"Targeting receptor interactions with SVEP1, a circulating biomarker of longevity in humans", Nathan Stitziel

Research Plan

A. Specific Aims

Atherosclerotic cardiovascular disease (CVD) is a leading cause of death worldwide and increases in prevalence with age; beginning in early adulthood, the lifetime risk of developing CVD is 50% for men and 33% for women. CVD has remained a leading cause of morbidity and mortality despite effective lipid-lowering therapies, highlighting a need for non-lipid therapeutic targets. We previously discovered a highly conserved missense polymorphism (D2702G) in the gene *SVEP1* that associated with risk of coronary artery disease (CAD) at genome-wide significance in humans independent of plasma lipid concentrations. In a series of complementary genetic studies, we have demonstrated that SVEP1 is a causal factor for atherosclerosis in humans and mice. Our Mendelian randomization (MR) studies also suggest that SVEP1 is causally related to hypertension and type 2 diabetes, two additional diseases which increase in prevalence with age.

Several other studies additionally found that the circulating level of plasma SVEP1 positively associates with aging, that its levels accelerate with increasing age, and that increased plasma SVEP1 is causally associated with dementia. Genetic variation in SVEP1 is associated with longevity and recent study found that a genetic variant in the transmembrane protein LHFPL6 which associated with increased longevity also associated with decreased plasma SVEP1 levels in humans. Taken together, these data suggest that increased SVEP1 is a robust biomarker of aging, that it promotes multiple chronic diseases of aging, and that decreasing its levels may ameliorate these conditions, thereby promoting longevity.

SVEP1, also known as polydom, is an extracellular matrix protein that contains complement binding motifs, von Willebrand factor type A, EGF and EGF-like Ca2+ binding, and pentraxin domains, many of which are known to be involved in cell growth and differentiation. Its only published interacting protein is integrin α9β1 (Itgα9β1) yet our preliminary results found that depleting Itgα9β1 did not phenocopy SVEP1 depletion in a mouse model of atherosclerosis, suggesting the presence of additional receptor(s) that are responsible for mediating the effect of SVEP1 on vascular disease.

Our studies from Year 1 of funding found that SVEP1 is a high affinity ligand for Platelet and Endothelial Aggregation Receptor 1 (PEAR1), an orphan receptor tyrosine kinase-like protein expressed by platelets, endothelial cells (ECs), and vascular smooth muscle cells (VSMCs). SVEP1 physically interacts with PEAR1 and their binding results in robust AKT/mTOR activation. This finding is of particular interest since AKT/mTOR signaling plays a key role in the disease processes associated with SVEP1 and has been associated with cell senescence and vascular aging, thereby providing a parsimonious mechanistic link between SVEP1 and longevity. The overall goal of this proposal is to further characterize the role of SVEP1, thereby generating foundational preliminary data which will be used to pursue additional funding in addressing how SVEP1 may be targeted in myriad diseases of aging. This goal will be pursued through the following aims:

Aim 1. To determine which region(s) of SVEP1 and PEAR1 mediate their interaction. The full length recombinant SVEP1 and PEAR1 bind with high affinity (K_D=9nM). We hypothesize this interaction is mediated by one or more critical regions of each protein and that identifying these regions will generate hypotheses on how to therapeutically target this interaction. To test this, we will generate multiple hypothesis-driven variant constructs lacking different portions of the proteins which we predict will abrogate their interaction. We will produce these variant proteins in mammalian cells, purify them, and assess their ability to bind to their respective wild type partner using biolayer interferometry (BLI). We will use cellular assays to validate that the constructs lacking the critical domains do not activate AKT/mTOR signaling.

Aim 2. To develop a method for quantitatively measuring SVEP1 levels. SVEP1 is emerging as a robust biomarker of aging and multiple chronic diseases, yet there is no direct assay other than expensive multiplexed platforms such as SomaScan for quantitatively measuring its levels. The inability to accurately measure SVEP1 hinders efforts to fully study its role in aging and age-related diseases. In this Aim, we will use the minimal binding region of PEAR1 identified in Aim 1 to develop a quantitative binding assay for measuring SVEP1 levels. We will produce a biotinylated peptide of the minimal SVEP1 binding region of the PEAR1 ectodomain and mount it on a BLI biosensor optimized for quantification. We will validate the specificity of our approach using tissue lysates from *Svep1*$^{-/-}$ mice and its sensitivity by using a recombinant SVEP1 dilution series. As an alternative approach, we will iteratively enrich a commercially produced library of DNA aptamers to identify an aptamer that binds with high affinity and mount it on the BLI biosensor for quantification.

This proposal is directly relevant to LLF's mission which, in part, is to support the study of factors that either predict mortality and morbidity or influence longevity and health. Understanding how SVEP1 interacts with PEAR1 and developing a method to measure SVEP1 protein levels will advance our understanding of SVEP1's role in disease and potential therapeutic impact.

B. Scope of work and relevance of the project to the mission of the Foundation

As reviewed below in **Background and Significance**, a series of recent human proteomic studies have identified SVEP1 as a robust plasma biomarker of aging[1,2] along with multiple age-associated chronic diseases including atherosclerosis, hypertension, and diabetes[3,4], along with dementia[5] and glaucoma[6]. Since 1) circulating plasma levels of SVEP1 are associated with aging; 2) most SVEP1-associated diseases can be classified as hallmarks of vascular aging; and 3) our new preliminary studies have identified PEAR1 as a novel receptor for SVEP1; and 4) SVEP1/PEAR1 interaction activates the AKT/mTOR signaling pathway which is associated with cardiometabolic disease[7-10] and longevity[11], we believe that chronic mTOR activation in the vasculature provides the mechanistic link between SVEP1 and its promotion of vascular aging. In that context, the scope of work in the current proposal will dissect how SVEP1 interacts with PEAR1. Completing these aims will provide robust preliminary data with which we can apply for future funding to fully delineate the role of SVEP1 in myriad diseases of aging. Our work is highly aligned with the mission of the Foundation which is, in part, to support the study of factors that either predict mortality and morbidity or influence longevity and health.

C. Background and Significance

Atherosclerosis and myocardial infarction are the leading causes of death and disability worldwide[12]. The cost in dollars and impacted lives is staggering; of individuals older than 40, one in two men and one in three women will suffer a coronary event during their lifetime[13] and in 2020, coronary heart disease is predicted to cost the US economy >$200 billion[12].

Plasma low-density lipoprotein cholesterol (LDL-C) concentration is a causal risk factor for coronary artery disease (CAD) and lowering LDL-C has proven therapeutic benefit. For example, when combined with statin medications, PCSK9 inhibitors[14,15] reduce the absolute risk of recurrent CAD events from ~15% to ~13%. However, this indicates that many individuals on these two therapies – despite many participants achieving LDL-C ≤25 mg/dL – harbor significant residual risk of disease and highlights the critical need for additional therapies targeting non-lipid mechanisms that are causally related to disease.

Human genetics is a powerful approach to identify novel therapeutic targets[16]. To identify novel genes underlying coronary artery disease (**CAD**) and CAD risk factors, we previously led a large-scale association study of low-frequency protein altering variation across the genome[4]. We found a highly conserved missense polymorphism in *SVEP1* (p.D2702G, minor allele frequency = 3.6%) that associated with a significantly increased risk of CAD in humans (odds ratio for CAD = 1.14, $P = 4\times10^{-10}$). The 2702G allele (which associated with increased risk of CAD) did not have any association with plasma lipids but was associated with increased blood pressure and risk of T2D, suggesting that *SVEP1* may represent a novel, non-lipid gene for CAD.

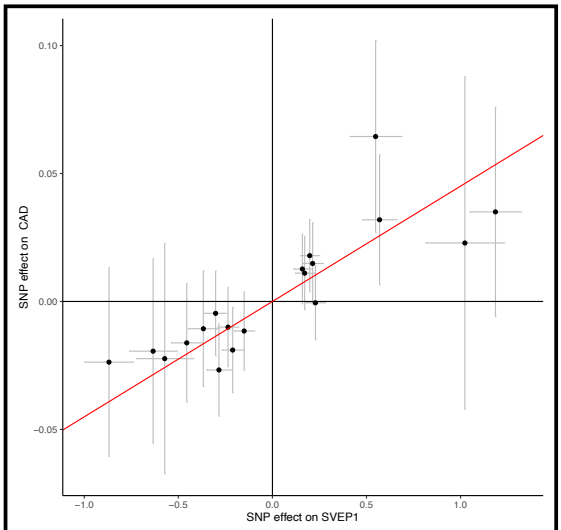

Figure 1. SVEP1 protein levels are causally associated with CAD in humans. Estimated effect (with 95% confidence intervals) of each variant in the SVEP1 Mendelian Randomization analysis is plotted for protein level and CAD risk. Red line indicates the causal effect estimate ($P = 7 \times 10^{-11}$).

Despite a robust statistical association, however, it was unclear if *SVEP1* was the causal gene in this locus. To generate additional human evidence linking SVEP1 with CAD, we first wondered if the 2702G allele that associated with increased disease risk might associate with altered SVEP1 levels. Although we did not find evidence that 2702G (or other alleles in linkage disequilibrium with 2702G) were significantly associated with levels of *SVEP1* mRNA (https://gtexportal.org), we did find that the 2702G risk variant was associated with a significant increase in circulating plasma SVEP1 protein levels ($P = 8\times10^{-14}$) in the INTERVAL study[2], suggesting that increased SVEP1 protein levels were associated with increased risk of CAD. To determine if this was true for other genetic variants influencing SVEP1 protein levels, we used data from the INTERVAL study[2] to identify 19 *cis*-acting variants that independently associated with SVEP1 protein levels at a genome-wide level of statistical significance ($P < 5\times10^{-8}$). We estimated each variant's effect on risk of CAD using summary statistics from a meta-analysis of CARDIoGRAMPlusC4D and UK Biobank[17]. Using these data, we performed a two-sample Mendelian Randomization[18] analysis and found that increased SVEP1 protein levels were causally related to increased CAD risk ($P = 7\times10^{-11}$; **Fig. 1**). We also found that SVEP1 protein levels were causally related to both hypertension ($P = 2\times10^{-15}$) and type 2 diabetes ($P = 0.0004$) as summarized in our recent publication[3].

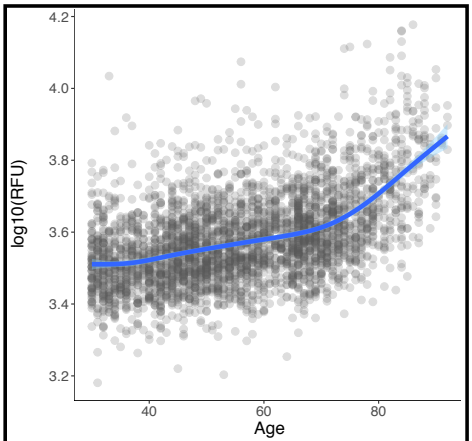

Figure 2. Plasma SVEP1 levels increase with age in humans. Log10 (relative units) of SVEP1 from Lehallier et al.[1] are plotted according to age. The loess regression estimate for age and SVEP1 level ($P = 4.2\times10^{-114}$) is shown in blue.

In addition to an association with CAD, we also observed that plasma SVEP1 level was highly significantly and positively associated with age ($P = 9.6\times10^{-22}$) in the INTERVAL study (Supplementary Table 2 in Sun et al.[2]). This association was replicated in a recent plasma proteomic study[1] which also found that plasma SVEP1 level was significantly associated with overall age ($P = 4.2\times10^{-114}$). Beyond an association with overall age, SVEP1 was the **topmost associated protein** in an analysis of proteins that accelerate with increasing age (Figure 3d in Lehallier et al.[1] and **Fig. 2**).

Increased plasma SVEP1 also associates with decreased parental longevity (Figure 3a in Perrot et al.[19]) which appears to be a causal relationship based on their Mendelian Randomization analysis[19]. The association with longevity is supported by a recent genome-wide association study of 1,320 centenarians which found a genetic variant in *LHFPL6* that significantly associated with both increased longevity and decreased plasma levels of SVEP1[20]. In addition to the disease associations with atherosclerosis, hypertension, and diabetes, genetic variation in *SVEP1* associates with glaucoma[6], and a recent plasma proteomics study in humans finds that SVEP1 levels are also causally related to risk of dementia[5].

Why do plasma SVEP1 levels so robustly associate with age? Although we do not yet know the source of circulating plasma SVEP1, we have shown that *SVEP1* is expressed in human arterial tissues and that it is induced in the vascular wall under atherogenic conditions[3]. Other extracellular matrix proteins can be detected in the plasma of patients with atherosclerotic disease, suggesting that plasma concentrations of these proteins may reflect tissue concentrations and atherosclerotic remodeling[21,22]. The Mendelian Randomization results from us and others summarized above suggest that the plasma levels of SVEP1 represent a causal factor in these diseases and not just a correlative observation. Thus, the available evidence suggests that increased SVEP1 plays a causal role in the development of multiple chronic diseases which increase with age; these increases in risks of disease may induce the robust association between SVEP1 and overall age. We believe the causal influence of SVEP1 on multiple age-associated chronic diseases also explains the causal association that is observed between increased SVEP1 levels and decreased longevity[19].

What role might plasma SVEP1 play in these age-associated diseases? Although we do not know what functions circulating plasma SVEP1 might have, vascular dysfunction and vascular aging is a unifying theme across the SVEP1-associated diseases. Our preliminary data showing that SVEP1 can activate mTOR signaling within endothelial cells and vascular smooth muscle cells may provide the link between the circulating protein and these vascular diseases. Completing the aims of the current proposal will define how SVEP1 interacts with the novel receptor PEAR1, thereby generating testable hypotheses on how it influences age-associated diseases, longevity, and ways to therapeutically target these interactions.

D. Preliminary data and progress in first year of LLF funding

SVEP1 is a secreted protein with multiple domains involved in cell growth and proliferation. SVEP1 (**S**ushi, **V**on Willebrand factor type A, **E**GF and **P**entraxin domain containing **1**), also known as polydom for its many (poly) domains, is a large (>300 kDa) protein. Several domains within SVEP1 are known to play critical roles in cell growth and proliferation, including Von Willebrand factor type A, EGF-like, Laminin-G, and integrin binding domains. SVEP1 is highly expressed in the bone marrow[23] and placenta[24] and has been shown to be critical for epidermal differentiation and lymphatic development[25,26]. It is cleaved after secretion into two products[27] and its only known binding partner is integrin α9β1 (Itgα9β1)[27].

Itgα9β1 does not mediate the pro-atherogenic effect of SVEP1. Our previous investigation of SVEP1 raised the possibility that it might interact with Itgα9β1 on vascular smooth muscle cells (VSMCs) and myeloid cells to promote atherosclerosis[3]. To determine if Itgα9β1 was indeed responsible for mediating the pro-atherogenic effect of SVEP1, we generated atheroprone animals with tissue specific Itga9 depletion in VSMCs and myeloid cells. Unexpectedly, we did not observe any differences between genotype groups in the size or complexity of atherosclerotic plaques in either model (data not shown due to space limitations), raising the likely possibility of another cellular receptor that is responsible for SVEP1’s impact on vascular disease.

PEAR1, an orphan cell surface receptor, is a trans-pQTL for SVEP1. To identify possible additional interacting proteins, we performed a protein quantitative trait locus (pQTLs) analysis of SVEP1 levels by conducting a genome-wide association study of the human plasma proteomic data from the INTERVAL study[2].

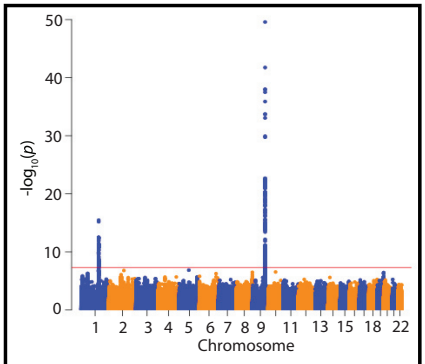

Figure 3. Manhattan plot of genome-wide association study for plasma levels of SVEP1. The p-value for each SNP’s association with SVEP1 level is plotted according to genomic location. Red line indicates genome-wide level of significance ($P<5x10^{-8}$).

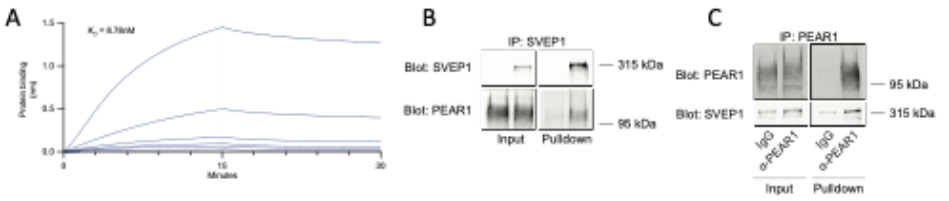

Figure 4. SVEP1 and PEAR1 physically interact. (A) Biolayer interferometry sensograms. A dilution series of SVEP1 was analyzed by sensors loaded with PEAR1ECD. The dashed line represents the end of the association step and the beginning of the dissociation step. (B,C) Immunoblots of the indicated proteins after co-immunoprecipitation. Negative controls included no SVEP1 (B), or non-specific IgG (C).

Genetic variation in two loci associated with SVEP1 levels at genome-wide significance (**Fig. 3**). The strongest signal for SVEP1 levels was a cis-pQTL in the SVEP1 locus on Chromosome 9. The other notable significant association with SVEP1 levels was a trans-pQTL in the Platelet and endothelial aggregation receptor 1 (*PEAR1*) locus on Chromosome 1. PEAR1 is an orphan receptor tyrosine kinase-like protein that is highly expressed by both platelets and endothelial cells. Although the ligand for PEAR1 has not been reported[28], the finding that genetic variation in this cell surface receptor is associated with altered levels of SVEP1 suggests the two proteins may interact.

SVEP1 and PEAR1 interact with high affinity. We tested the hypothesis that PEAR1 influences SVEP1 levels due to a physical interaction by using biolayer interferometry (BLI) to perform label-free, protein-binding analysis between recombinant PEAR1 and SVEP1. A global fitting of kinetic curves from a dilution series of SVEP1 (2.5-80nM) yielded a calculated K_D of 8.78 ± 0.03nM (**Fig 4A**). The extracellular domain (ECD) of PEAR1 (PEAR1ECD) also co-immunoprecipitated with recombinant, Myc-tagged SVEP1 in pulldown assays (**Fig 4B**). Reciprocally, SVEP1 coimmunoprecipitated with the PEAR1ECD (**Fig 4C**). These results suggest SVEP1 and PEAR1 physically interact with an affinity similar to tyrosine kinases and their ligands[29].

SVEP1 signals through PEAR1 to activate AKT signaling. Upon dimerization and activation, PEAR1 is phosphorylated by a Src family kinase (SFK)[30,31]. To test if SVEP1 induces PEAR1 activation and phosphorylation, we exposed human umbilical vein ECs (HUVECs) to immobilized bovine serum albumin (BSA, a nonspecific negative control protein), immobilized SVEP1, or soluble PEAR1 polyclonal antibody (pAb, a positive control)[31,32]. Immunoblots revealed a robust phospho-tyrosine signal corresponding to 140kDa, the expected mass of PEAR1, after pulling down PEAR1 from lysates of cells exposed to SVEP1 and PEAR1 pAb but not BSA (**Fig 5A**); this result is consistent with activation of PEAR1 by SVEP1[30]. AKT signaling was also activated upon exposure to SVEP1 and PEAR1 pAb (**Fig 5B**) relative to BSA controls. Together, these findings are consistent with the hypothesis that SVEP1 signals through PEAR1 to activate AKT. To directly test this hypothesis, we performed transient PEAR1 knockdown in human coronary artery smooth muscle cells (hCASMCs) using small-interfering ribonucleic acid (siRNA), since hCASMCs express PEAR1 and are readily transfectable with siRNA. Cells treated with *PEAR1* siRNA had diminished PEAR1 protein levels compared to negative controls (**Fig 5C**) and were unable to activate AKT upon exposure to SVEP1 and PEAR1 pAb. Serum-containing media was able to activate AKT signaling regardless of siRNA treatment however, demonstrating an intact AKT signaling axis. These data demonstrate that SVEP1-induced AKT signaling is dependent on PEAR1.

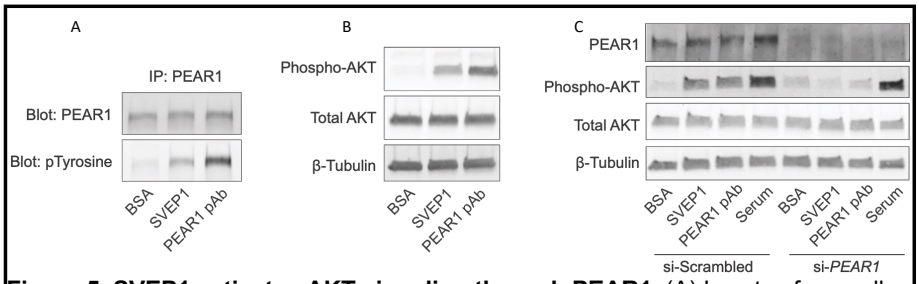

Figure 5. SVEP1 activates AKT signaling through PEAR1. (A) Lysates from cells exposed to BSA, SVEP1, or soluble PEAR1pAb for 15 min. PEAR1 was immunoprecipitated prior to immunoblotting for PEAR1 and pTyrosine. (B) Immunoblot of cell lysates from same conditions as (A). (C) Cells were transfected with scrambled siRNA or anti-*PEAR1* siRNA prior to exposure to listed stimuli.

SVEP1 and PEAR1 activate downstream mTOR signaling. AKT is a central regulator of numerous signaling pathways; however, little is known about which pathways downstream of AKT are activated by PEAR1. We screened for AKT-related pathways that may be influenced by SVEP1/PEAR1 signaling using an AKT pathway phospho-array and found that multiple phospho-proteins in the mammalian target of rapamycin (mTOR) signaling pathway were elevated after cells were

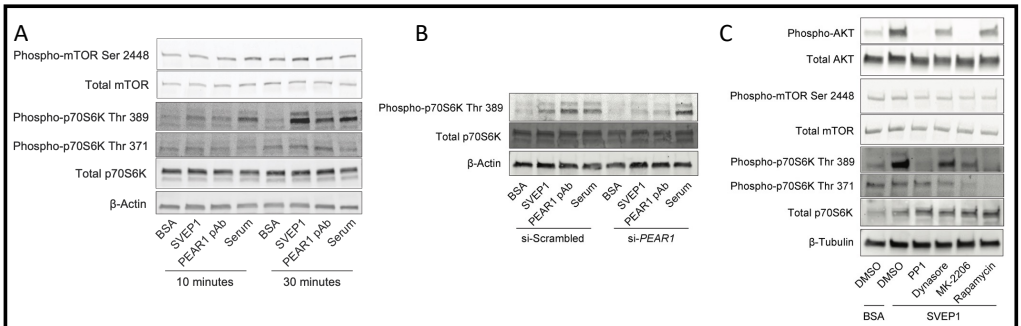

Figure 6. mTOR signaling is activated by SVEP1-induced PEAR signaling. (A) Immunoblots for the indicated proteins were performed on lysates from HUVECs exposed to immobilized BSA, SVEP1, soluble PEAR1 pAb, or serum for 10 or 30 min. (B) Immunoblots for the indicated proteins were performed on lysates from hCASMCs transfected with scrambled siRNA or anti-*PEAR1* siRNA prior to exposure to the listed stimuli. (C) Immunoblot assays for the indicated proteins after cells were pretreated with indicated agents (see text for details) prior to exposure to BSA or SVEP1.

exposed to SVEP1 for 30 minutes (screening data not shown due to space limitations). Immunoblot assays of phosphorylated mTOR (Ser 2448) and the mTOR-regulated residue Thr 389 of p70S6K[33] further support an activation of mTOR signaling by SVEP1 (**Fig 6A**). Phosphorylation of p70S6K on residue 389 was also increased by the PEAR1pAb and serum after 30 minutes of exposure (**Fig 6A**). Transient knockdown of PEAR1 by siRNA abrogated mTOR activation by SVEP1, as determined by immunoblot assay of p70S6K phospho-Thr 389 relative to total p70S6K (**Fig 6B**). Small molecule inhibitors of SFK (PP1), endocytosis (Dynasore), AKT (MK-2206), and mTOR (Rapamycin) were added to cells prior to SVEP1 exposure to test whether SVEP1-induced AKT/mTOR signaling was dependent on the respective protein or cell process. Activation of AKT by SVEP1 was completely abrogated by inhibition of SFK and AKT and partially abrogated by endocytosis inhibition. Phosphorylation of p70S6K Thr 389 was dependent on SFK and mTOR and partially dependent on endocytosis and AKT (**Fig 6C**). Taken together, these data suggest that activation of PEAR1 by SVEP1 induces AKT and downstream mTOR signaling.

E. Research Design and Methods

Aim 1. To determine which region(s) of SVEP1 and PEAR1 mediate their interaction.

Rationale and overall strategy: Our preliminary data find that SVEP1 activates AKT/mTOR signaling by activating PEAR1. To begin an approach on understanding how a large protein like SVEP1 activates the cell surface receptor PEAR1, we first need to understand which regions of the proteins mediate this interaction. These results will be critical if we are to move toward therapeutically inhibiting the interaction of SVEP1 with PEAR1. We will address this by generating variant SVEP1 proteins to determine their ability to bind to PEAR1 and induce downstream signaling. These studies will provide insight into the contribution of specific domains to receptor affinity and SVEP1 function and test which regions/interactions of the proteins may be strategically targeted for therapeutic intervention.

Specific techniques: Generating SVEP1 variant constructs. We have successfully generated a wild type *SVEP1* cDNA construct in an expression vector containing a C-terminal Myc and poly-His tag to enable both antibody identification and protein purification. Using site directed mutagenesis, we have also generated a cDNA construct containing the human SVEP1 CAD risk variant (2702G) and have found that this allele does not alter mRNA expression or protein secretion (data not shown). We have expressed both SVEP1 variants in 293-F eukaryotic cells and purified the recombinant proteins on an IMAC affinity column followed by additional purification with a Superose 6 size exclusion column. The purity of the protein was evaluated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) 4-15% stained with Coomassie brilliant blue. More of the 95% of the protein in the gel corresponded to a single high molecular weight band (**Fig 7A**). Further analysis by mass spectrometry found that >95% of the peptides detected corresponded to SVEP1.

To address which portions of SVEP1 are critical for PEAR1 binding, we will generate several SVEP1 variant constructs (**Fig 7B**). The variant proteins will be isolated from 293-F cells and will contain a C-terminal Myc and poly-His tag to enable antibody identification and purification. We use this eukaryotic cell expression system to retain post-translational modifications needed for protein function. Protein concentration and purity will be determined using a bicinchoninic acid (BCA) assay and Coomassie stain after SDS-PAGE.

Construct 1, which we have already generated, is the wild type SVEP1 variant which binds to PEAR1 with high affinity and will serve as a control for the subsequent experiments. SVEP1 is cleaved into two products after secretion. Although a prior study found that the two products appear to retain physical interaction[27], it is not clear which product is responsible for receptor binding or if retaining the physical interaction of the two products is needed. We will address these questions by producing the naturally occurring N-terminus and C-terminus cleavage products in isolation using Constructs 2 and 3, respectively. The C-terminus product does not contain the native signal peptide so we cloned this construct into a pSecTag2 vector (Invitrogen). It is also unknown if cleavage is necessary to activate SVEP1 and we will test this by creating Construct 4 in which we

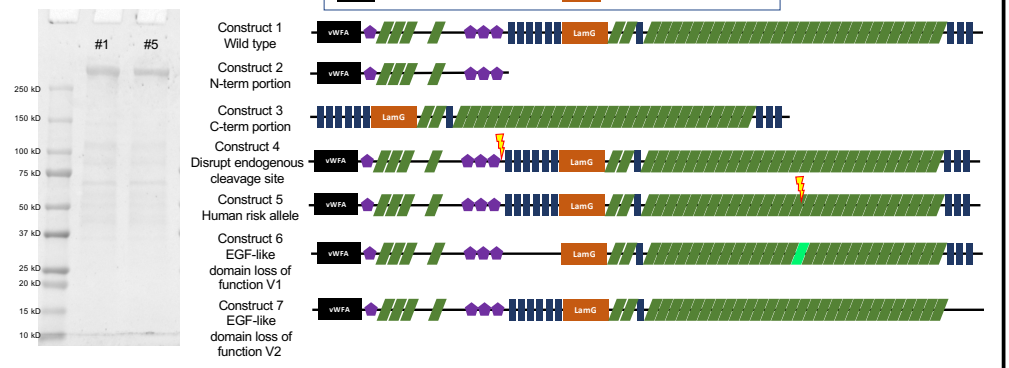

Figure 7. Variant SVEP1 constructs that will be used in Aim 1. (**A**) Coomassie-stained gel of protein preparations from Constructs #1 and #5 demonstrating high purity of the expected molecular weight band for each. High purity (>95%) was confirmed by mass spectrometry. (**B**) Constructs that will be generated for the functional assays in Aim 1 (see text for details).

will alter the previously mapped cleavage site[27] to disrupt endogenous protein cleavage. Construct 5, which has been generated, will be used to study how the human CAD risk allele 2702G impacts SVEP1 function. Construct 6 will contain a deletion of the first six repeat EGF domains while Construct 7 will delete the last three. We believe one or both of these regions may be needed to bind PEAR1 since the entire PEAR1 ectodomain is comprised of repeat EGF domains.

Binding assays with variant proteins. We will test the binding affinity of the six variant SVEP1 constructs (with Construct 1 serving as the control) for wild type PEAR1 using BLI as in Fig. 4. Using cellular assays, we will validate that any construct which does not bind PEAR1 will be unable to induce AKT/mTOR. In parallel to the constructs above, we will systematically delete EGF domains from the PEAR1ECD to determine the minimal SVEP1 binding region of the PEAR1 ectodomain.

Aim 2. To develop a method for quantitatively measuring SVEP1 levels.

Rationale and overall strategy: SVEP1 is emerging as a robust biomarker of aging and multiple chronic diseases, yet there is no direct assay for quantitatively measuring its levels other than expensive multiplexed platforms (e.g. SomaScan) which hinders efforts to fully study its role in aging and age-related diseases. In this Aim, we will develop a quantitative binding assay which will allow us to measure tissue levels of SVEP1.

Specific techniques: PEAR1-based quantification assay. We will use the minimal SVEP1 binding region of the PEAR1 ectodomain to develop a quantitative SVEP1 assay. First, we will create a cDNA construct of the binding region that contains a C-terminal bio domain (as in the PEAR1ECD construct above) which will be co-transfected in 293-F cells with a secretedBirA-8his plasmid to enable biotinylation of the bio domain. We will mount the biotinylated PEAR1 fragment to streptavidin biosensors and confirm high affinity binding to SVEP1 using BLI (as in Fig 4). We will assess specificity by measuring binding affinity in cell lysates from murine embryonic fibroblasts (MEFs) derived from *Svep1*$^{-/-}$ embryos. We will assess sensitivity by measuring binding affinity across a dilution series of recombinant SVEP1. The biotinylated PEAR1 fragment will be mounted to biosensors optimized for quantification on our BLI instrument. A standard curve will be generated from a dilution series of recombinant SVEP1 and we will validate our assay by spiking in varying amounts of recombinant SVEP1 protein to cell lysates harvested from *Svep1*$^{-/-}$ MEFs.

Aptamer-based quantitation assay. As a complementary approach for developing a quantitative SVEP1 assay (and in the event that the PEAR1 fragment is difficult to identify or not specific to SVEP1), in parallel we will pursue an aptamer-based approach. In this approach, we will obtain a commercially available library of ssDNA aptamers (Integrated DNA Technologies, Coralville IA). We will use Pierce Anti-c-Myc magnetic beads with our recombinant SVEP1 construct to perform multiple rounds of systematic evolution of ligands by exponential enrichment (SELEX) using published techniques[11]. Once an aptamer has been identified, we will produce a biotinylated version to mount on biosensors and validate as above.

Plans for obtaining additional extramural funding arising from the proposed research

We plan to use the results generated from this proposal to apply for NIH funding (R01 and/or R21) which will focus on validating the disease relevance of the novel receptor/ligand interaction we discovered in the first year of LLF funding. Although we will not be able to complete these studies in the timeframe of the current proposal, we have recently obtained the Pear1$^{-/-}$ mouse and have begun breeding these animals with Apoe$^{-/-}$ mice to study the effects of Pear1 deficiency in a mouse model of atherosclerosis. Based on our negative Itgα9β1 atherosclerosis study and the role of AKT/mTOR signaling in atherosclerosis[8], we believe depleting *Pear1* will phenocopy *Svep1* depletion, thus confirming the therapeutic relevance of their interaction. Biophysical protein and molecular signaling studies will be proposed to clearly define the mechanisms by which SVEP1/PEAR1 signaling axis influences endothelial and vascular smooth muscle cell biology and pathophysiology.

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