De novo design of a biologically active amyloid

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ABSTRACT

We report on the *de novo* design of a biologically active amyloidogenic peptide that inhibits VEGFR2 function and reduces VEGFR2 dependent tumor growth in mice xenografts upon intravenous administration. This peptide, consisting of a tandem repeat of an amyloidogenic fragment of VEGFR2, forms prefibrillar oligomers, protofibrils and mature fibrils, penetrates cells and seeds the aggregation of VEGFR2 by direct interaction. Our results therefore show that a short amyloidogenic protein fragment can induce the aggregation of a protein normally not associated to amyloidosis in a manner that recapitulates key biophysical and biochemical characteristics of natural amyloids. In addition we find that amyloid toxicity is observed only in cells that both express VEGFR2 and are dependent of VEGFR2 for survival. Thus rather than being generic, amyloid toxicity here appears to be both protein specific and conditional, i.e. determined by VEGFR2 loss-of-function in a biological context where target protein function is essential.

INTRODUCTION

Amyloid aggregation of proteins is driven by short amyloidogenic sequence segments within a protein chain (*1, 2*) that have the potential to self-assemble into β-sheet ribbons to form the characteristic cross-beta structured spine of amyloid structures (*3, 4*). It has been showed that most proteins do in fact possess such amyloidogenic sequence segments (*5, 6*). Still, only about 30 human proteins are known to be involved in amyloid-associated diseases (*7, 8*). Moreover it is still not clear what determines amyloid toxicity in these diseases (*8, 9*). Here we investigate whether an endogenously expressed protein that possesses amyloidogenic potential but aggregates neither under normal nor pathological conditions, can be induced to do so by seeding with a peptide consisting of an amyloidogenic fragment of its own sequence. The use of amyloidogenic fragment peptides is motivated by the observation that aggregation of disease–associated amyloidogenic proteins can be seeded by such peptides *in vitro* (*10, 11*) and that truncations of amyloid proteins have been associated with increased seeding potential *in vivo* (*12, 13*). Moreover it has been shown that amyloidogenic peptides and proteins are generally much more efficient at seeding aggregation of homotypic sequences (*14-16*) although examples

of cross seeding do exist (*17-19*). Indeed, seeding of protein aggregation *in vitro* appears to work universally, and fits with the structural model of aggregation as the addition of new strands to a growing amyloid fibril (*8*). This imparts sequence specificity to the seeding process as the incorporation of non-homologous sequences into the highly ordered in-register stacking of identical side chains in the fibril core is likely to be energetically disfavoured (*13, 20, 21*). The seeding concept appears to hold true both in cell culture and *in vivo*, even for non-prion aggregation-associated peptides and proteins, which have hence been called prionoids (*22*). As a target protein we choose VEGFR2 as the function of this protein is well characterized. To ensure efficient seeding we designed an amyloidogenic peptide termed vascin consisting of a tandem repeat of an amyloidogenic sequence segment in the VEGFR2 signal peptide.

We find that vascin is a *bona fide* amyloidogenic peptide that forms mature cross-beta fibrils along with prefibrillar intermediates including soluble oligomers and protofibrils. Moreover we find that the peptide is able to enter cells and reach the cytoplasmic compartment and specifically induce the aggregation of endogenous VEGFR2 thereby inhibiting its function in Human vein endothelial cells (HUVEC) *in vitro* and reducing VEGFR2-dependent tumor progression *in vivo*.

It remains unclear what determines amyloid toxicity in amyloid diseases and whether cell death results from a consequence of direct amyloid toxicity (gain-of-function) or whether it is a consequence of loss-of-function (*23, 24*). However, our detailed understanding of real amyloid disease models is often insufficient to address these questions directly. Indeed for many disease associated amyloidogenic proteins –such as the Aβ peptide in Alzheimer disease (*25*) and α-synuclein in Parkinson disease (*26*)- we still have insufficient understanding both of their physiological role as well as the cellular interactions of the amyloid conformation in disease (*26, 27*). We do however have a better understanding of the structural and biochemical characteristics that are common to most amyloid diseases. These features include the cross-beta structural organization of the spine of the amyloid fibrils formed by short segments of the sequence, the population of prefibrillar intermediates including soluble oligomers and protofibrils, and the capacity of amyloids to seed aggregation of the native conformation. While our artificial amyloid model recapitulates these

key structural and biochemical features of natural amyloids, it is also simple enough to investigate the relationship between protein loss-of-function and amyloid toxicity.

Our results show that vascin amyloids are not inherently toxic but that the emergence of amyloid toxicity is dependent on biological context. Vascin is not toxic to cells that do not express VEGFR2 or to cells expressing VEGFR2 but that are not dependent on VEGFR2 function. However when introduced in VEGFR2 dependent cells we find association of vascin amyloid toxicity and VEGFR2 loss-of-function. Our model system therefore demonstrates that amyloidogenic protein fragments can induce aggregation of non amyloidogenic proteins and that under these conditions amyloid gain of function is a phenotypic effect resulting from cell context specific loss-of-function.

RESULTS

Design of vascin, an amyloidogenic peptide derived from a VEGFR2 fragment

We analyzed the VEGFR2 receptors from mouse and human using the statistical thermodynamics algorithm TANGO (*28*) (Suppl Fig 1A). Since we envisaged testing the sequence ultimately in a mouse model, we opted for maximal compatibility with this version of the protein. Moreover, the two homologs share 84% sequence identity overall and 90% in the tango regions. To derive peptide sequences that are likely to form amyloid structure in isolation, but also have a high potential for forming soluble oligomers (*29*), we devised a strategy (*30, 31*) that makes use of a sequence feature of functional amyloids and yeast prions, which often contain several aggregation prone segments (APR) (*32*) closely connected by disordered regions (*33*). Hence we placed two ARPs in a peptide, separated by a rigid Pro-Pro linker, mimicking these repeat patterns. In order to maintain colloidal stability and solubility of the sequence, we supercharged the peptides by flanking the APRs with either negatively charged aspartate or positively charged arginine residues. Given the length limitations imposed by the efficiency of solid phase peptide synthesis, this design scheme imposes a length limitation on the APRs of 7 amino acids. Hence we selected 10 such high scoring sequences (Suppl Table 1) and generated the 38 peptide sequences listed in table 2, which explore both tandem repeats of the same APR as well as fusions of different APRs (Suppl Table 2). Peptides were screened for their ability to inhibit VEGF signalling in Hek293 cells transfected with mouse VEGFR2. To this end,

cells were treated overnight with an apparent concentration of 20 μM peptide (assuming 100% synthesis efficiency) and ERK phosphorylation was determined after stimulation for 5 min with 50 ng/mL VEGF (Suppl Fig 1B). At this concentration, we observed inhibition only with 2 peptides (B8 and B12). The effect was most pronounced with the peptide B8, which was based on a tandem repeat of the first high scoring aggregation prone region in the sequence that derives from the signal peptide and has the sequence $L_6AVALWF_{12}$ (Supplementary Figure 2A), resulting in the sequence DLAVALWFDPPDLAVALWFD (pI = 3.38, M.W. = 2272.15 Da). We termed this peptide vascin (Supplementary Figure 2B), obtained additional material by solid phase peptide synthesis followed by HPLC purification (Supplementary figure 2B), and confirmed its identity by mass spectrometry (observed mass: 2272.4, supplementary figure 2D).

Vascin forms soluble beta-structured oligomers that mature into amyloid fibrils

To determine the amyloidogenic nature of the peptide, vascin was dissolved to a final concentration of 300 μM in 1% (w/v) ammonium bicarbonate in ddH_2O. After 24 hours incubation Transmission Electron Microscopy (TEM) revealed typical amyloid fibrils of about 10 nm in width consisting of protofilaments of 4-5 nm (Figure 1A-D and Supplementary Figure 3). Additionally, vascin fibrils bind amyloid sensor dyes, including Thioflavin-T and the amyloid specific oligothiophene h-HTAA (*34*) displaying the characteristic emission spectra of beta amyloids (Figure 1E). X-ray diffraction of aligned bundles of vascin fibrils confirmed their cross-beta nature, with characteristic diffractions at 4.7 and 10.0 Å (Figure 1F). Together these data confirm the amyloidogenic nature of vascin. In order to follow amyloid formation kinetics we filtered dissolved 300 μM vascin 1% (w/v) ammonium bicarbonate in ddH_2O through a 0.2 μm regenerated cellulose filter and monitored particle size distribution using electrospray ionisation-mass spectrometry linked to ion mobility spectrometry (ESI-IMS-MS)(*35*) (Figure 1G) and Dynamic Light Scattering (DLS) (Figure 1H). At time zero, the MS showed a mixture of monomers and multimers up to X (Figure 1G), whereas the particle sizes visible in the DLS ranged from 5-100 nm (assuming linear polymer particles). After 6 h particles reached sizes over 1 μm (Figure 1H). This indicates that the filtered vascin solution contains soluble

oligomeric aggregates already at time zero. The fact that no lag phase was observed in the DLS autocorrelation function (Supplementary Figure 4A & B) further suggests that these soluble oligomers are able to directly proceed to amyloid fibril formation. In order to probe the secondary structure of these soluble aggregates we monitored the same aggregation kinetics using Fourier Transform Infrared Spectroscopy (FT-IR). The spectrum at time zero was dominated by maxima near 1630 and 1690 cm^{-1} (Figure 1I), characteristic of β-sheet structure. Over the following 6 h the intensity of these peaks increased markedly while the center of the peaks shifted gradually to 1622 and 1692 cm^{-1}, respectively (Figure 1I&J). These peaks were quite narrow and regions outside the peaks showed very little absorption, suggesting that most of the peptide sequence is involved in β-structured hydrogen bonding. FT-IR kinetics therefore indicate that vascin largely adopts a β-structured conformation upon solubilization in 1% bicarbonate and that these species mature into amyloid fibrils over time. Finally we measured binding to 8-Anilino-1-naphthalenesulphonic acid (ANS) during vascin amyloid formation (Supplementary Figure 4C), revealing high binding of this dye at time zero. The latter is a typical feature of interaction-prone cytotoxic prefibrillar oligomers that present a high degree of solvent exposed hydrophobic surface (*36*). The binding of ANS increased over time, suggesting hydrophobic surfaces stay exposed upon fibril formation. Given that the above observations were made at 300 μM peptide where the signal to noise ratio for biophysical characterisation is optimal, we also verified that vascin readily formed amyloid fibrils at the lower concentration of 30 μM (Supplementary Figure 5). Compared to vascin a scrambled version of the peptide (Supplementary Note 1) formed small soluble aggregates with hydrodynamic radii smaller than 100 nm by Dynamic Light Scattering, DLS (Supplementary Figure 6A&B) that exhibited a non-fibrillar morphology via TEM (Supplementary Figure 6C), and displayed marginal affinity for the amyloid sensor dyes with no specific emission spectrum for amyloid fibers (Figure 1E). Similar observation were made with a vascin variant in which proline mutations were introduced to break the beta-sheet propensity of the APRs in vascin (Supplementary Note 2, Supplementary Figure 7). In contrast, a version of vascin based on the human sequence (hVascin, DLAVALWLDPPDLAVALWLD), containing a single mutation of Phe to Leu at position 7 of the APR, displayed similar amyloid formation as the original mouse sequence (Supplementary Figure 8).

In conclusion the biophysical characterization above confirms that vascin is an amyloidogenic peptide that readily forms β-structured soluble oligomeric aggregates that mature into cross-β structured amyloid fibrils in a broad concentration range.

Vascin inactivates VEGFR2 in human vein endothelial cells by inducing its aggregation

As vascin is an amyloidogenic peptide derived from a VEGFR2 fragment we investigated whether vascin displays biological activity towards VEGFR2 in cultured cells. First, we monitored cellular uptake of vascin using carboxyfluorescein labelled vascin (CF-vascin) in human vein endothelial cells (HUVEC). We observed cellular uptake during the first hour of incubation as small vesicles or inclusions that contained diffuse homogeneous peptide (Figure 2A). Moreover, these inclusions were positive for the amyloid sensor dye pFTAA (Figure 2B&C), showing that the peptide remains in an amyloid-like conformation inside the cells. Co-staining for the ER protein Calnexin reveals that the peptide is on the cytoplasmic side of the ER (Figure 2D), where it partially overlaps with staining for ribosomes (Figure 2E). Proximity ligation (Duo-Link) using antibodies against VEGFR2 and the carboxyfluorescein-label on the peptide (Figure 2F), quantified by image analysis from high content microscopy demonstrated direct interaction between vascin and VEGFR2 (Figure 2G). This result was further confirmed by two color direct stochastic optical reconstruction microscopy (*d*STORM) super-resolution imaging(*37*) using 0.5μM of Alexa647 labeled vascin and Alexa568 labeled VEGFR2 (Figure 2H&I).

Co-immunoprecipitation of VEGFR2 from lysates of vascin-treated cells was performed with PEG-biotin labeled peptide again demonstrating direct interaction between vascin and VEGFR2 (Figure 3A). To investigate the consequences of the interaction between vascin and VEGFR2 on the aggregation status of the receptor, we determined the difference in distribution of VEGFR2 between soluble and insoluble lysate fractions of HUVECs treated with 20 μM of vascin. Upon vascin treatment we could observe a clear redistribution of full length and partially degraded VEGFR2 towards the insoluble fraction whereas this was not the case upon treatment with scrambled vascin (Figure 3B). In addition, the induced insoluble VEGFR2 displays

partial resistance to the ionic detergent SDS, a hallmark of amyloid-like aggregation (Figure 3C). To confirm that vascin mediated aggregation of VEGFR2 in HUVECs leads to its loss function we determined the dose response curve of vascin on VEGFR2 autophosphorylation and ERK phosphorylation (MSD ELISA assay) after stimulating HUVECs for 5 min with 1.3 nM VEGF. HUVECs displayed a clear dose responsive inhibition by vascin with an IC50 of 6.8 +- 0.5 μM for receptor autophosphorylation and 8.3 +- 0.4 μM for ERK phosphorylation (Figure 3D&E). The human version of vascin did show similar inhibition, which was not observed using the scrambled or proline controls (Figure 3F). Using fluorescence-activated cell sorting (FACS) and immunofluorescence microscopy we observed a concomitant reduction in the surface expression of VEGFR2 in HUVECs, but not of the unrelated cell-surface protein CD29 (Figure 3G & Supplementary Figure 9), confirming loss of VEGFR2 function in HUVECs. Together these data show that vascin is internalized by HUVECs and reaches the cytoplasmic compartment where it directly binds to VEGFR2 and localizes with ribosomes, resulting in the functional inactivation through aggregation of VEGFR2. This effect seemed to be specific as indicated by the CD29 result. Moreover, we observed no effect of vascin on EGF signalling in HeLa cells, which do not endogenously express VEGFR2 but the functionally homologous EGF receptor ErbB2. Treatment of this cell line with 20 μM vascin showed no inhibition of ERK phosphorylation in resonse to stimulation with EGF, showing that the effect is specific to VEGFR2 (Figure 3H). To test if known amyloidogenic proteins were affected by vascin, we investigated the effect of adding vascin to solutions of the Alzheimer β-peptide 1-42 (Aβ, Figure 3I) or the human Prion protein (PrP, Figure 3J). The mean lag time for spontaneous conversion of Aβ1-40 was 350 minutes whereas addition of preformed Aβ fibrils decreased the lag time to 130 minutes. Addition of B8 fibril variants did not lead to significant decrease of fibrillation lag times other than for B8 Scr3 where the significance level in a paired T-test was p>0,03. The mean lag time for spontaneous conversion of HuPrP23-231 was 1100 minutes. The conversion rate upon addition of preformed HuPrP90-231 fibrils was shortened to 135 minutes whereas addition of preformed B8 fibril variants did not lead to significant alteration of lag time.

Vascin reduces VEGFR2-dependent tumor growth in mice

To establish the effect of vascin *in vivo* we turned to a functional angiogenesis model that is sensitive to inhibition of VEGFR2 *in vivo* and used a subcutaneous B16 melanoma syngenic tumor model in C57BL/6 inbred mice. Tumor growth of this line is strongly reduced by VEGFR-specific inhibition approaches, such as the tyrosine kinase inhibitor PTK 787/ZK 222584 (*38*), providing a sensitive phenotypic readout for the anticipated effect of vascin. To assess if vascin administration would be tolerated by C57BL/6 mice we first performed a dose-escalation study by daily tail vein injections in two six-week-old, inbred C57BL/6 mice, starting from 1 mg/kg to 10 mg/kg, which corresponded to the highest stock concentration of the peptide that we could reach with the available material. No adverse effects to the basic physiological and behavioral parameters of the animals was apparent across this concentration range, including body weight, food and water consumption, home cage activity and locomotion. To test the effect of vascin on tumor growth, B16 cells were injected subcutaneously in the right dorsal flank of eight-week-old C57BL/6 mice. Starting from three days post tumor injection and until day 17, mice were treated daily by intravenous delivery of 10 mg/kg vascin (N=10) or scrambled vascin as the negative control (N=10). Another negative control group received intravenous delivery of the vehicle (50 mM Tris pH7.5, N=10) and the positive control group (N=5) received the kinase inhibitor PTK787 orally (75mg/kg). Tumor growth was similar in animals treated with scrambled vascin or vehicle, and markedly reduced in animals that received the PTK inhibitor. In the vascin treated group tumor growth was significantly inhibited compared to the negative controls over the entire experiment (ANOVA with Tukey post-hoc) and up to day 14 to a similar extent than the PTK treated group (Figure 4A).

In order to exclude overall toxicity effects of vascin on tumor growth, we executed a short term toxicology study in which we treated groups of 5 mice daily with 10 mg/kg of vascin (group A), scrambled (group B) or vehicle (50 mM Tris pH 7.5) for 14 days (the same duration as the tumor growth experiment). Gross examination at necropsy did not reveal any macroscopic changes (Supplementary Figure 10A). Concerning organ weights (Supplementary Figure 10B), hematology (Supplementary Figure 10C) and clinical chemistry (Supplementary Figure 10D), no statistically significant differences were observed among groups. Also no significant variations

were observed among the experimental groups in terms of lesion spectrum, frequency and severity. In addition, we examined brain sections from all the animals for amyloid deposition and associated astrogliosis. Thioflavin-S-positive deposits in the neuroparenchyma were not detected (Supplementary Figure 11 A, B & C) and co-staining with GFAP (glial fibrillary acidic protein) did not show any morphological evidence of astrogliosis, in sharp contrast to the positive control (Supplementary Figure 11D), for which we employed a transgenic Alzheimer disease mouse model that has marked amyloid deposition throughout the cortex and hippocampus (*39*).

In order to demonstrate the arrival and presence of B8-FITC at the tumor site, two groups of tumor-bearing mice were injected i.v. with B8-FITC or vehicle (PBS, negative control) and imaged with whole-body fluorescence imaging. Because the melanin-expression by the tumors strongly absorbs light hampering the *in vivo* detection of fluorescence, tumors were isolated and imaged *ex vivo*. The resulting fluorescence images showed strong green fluorescence emission for tumors isolated from B8-FITC-injected mice, whereas no fluorescence could be detected from tumors isolated from vehicle-injected control mice (Supplementary Figure 12A), supporting the presence of B8-FITC at the tumor site based on macroscopic FITC-fluorescence measurements. To further verify the presence of B8-FITC inside the tumor tissue with microscopic resolution, we additionally examined the presence of fluorescently tagged B8 with fibered confocal fluorescence microscopy (FCFM) on tumor samples from the same mice. Inserting the fibre-optical probe inside the tumor tissue sample of vehicle-injected control mice did not show any fluorescence signal, whereas the tumor samples of B8-FITC-injected mice were clearly positive for green fluorescence (Supplementary Figure 12 B). To quantify B8 in B16 tumors, the specific light absorption by melanomas was calibrated through the addition of different known quantities of B8-FITC to series of tumor tissue dilutions and compared to a standard curve of fluorescence intensity for a dilution series of pure B8-FITC. Both were found to be linear in a large concentration range and for two different fluorescence imaging modalities, thereby cross-validating the results (Supplementary Figure 13A & B). The tumor-specific fluorescence attenuation was then used to estimate the concentration of B8-FITC in tumors isolated from B8-FITC-treated mice, which was established to be 26.4 ± 10.5 μM, a concentration that is well above the IC50.

Taken together these data are consistent with a direct inhibitory activity of vascin on VEGFR2 function *in vivo* in the same manner that was observed in cells.

Amyloid toxicity is conditional to VEGFR2 dependence of cells

We determined loss of VEGFR2 function and vascin amyloid toxicity in several cell lines. CellTiter Blue cytotoxicity assays (Figure 4B) revealed that in HUVECs loss of function of the receptor (IC_{50} of 6.8 +- 0.5 μM for receptor autophosphorylation and 8.3 +- 0.4 μM for ERK phosphorylation) goes hand in hand with cytotoxicity. On the other hand in HEK293 or U2OS cells neither 2.5-100 μM of vascin or its scrambled counterpart were found to be toxic to either (Figure 4C and D). Upon transfection of VEGFR2 in HEK293 we observed that, after VEGF stimulation, ERK phosphorylation was inhibited upon treatment with 20 μM vascin (Supplementary figure 14A) which induced the aggregation of the VEGFR2 as evidenced on the fractionation assay (Supplementary figure 14B). However, no noticeable toxicity of vascin to HEK293 cells was observed in the range of 2.5 - 100 μM (Figure 4E). This demonstrates that vascin is not toxic to HEK293 cells in the absence of VEGFR2 but also that aggregation of transiently expressed VEGFR2 in these lines does not affect cell viability. Similar observations were made with U2OS cells transfected with VEGFR2 (Figure 4F). Thus vascin does not display generic amyloid toxicity and the aggregation of VEGFR2 by vascin is in itself also not toxic. Finally, as neurons are thought to be particularly sensitive to aggregate toxicity we also compared them with primary cortical neurons, which do not express VEGFR2. Although with these neurons we observed significant toxicity upon peptide treatment (Figure 4G), CellTiter Blue reaction levels were similar for the vascin and its scrambled non-amyloid variant, suggesting that the toxicity was not amyloid-specific but rather reflects the high sensitivity of these cultures, which also resulted in a large variability in the assay. To control for this we turned to human iPSCs differentiated to a cortical neuronal phenotype, which was verified using q-RT-PCR for genes specific for this cell type (Supplementary Figure 15). In these cultures we observed a lack of amyloid-specific toxicity, although there is 10% toxicity associated with administration of both vascin and the scrambled peptide.

Together these data suggest that amyloid toxicity is not dependent on cell type or even on target protein aggregation but that amyloid toxicity is mainly

determined by the biological context in which the target protein is inactivated by aggregation. As HUVEC survival is dependent on VEFGR2 function, its molecular loss of function by aggregation translates in gain of toxic phenotype.

DISCUSSION

About thirty amyloidogenic proteins are known to contribute to human disease. These diseases include neurodegenerative diseases such as Alzheimer's or Parkinson's disease but also organ-specific and systemic amyloidosis such as diabetes mellitus type-2 or light chain amyloidosis(*7*). Although the pathophysiological profiles of these diseases are disparate, involving the aggregation of different proteins, affecting distinctive cell types or tissues and having very different progression rates, they also share common structural, biochemical and biological features suggesting that amyloids might also have similar modes of interaction with cellular components(*8*). Although amyloids of different proteins have been shown to interact with lipids(*40*), proteins(*41*) and nucleic acids(*42*), it is currently unclear which of these interactions are relevant for disease. While some amyloid interactions are rather unspecific, e.g. with biological lipids(*43*) other amyloid interactions are highly specific(*20*). The most prominent of these is the self-interaction of amyloidogenic sequences during amyloid fibril formation which includes both amyloid nucleation and fiber elongation(*44, 45*). Proteome wide studies using amyloid prediction algorithms suggest that most proteins possess amyloidogenic sequence segments within their structure even though they do not form amyloid under normal conditions(*5, 6*). Given the sequence specificity of amyloid seeding this suggests that amyloid aggregation should be specifically inducible in non-amyloid associated proteins by exposing them to amyloid seeds consisting of amyloidogenic peptides derived from their own sequence.

Here we demonstrate that aggregation of endogenously expressed VEGFR2 can be induced under physiological conditions by exposing it to vascin, a peptide consisting of a tandem repeat of an amyloidogenic sequence in its signal peptide. We find that vascin possesses all attributes of natural amyloids including cross-beta structure, the population of amyloid precursor aggregates, and the ability to reach the cytoplasmic compartment of cells, confirming that the signal peptide of VEGFR2

possesses a genuine amyloidogenic sequence. In addition we find that vascin is able to induce VEGFR2 aggregation by direct interaction with VEGFR2. As vascin is targeting the VEGFR2 signaling peptide, seeding of VEGFR2 aggregation is likely to be co-translational as suggested by ribosomal colocalization of vascin in the cytoplasm and the occurrence of partial VEGFR2 degradation along with its aggregation. This results in VEGFR2 inactivation *in vitro* but also *in vivo* where it inhibits VEGFR2 dependent tumor growth upon intravascular administration. Both the aggregation as well as the inhibition of VEGFR2 is specific. Indeed, scrambled vascin does not interact with VEGFR2, nor does it provoke VEGFR2 aggregation or inhibition. In addition, whereas vascin suppresses cell surface presentation of VEFR2, it does not affect trafficking of other receptors such as CD29. Finally, vascin-induced VEGFR2 aggregation is not inherently toxic as it does not affect the viability of VEGFR2 overexpressing HEK293 cells. Together these findings show that VEGFR2, a protein not associated with amyloid disease, can be specifically induced to aggregate in the presence of specific amyloid seeds that are derived from its own sequence. There may be multiple reasons why large scale amyloidosis is not observed under natural conditions despite the likely prevalence of potentially amyloidogenic sequences in many proteins. First, most amyloidogenic sequences are buried in globular protein domains, which is probably the main protective factor against amyloidosis(*46*). Moreover, most misfolded proteins will be actively degraded in the cell before they have the opportunity to aggregate(*47*). Secondly, even in unfolded or intrinsically disordered proteins most amyloidogenic sequences are generally still sufficiently protected from aggregation by structural mechanisms such as gatekeeping(*48*) (i.e. the inhibition of aggregation by charged residues adjacent to the amyloidogenic segment) and entropic bristles(*49*) (i.e. unstructured protein segments that entropically prevent the association of amyloidogenic sequences). Finally interaction with molecular chaperones will also contribute to the inhibition of aggregation(*50*). The reason why vascin can overcome these potential protective mechanisms is not yet clear but might reside in its design: Vascin comprises a tandem repeat of an amyloidogenic sequence, which exacerbates the aggregation propensity of this sequence and leads to the formation of soluble and stable oligomeric aggregates, and these are likely to provide efficient sites for seeding. Incidentally, yeast prions often consist of peptide sequence repeats(*32*) and the

stability of soluble oligomers consisting of tandem peptide repeats recently allowed their structure solvation by X-ray crystallography(*29*).

The toxic gain-of-function observed in many amyloid-associated diseases remains poorly explained. This is mainly due to the impossibility to relate loss-of-function effects to the aggregation process of a given protein. Indeed for many amyloid proteins, especially in neurodegeneration, the functional role of the affected protein is often complex and not entirely understood(*25, 26*), which makes it difficult to unequivocally identify loss-of-function effects. In addition the interactions of amyloid with cellular components in disease is also not clarified so that again gain-of-toxic function is not directly tractable at the molecular level. The availability of our artificial amyloid model provides an opportunity to study the relationship between amyloid toxicity and specific protein loss-of-function. Indeed while recapitulating essential amyloid features our model also allows to monitor the effect of amyloidosis on a functionally well-characterized protein. Moreover, the use of a VEGFR2 fragment allows assessing amyloid toxicity independently of VEGFR2 function.

Our results demonstrate that using this setup vascin does not display generic toxic properties but rather to the contrary. Vascin gain-of-function is specific (i.e. it is dependent on the presence of VEGFR2) but also conditional (i.e. VEGFR2 needs to be expressed in the cell but the cell also needs to be dependent on VEGFR2 for its survival/proliferation). Indeed in VEGFR2 dependent HUVECs vascin toxicity and VEGFR2 loss-of-function correlate in a dose-responsive manner whereas this is not the case in VEGFR2 expressing HEK293 cells. Together these results therefore illustrate how amyloid toxicity can result from a conjunction of protein specific and cell dependent protein loss-of-function.

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Figure Legends

Figure 1 – Biophysical characterization of the amyloids formed by *vascin*. (A-D) Transmission Electron Microscopy image of 300 μM *vascin* in 1% (w/v) NH_4CO_3 after 24h incubation at room temperature and negatively stained with 2% (w/v) uranyl acetate. **(E)** Tinctorial analysis of fibrils of 40 μM *vascin* in 1% (w/v) NH_4CO_3 after 18h incubation at room temperature. **(F)** Fiber x-ray diffraction pattern of fibrils formed in 300 μM *vascin* in 1% (w/v) NH_4CO_3. **(G)** ESI–IMS–MS Driftscope plot of the vascin monomer (1) through to the heptamer (7) present two minutes after diluting the monomer to a final peptide concentration of 100 μM in in 1% (*w/v*) NH_4CO_3. ESI–IMS–MS Driftscope plots show the IMS drift time versus mass/charge (*m/z*) versus intensity (z, square-root scale). **(H)** Histogram of size distribution of particles calculated from the DLS data recorded during *vascin* aggregation. using a linear polymer as particle model. **(E)** Fourier Transform Infrared Spectrum (FTIR) of 300 μM *vascin* in 1% (w/v) NH_4CO_3 at t= 5 min (red) and t=24h (blue). (J) Time progression of the intensity of the absorbtion peak in the FTIR spectrum in panel E around 1628 cm^{-1}.

Figure 2 – *Vascin directly interacts with VEGFR2 in cells* (A) Cellular distribution of carboxyfluorescein-labeled *vascin*^{K11} (CF-*vascin*^{K11}) (green) at 1 μM in HUVEC cells. **(B)** Overlay between the fluorescence of CF-vascin^{K11} (2,5 μM green) and the amyloid specific dye pFTAA (5 μM, red). Overlap is shown as yellow colouring. **(C)** Equivalent image as B for the Proline mutant of vascin. **(D)** HUVEC cells treated for 4 hours with CF-*vascin*^{K11} (green) at 2.5 μM and co-immunostained for the ER-specific marker calnexin (red). **(E)** HUVEC cells treated for 4 hours with CF-*vascin*^{K11} at 5.0 μM (green) and co-immunostained for the ribosomal protein S6 (red). **(F)** Proximity ligation (Duo-Link) between CF-*vascin*^{K11} at 5 μM and VEGFR2 performed in HUVEC cells treated for 4 hours with peptide. For panels A, D, E and F the nuclei are stained with DAPI in blue. **(G)** Quantification of the Duo-Link signal by high content microscopy of HUVEC cells treated with *vascin* as depicted in (E). For each condition about 1000 cells were analyzed and the average fluorescence intensity per cell is shown. **(H)** dSTORM image of HUVEC cells treated for 4 hours with 0.5 μM Alexa647

labeled *vascin*^{K11} (purple) and co-immunostained for VEGFR2 (green). Scale bar is 1 μm. **(I)** Magnification of image in G where arrowheads indicate the interaction of *vascin* and VEGFR2. Scale bar is 1 μm.

Figure 3 – *Vascin knocks down VEGFR2 activity*. (A) Western blot analysis for VEGFR2 co-immunoprecipitation in HUVEC cells treated for 4 hours with 20 μM biotin-PEG labeled *vascin*. **(B)** Western blot analysis of the partitioning of VEGFR2 between the soluble and insoluble fraction of HUVEC cells treated with 20 μM *vascin*. **(C)** Western blot analysis of the SDS-gradient extraction of the soluble and insoluble fraction of HUVEC cells treated with 20 μM *vascin.* **(D)** Dose-response curve of the effect of 2.5-100 μM *vascin* on VEGF stimulation of VEGFR2 autophosphorylation in HUVEC cells. **(E)** Dose-response curve of the effect of 2.5-100 μM *vascin* on VEGF stimulation of ERK phosphorylation in HUVEC cells. **(F)** VEGFR2 autophosphorylation and ERK phosphorylation in HUVEC cells treat with 20 μM vascin, human vascin (h.vascin), scrambled vascin and proline mutant vascin (Pro). **(G)** Quantification by Fluorescence Activated Cell Sorting (FACS) of the fraction of cells that display VEGFR2 and CD29 on the cell surface upon treatment with *vascin* or scrambled *vascin* at 20 μM. **(H)** Quantification of ERK phosphorylation level as determined by MSD assay upon EGF stimulation of Hela cells treated with 20 μM vascin or its scrambled version. **(I)** Lag phase of the aggregation kinetics of the Alzheimer β-peptide 1-40 observed with Thioflavin T fluorescence in the presence of 1% molar fraction of homotypic seeds, or the equivalent amount of seeds of vascin, human vascin, scrambled vascin or the proline mutant vascin. **(J)** Equivalent experiment to panel I for the human Prion protein (PrP).

Figure 4 – In vivo effect of *Vascin* and cell-type dependent cytotoxicity. (A) Tumor growth curves of subcutaneously inject B6 melanoma cells in C57BL6 mice treated with daily intravenous injections of 10 mg/kg *vascin* or its scramble. As a positive control we used the inhibitor of the VEGFR2 tyrosine kinase activity PTK787. **(B-H)** Dose dependent toxicity of *vascin* or its scrambled version tested from 2.5 μM – 100 μM by the CellTiter Blue assay on **(B)** HUVEC cells. **(C)** HEK293 cells. **(D)** U2OS cells. **(E)** HEK293 cells transiently overexpressing VEGFR2. **(F)** U2OS

cells transiently overexpressing VEGFR2. **(G)** Cortical primary neuron cells from mouse and induced pluripotent stem cells (iPSCs) after a neuronal differentiation protocol.