with our previous results, further implicating the role of intragenic complementation: while the V variant leads to a decrease in half-life, the expression of the S variant attenuates the phenotype when expressed together in the VS variant.

Altered Dimerization of KL Variants—Dimerization can affect processing of proteins (31, 32) and can be important in internalization of transmembrane proteins (33, 34). Since KL forms dimers (7, 8) and dimerization is important for the activity of many β -glycosidases, the effect of the KL variants on dimerization was investigated using Blue Native PAGE. Cells were transfected with KLWT or the variants and proteins were

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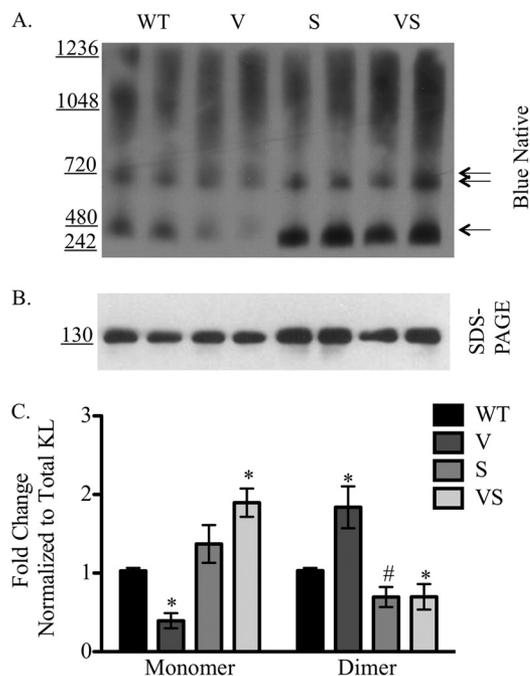


FIGURE 4. KL variants alter the levels of both monomeric and dimeric KL in cells. HEK 293 cells transfected with wild-type (WT) KL or variants (V, S, or VS) were assessed for differences in dimerization using Blue Native PAGE. *A*, Western blot illustrating differences seen in monomers and dimers of KL using the Blue Native-PAGE method. The *single arrow* points to KL monomers and *double arrows* indicate KL dimers. *B*, Western blot showing total KL amounts in denaturing and reducing conditions. *C*, bar graph depicting fold change across multiple experiments in monomer and dimer formation in the KL variants compared with wild-type normalized to total KL levels (error bars are \pm S.E.; $n = 3$, #, $p = 0.06$, *, $p < 0.05$, ANOVA). KLV leads to increases in dimeric KL and decreases in monomeric KL while both KLS and KLVs show the opposite effect leading to increased monomeric and decreased dimeric KL.

separated on Blue Native PAGE gels or in denaturing conditions by SDS-PAGE to compare the amount of KL found in dimeric and monomeric forms to total amount of KL expressed. While the amount of monomeric KLV was decreased compared with KLWT, dimerization was increased with this variant ($p < 0.05$, Fig. 4, A–C). On the other hand, both KLS ($p = 0.06$, Fig. 4, A–C) and KLVs ($p < 0.05$, Fig. 4, A–C) led to a decrease in the dimeric form and KLVs led to a significant increase in the monomeric form ($p < 0.05$, Fig. 4, A and B). Similar to the effects of the variants in the other experiments, the effect of the KLV variant is attenuated by co-expression with the KLS variant. However, for the first time we found a distinct effect of the VS variant compared with WT. When VS is overexpressed, more monomeric and less dimeric KL is found.

Because the genome-wide association studies (GWAS) on the KLVs variant suggest that there is a difference between heterozygous and homozygous carriers, the effect of the KLVs variant was further investigated through co-immunoprecipitation. Cells were co-transfected with combinations of KLWT and KLVs containing either a V5 or GFP tag (Fig. 5). Lysates were collected and the interaction of KLWT and KLVs were assessed by co-immunoprecipitation. A GFP antibody was used to pull down GFP-tagged KL and V5 conjugated beads were used to pull down V5-tagged KL. Alternatively, separate lysates were transfected with GFP- or V5-tagged KLWT or KLVs alone and were pulled down with the opposite antibody (KLV5 pulled

down with GFP antibody and *vice versa*) in order to assess whether there was any nonspecific pull-down because of antibody cross-reactivity. After pull-down, samples were separated by SDS-PAGE and the amount of interaction partner was determined by Western blotting. By this method, we could ascertain differences in levels of dimerization between KLWT and KLVs either to themselves or each other. The presence of a glycosylated form of KL previously discussed by Imura *et al.* can be seen in some of the KL blots (8). Results show that when compared with levels of homodimerization of KLWT, dimerization of KLWT to KLVs and of KLVs to KLVs are reduced irrespective of the antibody used to pull down the interaction partners ($p < 0.05$) (Fig. 5, A–D). KLVs dimerization to KLVs was reduced compared with wild type confirming the results from Fig. 5. The control immunoprecipitation also shows that these results are not due to pull-down of the opposite tag by nonspecific antibody interactions (Fig. 5, E and F). Interestingly, these results expand the Blue Native findings to suggest that regardless of binding partner, WT or VS, KLVs dimerizes less efficiently than WT.

Changes in Role of KL as FGF Co-Receptor—Changes in KL ability to self-dimerize may impact its function alone or in combination with other binding partners. We used KL function as a co-receptor with FGFR1c to transduce FGF23 signals (35–38) as a model to determine whether the altered dimerization capacity of KLVs could affect protein-protein interactions. HEK 293 cells were co-transfected with KLWT or KLVs-GFP and EV or FGFR1c-V5. Lysates were collected and the interaction of KL and FGFR1c was assessed by co-immunoprecipitation. A GFP antibody was used to pull down KLWT or VS and a V5 antibody was used to pull down FGFR1c. After pull-down, the amount of co-receptor was assessed by separating the immunoprecipitated proteins on SDS-PAGE followed by Western blotting for the interaction partner. Immunoprecipitation with either member of the complex showed a decrease in interaction between KLWT and FGFR1c compared with KLVs and FGFR1c ($p < 0.05$) (Fig. 6, A–D). Immunoprecipitation with non-immune mouse IgG (mIgG) showed this was not due to nonspecific antibody interactions (data not shown). These data suggest that either the VS variant is a better binding partner for FGFR1 or that the KL monomer is needed to function as a FGFR co-receptor.

Increased FGFR interaction would be predicted to impact FGF23 signal transduction. To assess whether KLVs affects FGF signaling, HEK 293 cells were transfected with KLWT or KLVs. After transfection, recombinant FGF23 was added to media overlaying cells. The resulting lysates were separated by SDS-PAGE and analyzed for the levels of phosphorylated (pERK) and total ERK (tERK), a kinase that is activated downstream of FGF23 receptor binding. Similar to the increase in binding of KLVs to FGFR1c, there is also a significant increase in ERK activation as measured by pERK/tERK expression ($p < 0.01$) (Fig. 7, A and B). Together these data indicate that while trafficking of the VS variant may be distinct from the wild-type, the most profound alteration of this polymorphism would be in enhancing FGFR heterodimerization and thus FGF23 signaling.

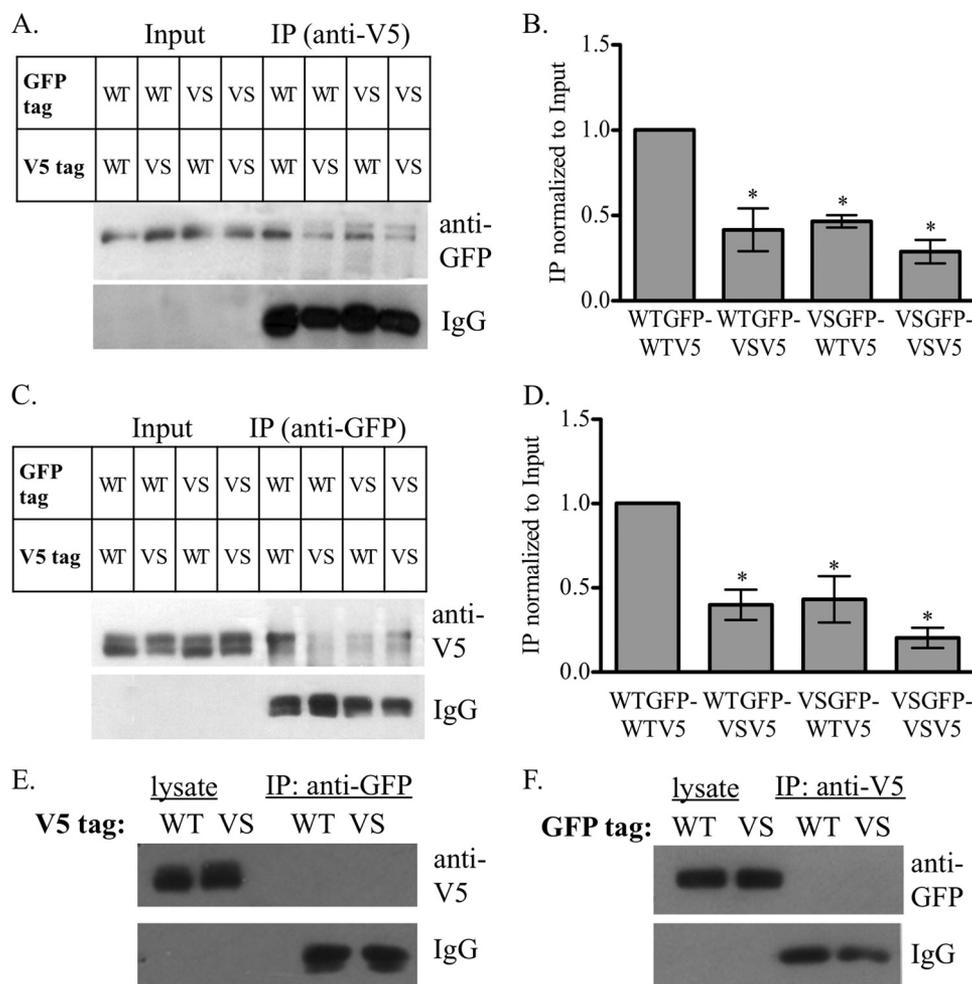


FIGURE 5. KL homodimerization is altered by the presence of the VS variant. HEK 293 cells were co-transfected with combinations of wild-type and/or VS KL containing either a V5 or GFP tag. Resulting lysates were assessed for differences in dimerization using co-immunoprecipitation (A) Western blot for GFP shows KL-GFP pulled down by interactions with co-expressed KLV5 variant through use of V5 beads. Non-precipitated lysate is shown as a control. B, bar graph depicting fold changes across multiple experiments in amount of GFP-tagged KL immunoprecipitated by V5 beads normalized to non-precipitated lysate. (Error bars are \pm S.E.; $n = 3$, $p < 0.05$, ANOVA). C, representative Western blot showing changes in pull-down of V5-tagged KL when associated with GFP-tagged KL. Non-precipitated lysate is shown as a control. D, bar graph depicting fold changes in amount of V5-tagged KL immunoprecipitated by GFP beads normalized to non-precipitated lysate (error bars are \pm S.E.; $n = 3$, $p < 0.05$, ANOVA). To assess whether there was any nonspecific pull-down between the tags used for co-immunoprecipitation, HEK 293 cells were transfected with either KL-GFP or KL-V5. E, resulting KL-V5 lysates were pulled down with GFP antibody and then were probed by Western blotting for presence of KL-V5 in the pull-down. No nonspecific pull-down of KL-V5 was seen. Non-precipitated KL-V5 lysate is shown as control. F, resulting KL-GFP lysates were pulled down with V5 antibody and then were probed by Western blotting for presence of KL-GFP in the pull-down. No nonspecific pull-down of KL-GFP was seen. Non-precipitated KL-GFP lysate is shown as control. The presence of KLV5 in KL dimers leads to an overall decrease in dimerization even if KLWT is present in the dimer.

DISCUSSION

In mice, KL deficiency results in a profound phenotype that shortens lifespan and dramatically impacts the normal function of nearly every organ system. To determine whether KL affects human lifespan and disease development, studies have focused on identifying polymorphisms that correlate with alterations in disease risk. Polymorphisms were identified in both coding and noncoding regions of the KL gene using either GWAS or by case studies identifying patients with unique mutations. One such case study identified the H193R polymorphism in a 13-year-old girl who presented with severe soft tissue and vascular calcifications and abnormalities in mineral homeostasis. The H193R polymorphism causes a decrease in the affinity of KL for FGF23 and FGFR1c (39). Although not a polymorphism, in a second case study KL was overexpressed due to a chromosomal translocation causing hyperphosphatemic rickets and

hyperparathyroidism (40). Although these dramatic changes in KL function are the result of rare occurrences, it is clear that extreme alteration of KL function is inconsistent with normal healthy life. GWAS have revealed more common alterations in KL that do not cause overt and disabling disease but do affect disease risk. Two of these variants, G395A in the promoter region and C1818T in exon 4 of KL, are associated with increased risk of cardiovascular disease, priapism, nephropathy, osteoarthritis, diabetes, and increased bone mineral density (41–49). Multiple studies have also shown an association of KLV5 with both longevity and disease risk. Although some do not report an association with VS, the disparity between these studies may be due to a number of differences including heterogeneity of the diseases and studied populations as well as different examined endpoints (25, 50–52). Overall the effects of these variants on disease risk suggest that further in-depth

Molecular Characterization of the KL VS Polymorphism

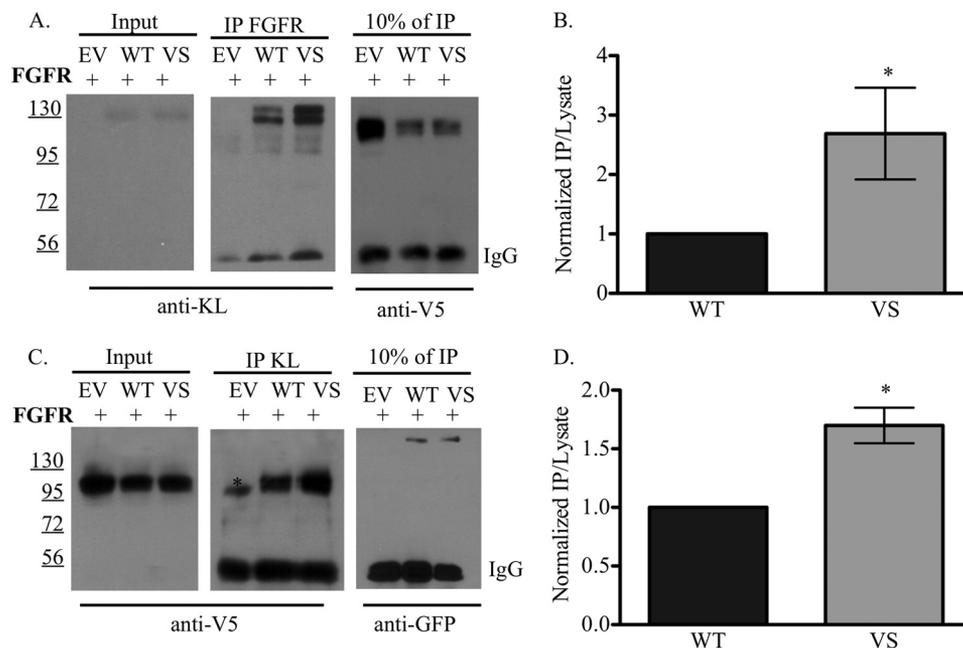


FIGURE 6. KLVS enhances interaction with FGFR1c. HEK 293 cells transiently transfected with FGFR1c-V5 (FGFR) and either empty vector (EV), wild type (WT), or mutant (VS) KL with (C-D) or without (A-B) a GFP tag. **A**, Western blot showing samples that were pulled down with V5 beads to isolate FGFR1c-V5 and probed for KL presence. Ten percent of IP was probed for FGFR-V5 presence to show equal immunoprecipitation. **B**, bar graph depicting fold change across multiple experiments in immunoprecipitation of KL normalized to IgG (error bars are \pm S.E.; $n = 3$, *, $p < 0.05$). **C**, Western blot of representative samples that were pulled down with GFP beads to isolate KL and probed for FGFR1c-V5 presence. Ten percent of IP was probed for KL presence to show equal immunoprecipitation (* marks nonspecific band seen in EV transfection). **D**, bar graph depicting fold change across multiple experiments in immunoprecipitation of FGFR1c-V5 normalized to IgG (error bars are \pm S.E.; $n = 3$, *, $p < 0.05$, **, $p < 0.01$).

examination of their actions may lead to a better understanding of KL function in humans.

Most of the GWAS studies examining the KLVS are focused on disease risk and do not attempt to examine mechanisms behind these risk changes. Consistent with our findings of altered shedding, the first study to examine mechanism indicated that the VS polymorphism does change the function of the protein (22). To do so, Arking *et al.* transfected the KL1 domain into HeLa cells and examined protein secretion. KLV led to a decrease in secretion while the expression of KLS and the combination of both substitutions led to an increase in secretion. This study also showed that the F352V substitution alone lead to a decrease in the enzymatic activity of KL1 through measuring the activity of a KL paralog with a corresponding amino acid substitution (22). However, the use of the KL1 domain alone is non physiologic as KL1 is only detected at the mRNA level and has been identified as a target for non-sense-mediated mRNA decay by NCBI (reference sequence: NM_153683.2).³ A second study examining KLVS mechanism also showed that the full-length KLV variant alone did not have the same growth inhibitory effect as KLWT in breast cancer cells (28).

We sought to confirm and expand upon the earlier studies to better understand the mechanism by which KLVS confers disease risk. To do so we generated full-length, transmembrane KL constructs containing the amino acid substitutions alone (V or S) and together (VS), as the full-length construct is the most

physiologically relevant form. Our initial assumption was that each individual substitution would contribute unique changes that were magnified or diminished when put together. As such, the F352V and C370S substitutions were anticipated to allow us to isolate the unique contribution of each substitution alone. Our results revealed that KLV did lead to alterations in shedding similar to that of KL1 (Fig. 2), which could be explained by the decreased half-life of the V variant (Fig. 3). Despite these changes in the individual variants, the F352V and C370S substitutions alone are not physiologically relevant because the changes observed individually are affected by intragenic complementation when the substitutions are together in the VS, as was also observed by Arking *et al.* (22). This suggests that the presence of the S variant is capable of correcting for the changes in processing the V variant causes.

Prior to our work, no functional changes have been described as unique to the KLVS isoform. In attempting to provide an answer as to why the KLVS would change protein function sufficient to alter disease risk we examined dimerization and FGF23 signaling. Dimerization is often necessary for proper trafficking and function of transmembrane proteins (31). Because KL is known to form dimers or higher oligomers in the blood and CSF (7, 8) it is plausible that the VS variant may lead to differences in dimerization, which could explain the alterations in function. This hypothesis was confirmed by the results in this study that show KLV leads to decreased monomers and increased dimers, while KLS and KLVS have increased monomers and decreased dimers (Fig. 5). This decreased homodimerization could be due to the loss of the cysteine at amino acid 370. Cysteines are known to be important for the formation of disulfide bonds, which are critical in

³Database, N. N. NM_153683.2. National Center for Biotechnology Information, U.S. National Library of Medicine 8600 Rockville Pike, Bethesda MD, 20894.

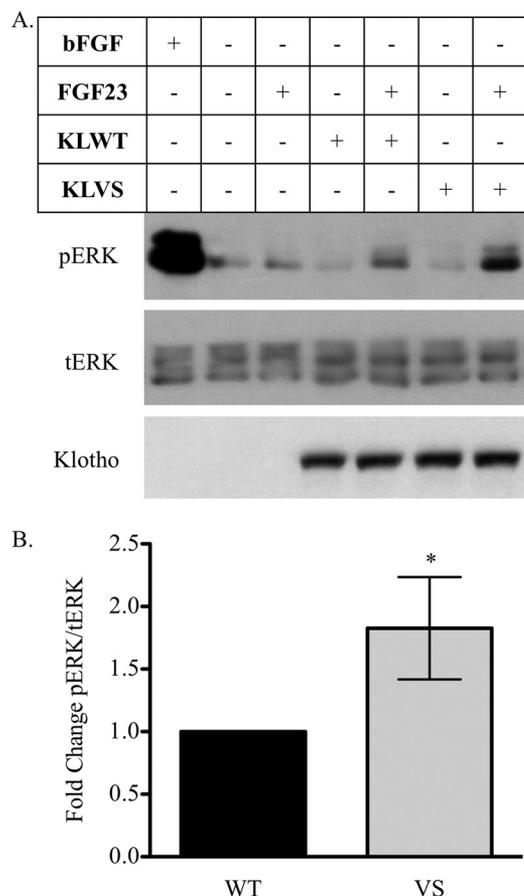


FIGURE 7. **KLVS enhances FGF23 signal transduction.** HEK 293 cells transiently transfected with KLWT or KLVS were treated with FGF23 to activate FGFR1c signaling. *A*, representative Western blot showing differences in ERK phosphorylation compared with total expression of ERK after transfection of KLWT or KLVS. *B*, bar graph depicting fold change across multiple independent experiments in ERK phosphorylation normalized to total ERK expression (error bars are \pm S.E.; $n = 3$, *, $p < 0.05$).

proper folding of proteins (54) and can play crucial roles in dimerization (55–58). In fact, the cysteine at amino acid 370 is predicted to form an intramolecular disulfide bond as determined by software designed to predict disulfide connectivity, DiANNA (59). The S substitution counteracts KLV's increase in dimerization, which leads to a correction of KLV aberrant shedding and half-life, but despite the normal processing of the VS variant, its dimerization is significantly different from that of KLWT. Co-immunoprecipitation studies analyzing the dimerization of KL to itself in the presence and absence of the VS variant suggest that this difference persists even when one molecule of KLWT is present in the dimer (Fig. 5). Thus the differences in disease risk seen between heterozygous and homozygous expressers of KLVS are not due to variances in dimerization. Interestingly, the presence of the higher glycosylated form of KL can be seen in the samples immunoprecipitated with V5 as well as in the lysate of the blot probed for V5. The increased presence of the more glycosylated form could be due to a difference in the recognition of glycosylated KL by the various antibodies. Overall these findings suggest that despite the correction of processing, the KLVS variant may still have altered function through altered protein-protein interactions.

KL interaction with FGFR as a co-receptor for FGF23 binding is the most well studied KL protein-protein interactions and is critical in proper kidney function (60, 61). The KL activity as an FGF co-receptor is vital to many of the hallmark functions of KL as suggested by the similarities of the KL $-/-$ and FGF23 $-/-$ mice (35) and drastic changes in KL ability to bind to FGFR can lead to devastating illness (H193R) (39). Thus, we decided to determine whether KLVS would alter interaction with FGFR1c and thus affect its subsequent FGF23 signaling cascade. The increased level of monomers observed with KLVS is associated with increased binding of KLVS and FGFR1c (Fig. 7). These results suggest that when KL is a homodimer, it is less accessible to FGFR1c resulting in fewer interactions between the two proteins. The decrease in dimerization seen with KLVS frees up more KL monomer that can then complex with FGFR1c leading to an increase in the number of interactions (Fig. 7). In line with the changes in binding, we found that KLVS also leads to an increase in downstream ERK activation. It is possible that the increase in FGF23 signaling which can lead to detrimental health effects such as hypophosphatemia, aberrant vitamin D metabolism, impaired growth and rickets/osteomalacia (62) is, at least, partially responsible for the increased disease risk found in homozygous carriers of KLVS. Hypophosphatemia, for example, has been linked to both cardiovascular disease and metabolic syndrome (53), which have both been associated with KLVS homozygosity (21, 23–25). However, our results do not explain why patients heterozygous for this polymorphism are often protected from disease, which could be due to a possible difference in the amount of change in FGF23 signaling or due to another yet undiscovered alteration in KL signaling caused by the VS polymorphism.

The opposite effects of KL homodimerization and FGFR1c binding (*i.e.* KL-FGFR1c heterodimerization) suggest that alternate dimerization states of KL may be important for different functions of the protein. While our results suggest the monomeric form of the protein may be critical for its transmembrane effects, the dimeric form may be necessary for proper circulatory functions of KL. This is suggested by evidence that the shed form of KL is often reported as a dimer or higher order oligomer (7, 8). It is also possible that KL dimerization regulates its transmembrane location therefore leading to the differences in FGF23 signaling. If dimerization is a signal for KL internalization and subsequent degradation, it could explain the increased dimerization and decreased half-life of KLV, which could lead to less KL on the plasma membrane where it is important for both shedding and FGFR1c co-receptor activities. This is a possible explanation for why KLVS has different effects on KL-KL dimerization compared with KL-FGFR1c interactions. Further studies could use the changes in dimerization of KLVS and other variants to determine which functions of KL may require dimerization and how dimerization may play a role in KL internalization.

In humans the KLVS polymorphism is the result of a collection of 6 SNPs that always occur together. Our study focused on the two SNPs that alter amino acids. We show alterations in shedding and trafficking but the effects are small overall and intragenic complementation may further minimize these effects *in vivo*. Despite the ability of intragenic complementa-

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tion to allow proper trafficking of the VS variant, we have identified for the first time, KLVs specific changes in KL function namely differences in the protein-protein interactions as illustrated by the dimerization and FGFR1c interaction and signaling experiments. Although we have identified clear KLVs changes, it is likewise possible that the four other SNPs contribute to disease risk in ways we have not appreciated. As the SNPs are near the intron/exon borders, splicing or splicing efficiency could be affected by the SNPs. A small decrease in proper splicing coupled to dimerization or other protein binding changes could compound to create a bigger problem than presently appreciated. Further studies are ongoing to determine other functions of KL that could be altered by the VS variant.

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