CYTOSKELETON

Vasohibins encode tubulin detyrosinating activity

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Tubulin is subjected to a number of posttranslational modifications to generate heterogeneous microtubules. The modifications include removal and ligation of the C-terminal tyrosine of α-tubulin. The enzymes responsible for detyrosination, an activity first observed 40 years ago, have remained elusive. We applied a genetic screen in haploid human cells to find regulators of tubulin detyrosination. We identified SVBP, a peptide that regulates the abundance of vasohibins (VASH1 and VASH2). Vasohibins, but not SVBP alone, increased detyrosination of α-tubulin, and purified vasohibins removed the C-terminal tyrosine of α-tubulin. We found that vasohibins play a cell type–dependent role in detyrosination, although cells also contain an additional detyrosinating activity. Thus, vasohibins, hitherto studied as secreted angiogenesis regulators, constitute a long-sought missing link in the tubulin tyrosination cycle.

Microtubules are crucial constituents of the eukaryotic cytoskeleton, a dynamic structure important for cell shape and intracellular transport, composed of polymerized α- and β-tubulin heterodimers. Extensive enzymatic alterations create heterogeneous microtubules decorated with a variety of posttranslational modifications including acetylation, (poly)glutamylation, (poly)glycylation, and polyamination (*1*). Most α-tubulin isoforms encode a tyrosine at their C terminus that can be proteolytically removed and religated. The incorporation of tyrosine, the first described posttranslational modification of tubulin, is carried out by the tubulin tyrosine ligase (TTL), which reverses the detyrosinated α-tubulin state to the translated form (*2*). However, the activity of the detyrosinating enzyme (*3*), which initiates the tyrosination cycle, remains unclear.

Tubulin detyrosination has been implicated in cardiac cell function (*4*), cell migration (*5*), mitosis (*6*), and trafficking in neurons (*7*). We applied a genetic approach in haploid human cells (*8*, *9*) to identify tubulin-detyrosinating enzymes. Detyrosinated α-tubulin could be detected in wild-type Hap1 cells, and this signal was increased in *TTL*-deficient Hap1 cells and in cells treated with the microtubule-stabilizing agent paclitaxel (Fig. 1A), indicating that the tyrosination cycle is active in Hap1 cells. Next, mutagenized Hap1 cells were stained with antibodies recognizing the detyrosinated form of α-tubulin after paclitaxel treatment, and cells displaying the highest and lowest 1% of detyrosinated α-tubulin levels were isolated by fluorescence-activated cell sorting (FACS). Gene-trap insertion sites were mapped to identify genes enriched for mutations in cells exhibiting either high or low levels of α-tubulin detyrosination (Fig. 1B) (*9*). *TTL* was identified as the strongest negative regulator of α-tubulin detyrosination (647 independent gene-trap insertion events mapped in the locus in the "high" cell population versus 11 mutations in the "low" population; Fig. 1C). In addition, we identified both subunits (KATNA1 and KATNB1) of the microtubule-severing protein complex katanin as negative regulators, and *CAMSAP2* and *MAP4* as positive regulators, in agreement with previous studies (*10*–*12*). Among the genes that were enriched by at least a factor of 4 for mutations in the "low" channel, SVBP (small vasohibin binding protein) was identified as the most significant hit ($P = 4 \times 10^{-10}$). Using an antibody from a different supplier to enrich for cells with high and low levels of tubulin detyrosination, mutations in *SVBP* were similarly enriched in the population displaying "low" detyrosination ($n = 62$ independent mutations), whereas no mutations in this locus could be identified in the population displaying "high" detyrosination levels (fig. S1A). Neither *TTL* nor *SVBP* scored as regulators in 10 unrelated genetic screens examining diverse protein phenotypes (fig. S1B), which suggests that SVBP has a specific function in α-tubulin detyrosination.

The interaction of SVBP, encoding a short peptide (66 amino acids), with vasohibins (*13*) further suggested a possible function of SVBP in tubulin detyrosination. Although vasohibins have a predicted transglutaminase-like protease fold (*14*), enzymatic activity has not been demonstrated and substrates have not been proposed. They are found in the cytosol but are considered to act in the extracellular milieu after secretion through a noncanonical pathway (*13*, *15*, *16*). Mammalian cells contain two vasohibin paralogs, VASH1 and VASH2 (fig. S1, C and D), that may act redundantly. To assess the function of SVBP and vasohibins in detyrosination, we expressed *SVBP*, *VASH1*, and *VASH2* in HeLa cells, a cell line with minimal levels of detyrosinated α-tubulin (*17*) (Fig. 1D). Whereas *SVBP* did not increase detyrosinated α-tubulin, expression of *VASH1* or *VASH2* modestly increased detyrosinated α-tubulin. Coexpression of *SVBP* with vasohibins increased the abundance (*13*) and solubility (fig. S2) of vasohibins and further increased detyrosination of α-tubulin. Thus, SVBP together with vasohibins can increase α-tubulin detyrosination.

To determine whether endogenous vasohibins affected detyrosination of α-tubulin, we generated single- and double-knockout cell lines (fig. S3). Loss of *VASH1* or *VASH2* led to a modest decrease in the amount of detyrosinated tubulin in Hap1 cells, and their combined loss led to a further decrease (but not absence) of detyrosinated tubulin (Fig. 2A). The presence of detyrosinated tubulin in vasohibin-deficient cells could not be attributed to expression of *TUBA4A* (fig. S4), an isoform lacking the C-terminal tyrosine. To investigate the role of the vasohibins in other cell types, we generated both *VASH1*-deficient and *VASH1*/*VASH2*-deficient cell lines in human embryonic kidney (HEK) 293T cells (fig. S5) as well as in the melanoma-derived cell line CHL-1 (fig. S6). In HEK293T cells, a minimal decrease in detyrosinated α-tubulin was observed in *VASH1* mutant cells, but a substantial decrease was observed in double-knockout cells (Fig. 2B). In CHL-1 cells, however, the double-knockout cells displayed undetectable levels of detyrosinated α-tubulin (Fig. 2B) and a modest increase in the amount of tyrosinated tubulin (fig. S7A). Thus, vasohibins are important for α-tubulin detyrosination and cells can also contain vasohibin-independent detyrosinating activity.

Having identified vasohibin-dependent and -independent activities, we next sought to determine whether these could affect the polymerized microtubule population. Paclitaxel stabilizes microtubules and thereby depletes the amount of free α/β-tubulin dimers that are the substrate for TTL (*18*). Treatment of Hap1, HEK293T, and CHL-1 cells with paclitaxel led to a robust increase in α-tubulin detyrosination. In Hap1 and HEK293T cells deficient for *VASH1* and *VASH2*, a similar response was observed; this was also the case when translation was inhibited by cycloheximide in HEK293T cells (Fig. 2C and fig. S8). These findings suggest that the vasohibin-independent activity affects paclitaxel-stabilized microtubules. Comparative immunoblot analysis showed that vasohibins mediate at least 97% of α-tubulin detyrosination in CHL-1 cells (fig. S9).

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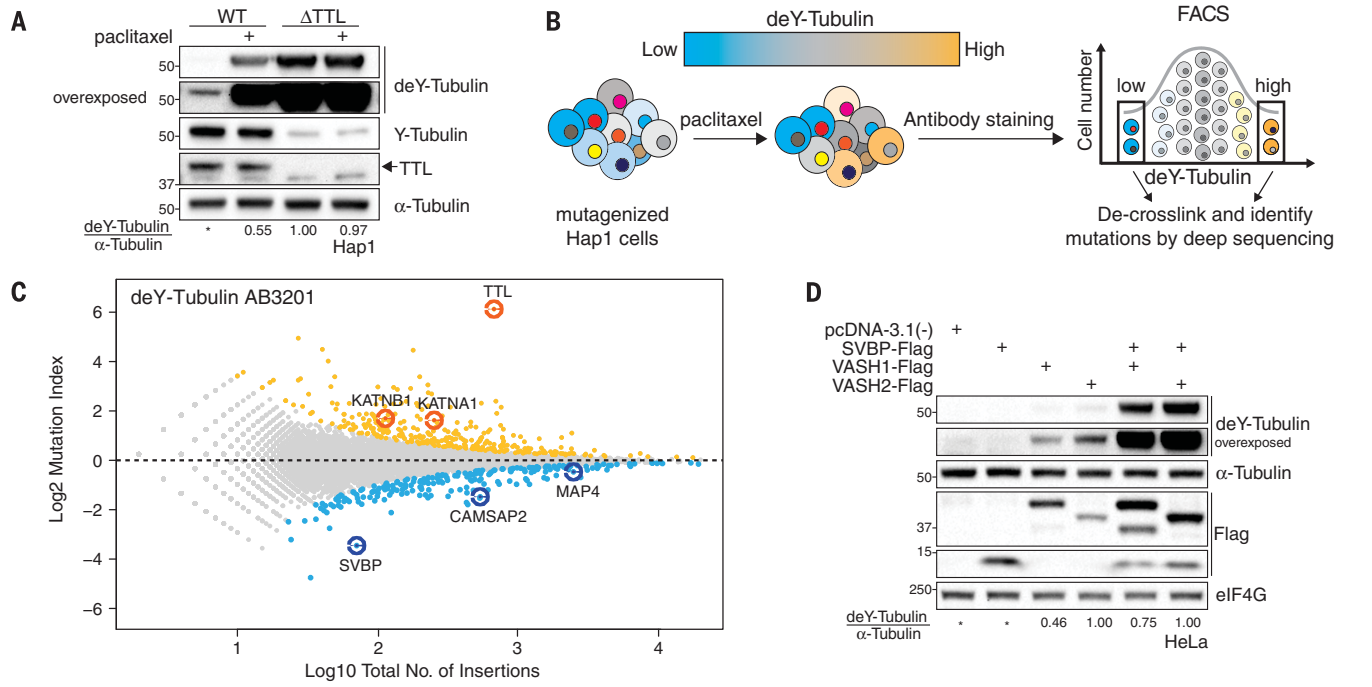

Fig. 1. Identification of genetic regulators of tubulin detyrosination in haploid human cells. (**A**) Wild-type Hap1 cells and Hap1 cells deficient for TTL were treated with paclitaxel and subjected to immunoblot analysis using antibodies directed against detyrosinated (deY) and tyrosinated (Y) tubulin and TTL. Total amounts of tubulin were used as a loading control. The relative ratios of detyrosinated versus total α-tubulin levels are indicated. Asterisk indicates that the detyrosinated tubulin signal is not quantifiable. (**B**) Schematic overview of the haploid genetic screen using antibodies to detect detyrosinated tubulin. (**C**) Result of the genetic screen for regulators of α-tubulin detyrosination. The relative mutation frequency in the "high" versus the "low" cell population (referred to as mutation index) was plotted against the total amount of insertions mapped per gene. Positive regulators are labeled in yellow, negative regulators in blue. (**D**) HeLa cells transfected with vectors directing the expression of FLAG-tagged *SVBP*, *VASH1*, *VASH2*, or combinations thereof, were subjected to immunoblot analysis.

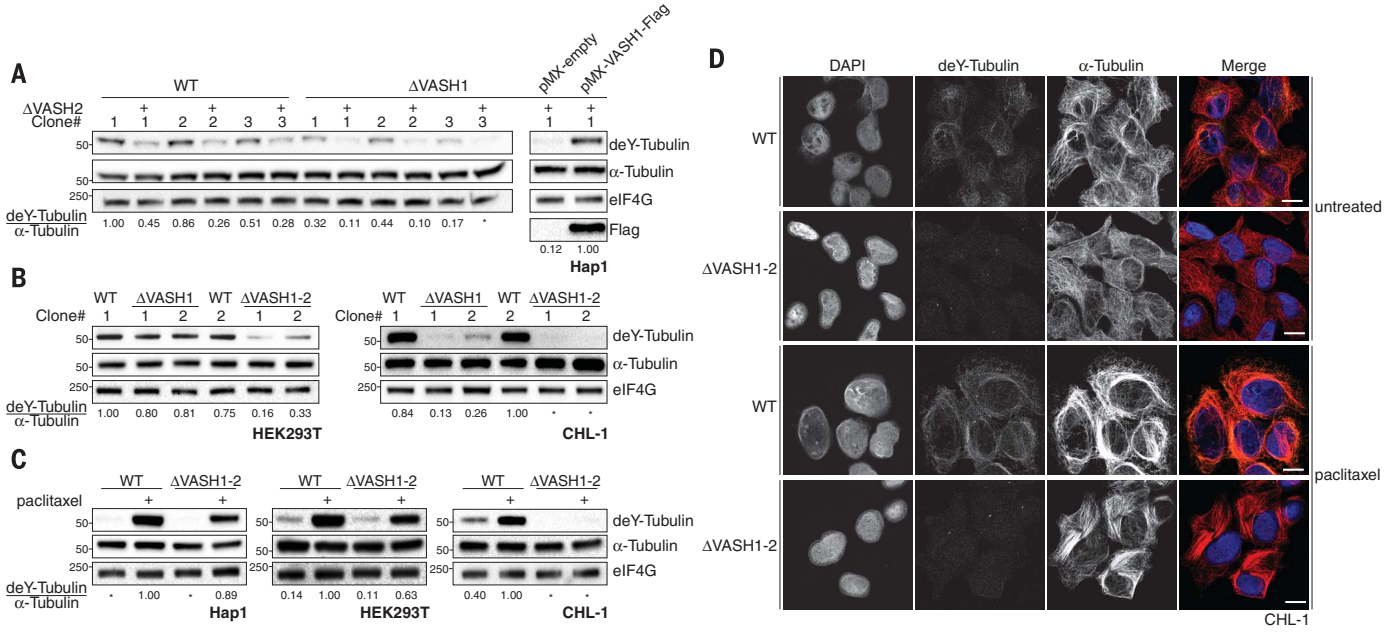

Fig. 2. VASH1 and VASH2 control tubulin detyrosination and affect the detyrosination status of polymerized microtubules. (**A**) Independent Hap1 cell lines deficient for *VASH1* or *VASH1* and *VASH2* were generated and subjected to immunoblot analysis. Relative ratios of detyrosinated versus total α-tubulin levels are indicated. (**B**) Independent HEK293T and CHL-1 cell lines deficient for *VASH1* and/or *VASH2* were generated and subjected to immunoblot analysis. (**C**) Wild-type cells and cell lines deficient for *VASH1* and *VASH2* were treated with paclitaxel and subjected to immunoblot assay as in (A). (**D**) Wild-type CHL-1 cells and CHL-1 cells deficient for *VASH1* and *VASH2* were treated with paclitaxel and stained with antibodies to detect detyrosinated α-tubulin (green) and α-tubulin (red). Blue indicates 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain. Paclitaxel treatment led to a factor of 1.89 increase in the detyrosination/total tubulin ratio of wild-type cells. Scale bars, 25 μm.

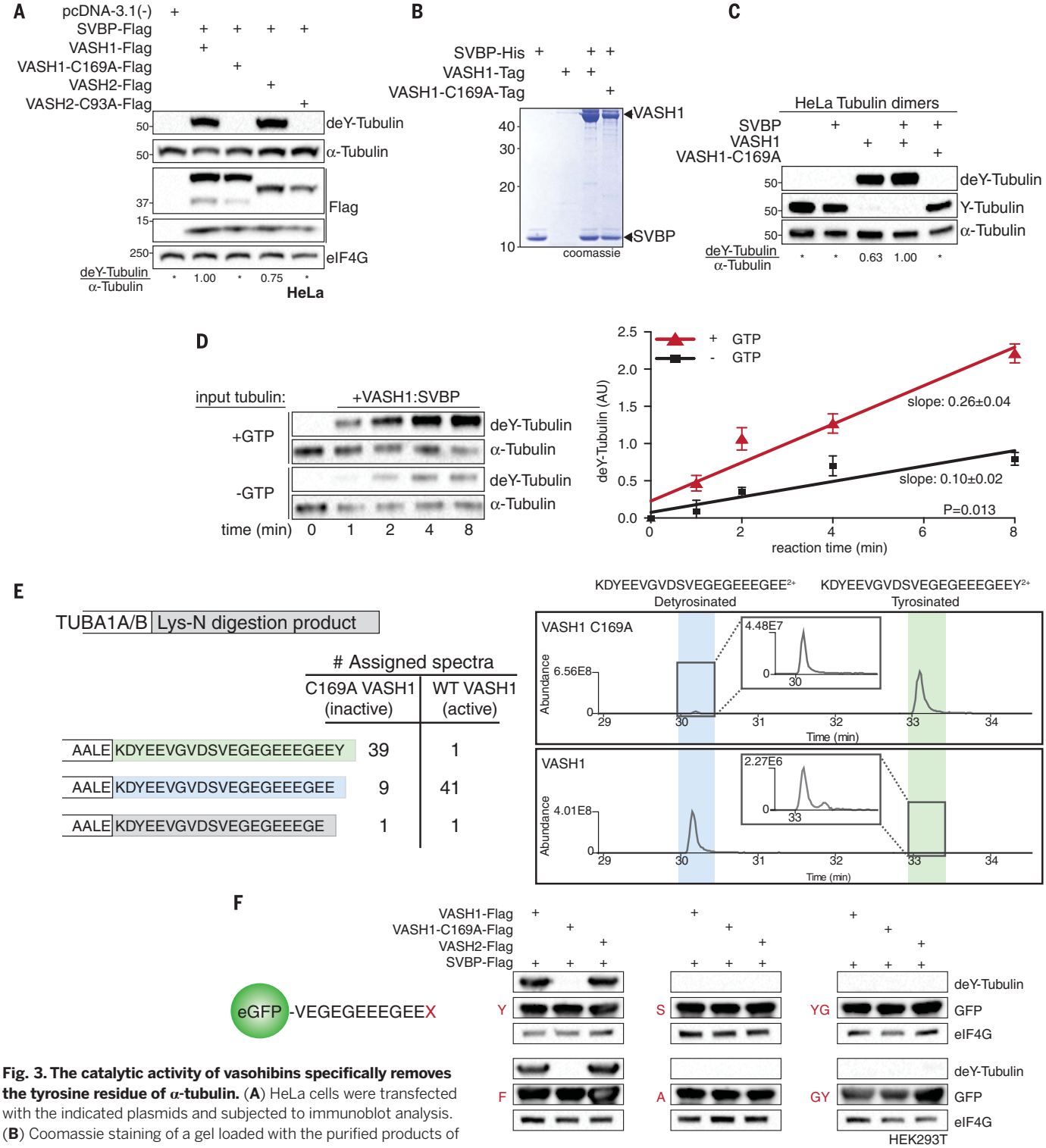

Fig. 3. The catalytic activity of vasohibins specifically removes the tyrosine residue of α-tubulin. (**A**) HeLa cells were transfected with the indicated plasmids and subjected to immunoblot analysis. (**B**) Coomassie staining of a gel loaded with the purified products of SVBP, VASH1, SVBP-VASH1, and SVBP-VASH1-C169A expressed in insect cells. (**C**) In vitro detyrosination assay, using recombinant SVBP, VASH1, VASH1-SVBP, and catalytic inactive VASH1-SVBP, with purified HeLa α/β-tubulin as substrate. Tubulin tyrosination and detyrosination levels were determined using immunoblot analysis. (**D**) Purified VASH1-SVBP was incubated with in vitro generated microtubules from HeLa cells, and immunoblot signals were quantified to establish the detyrosination rate relative to nontreated α/β-tubulin ($n = 3$). (**E**) NanoLC-MS/MS analysis of HeLa tubulin incubated with catalytic active or inactive VASH1-SVBP complexes. Extracted ion chromatograms of the detyrosinated and tyrosinated peptides are shown together with the number of assigned spectra of the respective peptides. (**F**) HEK293T cells were cotransfected with vectors encoding the expression of enhanced GFP molecules with C-terminal extensions corresponding to the C terminus TUBA1A/B with the indicated modifications as well as SVBP and VASH1, VASH2, or catalytically inactive VASH1. Transfected cells were subjected to immunoblot analysis. Amino acid abbreviations: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; K, Lys; L, Leu; S, Ser; V, Val; Y, Tyr.

Paclitaxel treatment also increased detyrosination in these cells, which suggests that vasohibins also affect the detyrosination status of polymerized microtubules. To address this further, we stained CHL-1 cells using antibodies directed against α-tubulin and tyrosinated and detyrosinated tubulin (Fig. 2D and fig. S7B). The signal for detyrosinated tubulin was absent in vasohibin-deficient cells, but it colocalized with microtubules in both interphase and mitotic wild-type cells (Fig. 2D and fig. S10). Thus, vasohibins affect the detyrosination state of polymerized microtubules, although their activity appeared not to be absolutely needed for chromosome congression (*6*).

To directly test whether vasohibins act as transglutaminase peptidases toward tyrosinated α-tubulin, we designed VASH1 and VASH2 mutants that affected their respective predicted catalytic sites, Cys^{169} and Cys^{93} (*14*). Coexpression of VASH1-Cys169Ala and VASH2-Cys93Ala with SVBP showed that these cysteines were essential for vasohibin-dependent induction of detyrosinated α-tubulin (Fig. 3A).

To study whether vasohibins can produce detyrosinated α-tubulin in vitro, we coexpressed the VASH1-SVBP complex in insect cells (Fig. 3B) and purified a stable and soluble complex. Small-angle x-ray scattering (SAXS) coupled to size exclusion chromatography revealed a well-folded, structurally robust, elongated complex with 1:1 stoichiometry (fig. S11). Purified VASH1-SVBP reduced the tyrosinated form of tubulin while increasing the detyrosinated form as examined by specific antibodies, with an apparent Michaelis constant K_M of ~700 nM on α/β-tubulin (fig. S12). VASH1 alone was expressed in small amounts, again suggesting that SVBP is needed for folding, and thus solubility, of VASH1.

Relative to nontreated α/β-tubulin, the in vitro detyrosination rate of VASH1-SVBP was higher using guanosine triphosphate (GTP)–induced polymerized stabilized microtubules as a substrate by a factor of ~2.5 (P = 0.013) (Fig. 3D). Whereas immunoblot analysis suggested that detyrosinated α-tubulin is generated by vasohibins, it is a possibility that other reaction products could also be generated, including the deglutaminated Δ2- or Δ3-forms of α-tubulin (*19*, *20*). Nano–liquid chromatography combined with tandem mass spectrometry (nanoLC-MS/MS) indicated that VASH1-SVBP detyrosinated α-tubulin without affecting the adjacent glutamic acid residues (Fig. 3E). Thus, VASH1 acts as a peptidase to catalyze removal of the C-terminal tyrosine of α-tubulin.

We next designed experiments to study the specificity of vasohibins. Tubulin isoforms encode different C-terminal tails. These tails were attached to the C terminus of green fluorescent protein (GFP) and coexpressed with *VASH1*, *VASH1-Cys169Ala*, and *VASH2*. All isoforms containing a tyrosine at their C terminus could be detyrosinated; *TUBA8A*, which encodes for a C-terminal phenylalanine, could also be modified by vasohibins (fig. S13). To further determine the substrate specificity, we generated mutants in the TUBA1A/B minimal substrate. Only variants with a C-terminal tyrosine or phenylalanine were processed by vasohibins (Fig. 3F), suggesting a requirement for an aromatic ring at the C-terminal position. Extension of the C terminus with a glycine prevented enzymatic conversion by vasohibins, suggesting that the terminal free carboxyl group is required and that vasohibins do not cleave internally. These experiments start to provide a rationale for the specific proteolysis of the C terminus of α-tubulin.

Previously, vasohibins have been studied as secreted molecules affecting angiogenesis, although the mechanism of secretion remains unclear (*21*–*23*). The enzymatic activity described here addresses long-standing questions about the nature of molecules that are able to start the detyrosination-tyrosination cycle. Additional studies are required to address whether certain isoforms or modified versions of vasohibins function specifically inside the cell to detyrosinate tubulin. Although detyrosination was envisioned as a simple reaction carried out by a carboxypeptidase, the identification of VASH1, VASH2, the regulating peptide SVBP, and a yet-unidentified activity extend our view on the complexity of this process.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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Tubulin carboxypeptidase identity revealed

Enzymes of the α-tubulin detyrosination/tyrosination cycle create landmarks on microtubules that are essential for their multiple cellular functions and are altered in disease. Tubulin carboxypeptidases (TCPs) responsible for detyrosination have remained elusive for 40 years (see the Perspective by Akhmanova and Maiato). Aillaud *et al.* identified vasohibins as enzymes that perform the TCP function and found that their small interacting partner SBVP was essential for their activity. Vasohibin/SVBP complexes were involved in neuron polarization and brain cortex development. The authors also developed an inhibitor targeting this family of enzymes. Using a completely different strategy, Nieuwenhuis *et al.* also showed that vasohibins can remove the C-terminal tyrosine of α-tubulin.

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