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Cobalamins Function as Allosteric Activators of an Angelman Syndrome-Associated UBE3A/E6AP Variant

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Genetic aberrations of the maternal UBE3A allele, which encodes the E3 ubiquitin ligase E6AP, are the cause of Angelman syndrome (AS), an imprinting disorder. In most cases, the maternal UBE3A allele is not expressed. Yet, approximately 10 percent of AS individuals harbor distinct point mutations in the maternal allele resulting in the expression of full-length E6AP variants that frequently display compromised ligase activity. In a high-throughput screen, we identified cyanocobalamin, a vitamin B12-derivative, and several alloxazine derivatives as activators of the AS-linked E6AP-F583S variant. Furthermore, we show by cross-linking coupled to mass spectrometry that cobalamins affect the structural dynamics of E6AP-F583S and apply limited proteolysis coupled to mass spectrometry to obtain information about the regions of E6AP that are involved in, or are affected by binding cobalamins and alloxazine derivatives. Our data suggest that dietary supplementation with vitamin B12 can be beneficial for AS individuals.

Loss of function mutations of *UBE3A*, which is located on chromosome 15q11-q13[1] and encodes the E3 ubiquitin ligase E6AP,[2] are the cause of Angelman syndrome (AS), a rare neurodevelopmental disorder.[3] While in most cells both alleles are expressed, in neurons the paternal UBE3A allele is not or only poorly transcribed due to genomic imprinting.[4] Consequently, gross deletions within the maternal 15q11-q13 region, as observed in about 75 percent of AS individuals, result in greatly reduced E6AP expression levels. Point mutations of the maternal *UBE3A* allele resulting in E6AP variants with compromised E3 activity represent the second most common cause of AS.[3c,d,5]

Besides regulation at the level of expression, several lines of evidence indicate that the E3 activity of E6AP is regulated. For instance, phosphorylation of T485 (numbering according to E6AP isoform 1[1b]) negatively affects the E3 activity,[6] while interaction with the E3 ligase HERC2 or the E6 oncoprotein of cancer-associated human papillomaviruses (HPVs) such as HPV-16 activates the E3 activity of E6AP.[7] Moreover, by cross-linking coupled to mass spectrometry (XL-MS) we showed that binding of the E6 protein affects the structural dynamics of E6AP.[8] In a simplified view, this indicates that E6AP exists in at least two different conformational states, an active and an inactive one, and that binding of E6 stabilizes the active state. In support of this assumption, we previously identified small molecule activators of wild-type (wt) E6AP by measuring E6AP auto-ubiquitination in a fluorescence polarization (FP)-based high-throughput screen using TAMRA-labeled ubiquitin.[9] Notably, the compounds did not only activate wt E6AP, but also rescued the E3 activity of E6AP-F583S (substitution of F583 by S), an AS-derived E6AP variant with compromised E3 activity. F583 is located in the N-terminal region of the N lobe of the catalytic HECT domain,[10] and substitution of F583 by S interferes with the ability of E6AP to covalently attach ubiquitin to substrate proteins and to itself.[9] Encouraged by this finding, we set out to screen for additional small molecule activators of E6AP-F583S. To do so, we first confirmed that the FP-based auto-ubiquitination assay is capable of measuring E6AP-F583S activity in a reproducible manner. As previously reported, auto-ubiquitination of E6AP-F583S was readily observed in the presence of the small molecule OF227 or of the E6 oncoprotein,[9] but it was only poorly auto-ubiquitinated in its absence (Figure S1). Furthermore, the dynamic range of the observed FP values results in Z′ values of ≥ 0.8 (Figure S1) indicating that the assay is highly robust and well suited to identify activators of E6AP-F583S in a high-throughput format.

We screened 62,761 compounds in total (Screening Center, University of Konstanz) at a concentration of 20 μM. Reactions in the presence of the E6 oncoprotein served as positive control. Compounds were scored as positive, when the reaction was stimulated by $\geq 50\%$ at reaction time $t_1 = 70$ min and/or $t_2 = 90$ min, with the respective values of the control reaction in the absence of compounds and of the E6-containing control reaction set to 0% and 100%, respectively. 49 compounds fulfilled these cut-off criteria (Figure S1; for structural information on the compounds, see Table S1). Notably, we found several compounds that contain alloxazine as common scaffold and that we previously identified as potent activators of wt E6AP (e.g. MDB1I08, termed OF232 in ref. [9]), underlining the

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reliability of the FP-based assay to identify small molecule activators of wt E6AP and AS-associated E6AP variants. The potentially most interesting compound identified was cyanocobalamin (activation at $t_1 = 74\%$ and $t_2 = 67\%$), since it is a manufactured form of the vitamin B_{12} family. Vitamin B_{12} consists of a central cobalt ion that coordinates the tetradentate corrin ring and two axial ligands, a benzimidazole group and one of four different ligands serving as the eponym of methyl-, 5′-desoxyadenosyl- (or briefly, adenosyl−), hydroxy- or cyanocobalamin (Figure 1A).[11] For validation of the hit compounds by secondary assays, we therefore focused on cyanocobalamin and included methyl- and adenosylcobalamin, as these are the biologically active forms of vitamin B_{12} and as in cells cyanocobalamin is presumably converted to these.

We initially validated the activating potential of the cobalamins in titration experiments employing the FP-based auto-ubiquitination assay. This showed that each of the cobalamins stimulates E6AP-F583S auto-ubiquitination up to 70% compared to the reaction in the presence of the E6 oncoprotein. As shown in Figure 1B, for cyanocobalamin the strongest activation was observed at a concentration of 20 μM. For the two other cobalamins, most efficient activation was achieved with 1 μM, while E6AP-F583S auto-ubiquitination was blocked at higher concentrations. This different behavior of the cobalamins was confirmed in additional titration experiments indicating that adenosylcobalamin and methylcobalamin reach their strongest activation potential at approximately 2 μM under the conditions used (Figure 1C). To prove that the cobalamins do not activate E3 ligases in general, we performed auto-ubiquitination assays with a truncated form of HUWE1 (HUWE1_trunc), which like E6AP is a member of the HECT E3 family, and HDM2, a member of the RING E3 family, and the RING domain of RLIM (RLIM_RING) (Figure 1D).[12] This showed that none of the cobalamins stimulates auto-ubiquitination of these E3s, indicating the specificity of cobalamins for E6AP. Furthermore, similar to the data obtained with E6AP-F583S, higher concentrations of adenosylcobalamin or methylcobalamin completely abolished auto-ubiquitination. Since auto-

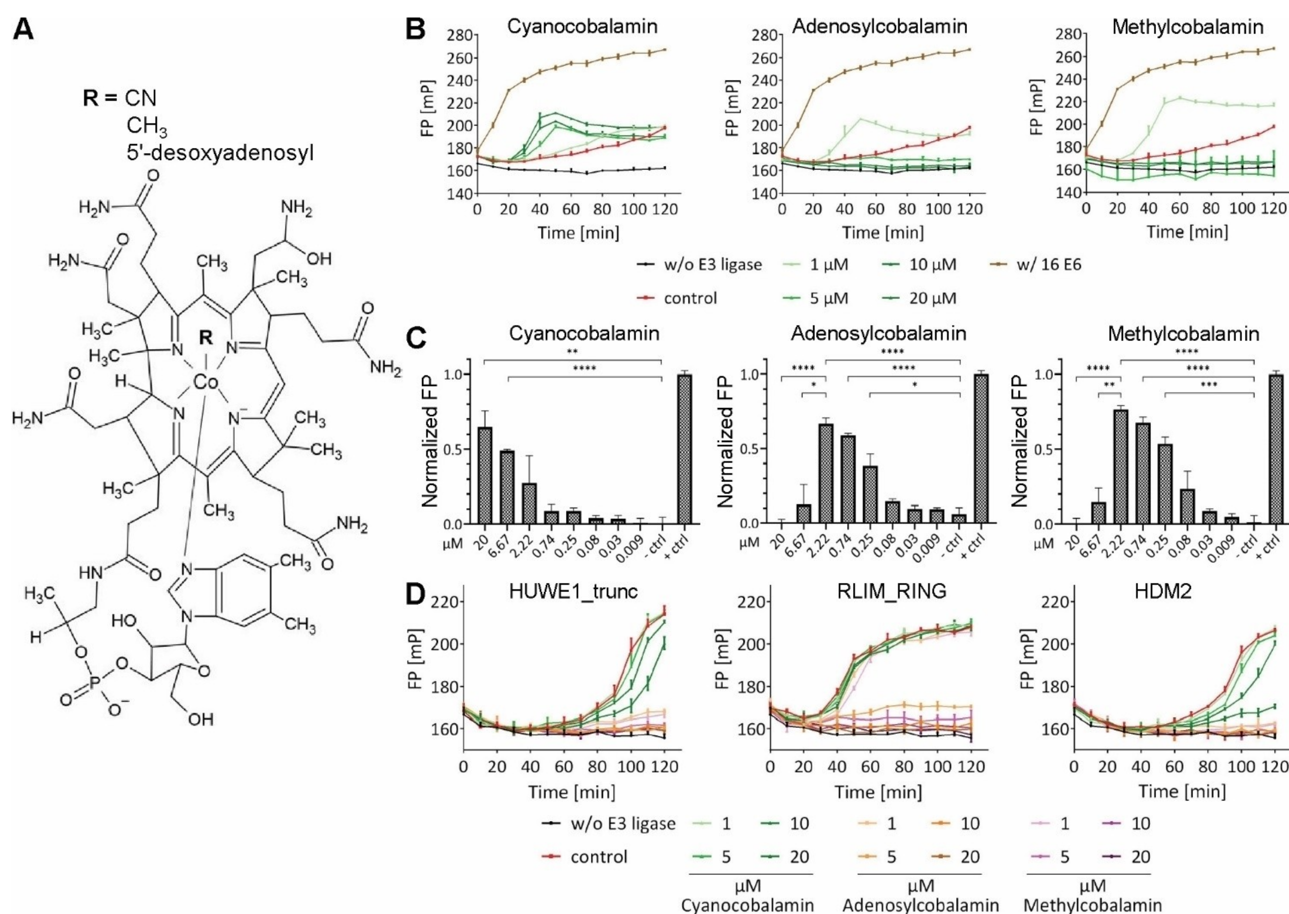

Figure 1. Cobalamins stimulate the activity of E6AP-F583S but do not stimulate E3 ligases in general. A) Structure of Cobalamin family members. B) FP-based auto-ubiquitination assay was performed with E6AP-F583S in absence (control) or presence of increasing concentrations of the different cobalamins or GST-16 E6 as positive control. FP values were measured every 10 min in triplicates. w/o E3 ligase, reaction in the absence of E6AP-F583S. C) FP-based auto-ubiquitination was performed with E6AP-F583S in absence (− ctrl) or presence of increasing concentrations of the different cobalamins or GST-16 E6 (+ ctrl). FP values (of triplicates) measured at 110 min were normalized and plotted as bar graphs using GraphPad Prism 8. The mean of the positive control (+ ctrl) was set to 1, while the mean of the negative controls (− ctrl) was set to 0. P-values were determined by Welch's t-test. *, p-value ≤ 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001. D) FP-based auto-ubiquitination assays were performed with the E3 ligases indicated in absence (control) or presence of increasing concentrations of the different cobalamins as indicated. FP values were measured every 10 min in triplicates. w/o E3 ligase, reaction in absence of the respective E3 ligase.

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ubiquitination of E3s generally requires the presence of the E1 ubiquitin-activating enzyme and a cognate E2 ubiquitin-conjugating enzyme (for the E3s tested, a member of the UbcH5 subfamily),[12] this indicated that the inhibitory effect occurs at the level of E1 and/or E2 rather than on the E3. Indeed, the cobalamins interfere with E1 activity in a dose-dependent manner, with adenosylcobalamin and methylcobalamin being more potent than cyanocobalamin (Figure S2).

To exclude the possibility that the effect of cobalamins on E6AP-F583S in the FP-based assay is an artifact of the use of TAMRA-labeled ubiquitin, we performed "standard" in vitro auto-ubiquitination assays using non-modified ubiquitin (Figure 2A). Also in this assay, the cobalamins stimulated E6AP-F583S auto-ubiquitination, as evidenced by the observation that bands representing the non-modified form of E6AP-F583S and free ubiquitin are significantly decreased with the concurrent appearance of a high-molecular mass smear representing poly-ubiquitinated forms of E6AP-F583S (note that cobalamins had no effect on the other E3s also in this type of assay; Figure S2). The inhibitory effect of higher concentrations of adenosylcobalamin and methylcobalamin was less pronounced than in the FP-based assay (Figure 2A; see also Figure S3). This may be explained by the notion that in "standard" auto-ubiquitination assays, E6AP-F583S as well as E1 and E2 have to be used at higher concentrations than in the FP-based assay, since the reaction products are analyzed by SDS-PAGE followed by Coomassie blue staining (i.e. the readout of the assay is less sensitive than that of the FP-based assay).

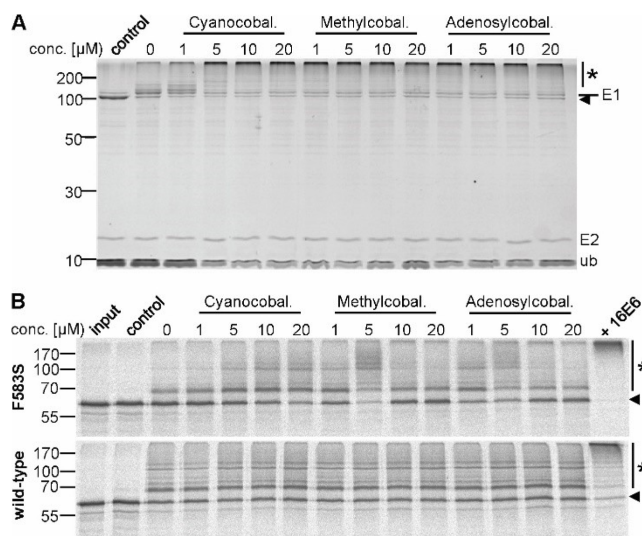

Figure 2. Cobalamins stimulate E6AP-F583S auto-ubiquitination as well as E6AP-F583S-mediated ubiquitination of Ring1B. A) E6AP-F583S was incubated in absence (0) or presence of increasing concentrations of the different cobalamins as indicated. Reactions were incubated for 90 min at 37 °C or 4 °C (control) and analyzed by SDS-PAGE followed by Coomassie Blue staining. Running positions of E1, E2 (UbcH7), free ubiquitin (ub), non-modified E6AP-F583S (arrowhead), and ubiquitinated forms of E6AP-F583S (asterisk) are indicated. B) In vitro translated radiolabeled Ring1B-I53S was incubated with wild-type E6AP or E6AP-F583S for 15 min at 30 °C and 90 min at 37 °C, respectively, in absence (0) or presence of increasing concentrations of the different cobalamins or GST-16 E6 as indicated. Reactions were analyzed by SDS-PAGE followed by fluorography. Running positions of non-modified Ring1B-I53S (arrowhead), and ubiquitinated forms of Ring1B-I53S (asterisk) are indicated. control, reaction at 4 °C; input, Ring1B-I53S used in the ubiquitination reaction.

Next, we performed in vitro ubiquitination assays with Ring1B, a known substrate of E6AP,[13] to determine whether cobalamins can also stimulate E6AP-F583S-mediated substrate ubiquitination or whether their stimulating effect is limited to auto-ubiquitination. To this end, in vitro translated radiolabeled Ring1B-I53S – an E3 ligase deficient mutant of Ring1B[13] – was incubated with E6AP-F583S in the absence and presence of increasing concentrations of the different cobalamins or the E6 oncoprotein as positive control. Subsequently, the reaction mixtures were analyzed by SDS-PAGE followed by fluorography (Figure 2B). In the absence of any compound, E6AP-F583S ubiquitinated only small amounts of Ring1B resulting mainly in mono-ubiquitinated Ring1B, while in the additional presence of E6, Ring1B was efficiently poly-ubiquitinated as expected.[9] Though less efficient than with E6, addition of increasing concentrations of cyanocobalamin increased both the amount of Ring1B subjected to ubiquitination (as evidenced by decreasing levels of the non-modified form of Ring1b) and the number of ubiquitin molecules attached to it (as evidenced by the migration behavior of the ubiquitinated forms). As in the FP-based assay, at low concentrations adenosylcobalamin and methylcobalamin were more potent than cyanocobalamin in stimulating E6AP-F583S-mediated Ring1B ubiquitination, while the stimulating effect was not observed at higher concentrations.

To determine if the cobalamins can also stimulate wt E6AP, we also performed Ring1B ubiquitination assays with wt E6AP (Figure 2B). However, no effect was observed under the conditions used. We therefore resorted to auto-ubiquitination assays using a hydrophobic patch mutant of ubiquitin (UbLIA; replacement of L8 and I44 by A) that is only poorly used by wt E6AP alone, a deficiency that is rescued by the E6 oncoprotein.[7b] This revealed that as for E6AP-F583S, the cobalamins stimulate wt E6AP auto-ubiquitination in a concentration-dependent manner, i.e. auto-ubiquitination was most efficient with 10–20 µM of cyanocobalamin and 1 µM of adenosylcobalamin and methylcobalamin (Figure S3). Similar results were obtained with E6AP-F583S and UbLIA, further corroborating the stimulating potential of cobalamins. However, it should be noted that as previously reported for alloxazine derivatives,[9] the stimulating/rescuing potential of cobalamins is presumably limited to distinct AS-associated E6AP variants, since they stimulated E6AP-E584Q less efficiently than E6AP-F583S (Figure S3).

By quantitative XL-MS (qXL-MS), we previously showed that the E6 oncoprotein and alloxazine derivatives affect the structural dynamics of wt E6AP and E6AP-F583S such that an E3-active conformation of E6AP is stabilized.[8–9] Thus, we next asked if this can also be shown for cobalamins. As qXL-MS is rather laborious and time-consuming, we focused our efforts on cyanocobalamin and compared the cross-links within E6AP induced by cyanocobalamin, N12 (an alloxazine derivative identified in this study; see Table S1 and Figure S3) or E6 to the cross-links found in the control reaction (i.e. E6AP alone). In comparison to N12 and E6, the presence of cyanocobalamin

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had a mild effect on both E6AP-F583S (Figure 3) and wt E6AP (Figure S4), but the pattern of up- and downregulated cross-links observed was similar to that of N12 (and other alloxazine derivatives[9]) insofar as mostly the interaction between the N and C terminus was affected.

The qXL-MS data are in line with the notion that the cobalamins physically interact with E6AP-F583S and wt E6AP and activate these by inducing structural rearrangements or stabilizing a distinct conformation or conformational ensemble. Since cyanocobalamin, alloxazine derivatives, and E6 appear to affect the structural dynamics of E6AP, though to different extents, we wondered if they all bind to similar or overlapping regions on E6AP. To study this, we employed limited proteolysis-coupled to mass spectrometry (LiP-MS).[14] The principle consideration underlying LiP-MS is that by binding to a protein, small molecules alter the pattern of peptides obtained by proteolytic digest by inducing conformational changes and by protecting the region to which they bind from the attack of proteases; i.e. the molecules leave a "footprint" on the interacting protein. Indeed, in presence of E6 a region of E6AP that comprises the primary interaction site for E6 (amino acids 378–395)[15] is protected from proteolytic digest (Figure 4C, Figure S5). This region is not or only mildly affected by cyanocobalamin or alloxazine derivatives indicating that they do not bind to the E6-binding region of E6AP (Figures 4A, 4B; Figure S5). Moreover, cyanocobalamin and in particular alloxazine derivatives protect mainly three regions of E6AP from proteolytic digest, the very N terminus, a region at the C terminus, and a region comprising approximately amino acids 230–255 (Figures 4A, 4B; Figure S5).

The protection of both the N terminus and the C terminus fits well with the data obtained by qXL-MS, supporting the notion that binding of cyanocobalamin and alloxazine derivatives stabilizes a conformation, in which these regions are in close proximity (see also ref. [9]). The third region protected (amino acids 230–255) is at the surface of E6AP[16] and may in turn represent the primary binding site for cyanocobalamin and alloxazine derivatives. Since this region contains an α-helix, we asked whether the α-helix may be part of the binding site of these compounds. To do so, we generated a respective deletion mutant of E6AP (deletion of amino acids 240–248) and an E6AP mutant, in which S241, N243, E245, and D247 were replaced by glycine. Both E6AP mutants turned out to be catalytically inactive (data not shown). While this does not prove that the α-

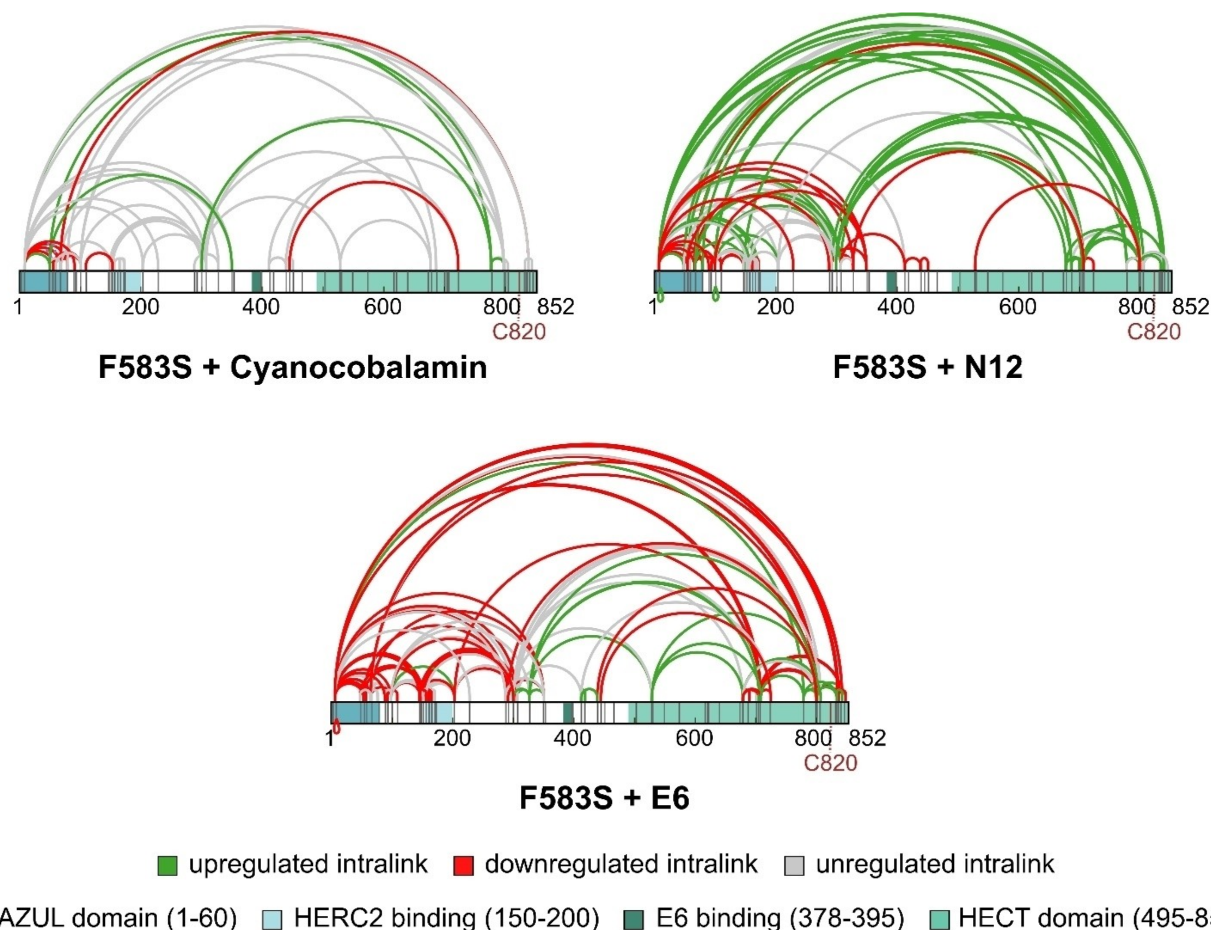

Figure 3. Cyanocobalamin induces structural rearrangements in E6AP-F583S. Patterns of intralink distribution within E6AP-F583S were determined by qXL-MS in the presence of cyanocobalamin, the alloxazine derivative N12, and GST-16 E6 as indicated. Quantification was performed relative to E6AP-F583S alone. Cross-links upregulated in the presence of cyanocobalamin, N12, and GST-16 E6 are depicted in green, while downregulated links are shown in red (defined as a log2 change of greater than or equal to ± 1.0 and p-value < 0.01). Cross-links with no significant change are depicted in gray. Lysine residues are shown in black. The catalytic cysteine residue of E6AP at position 820 is marked in red. For additional information, see Supporting Information.

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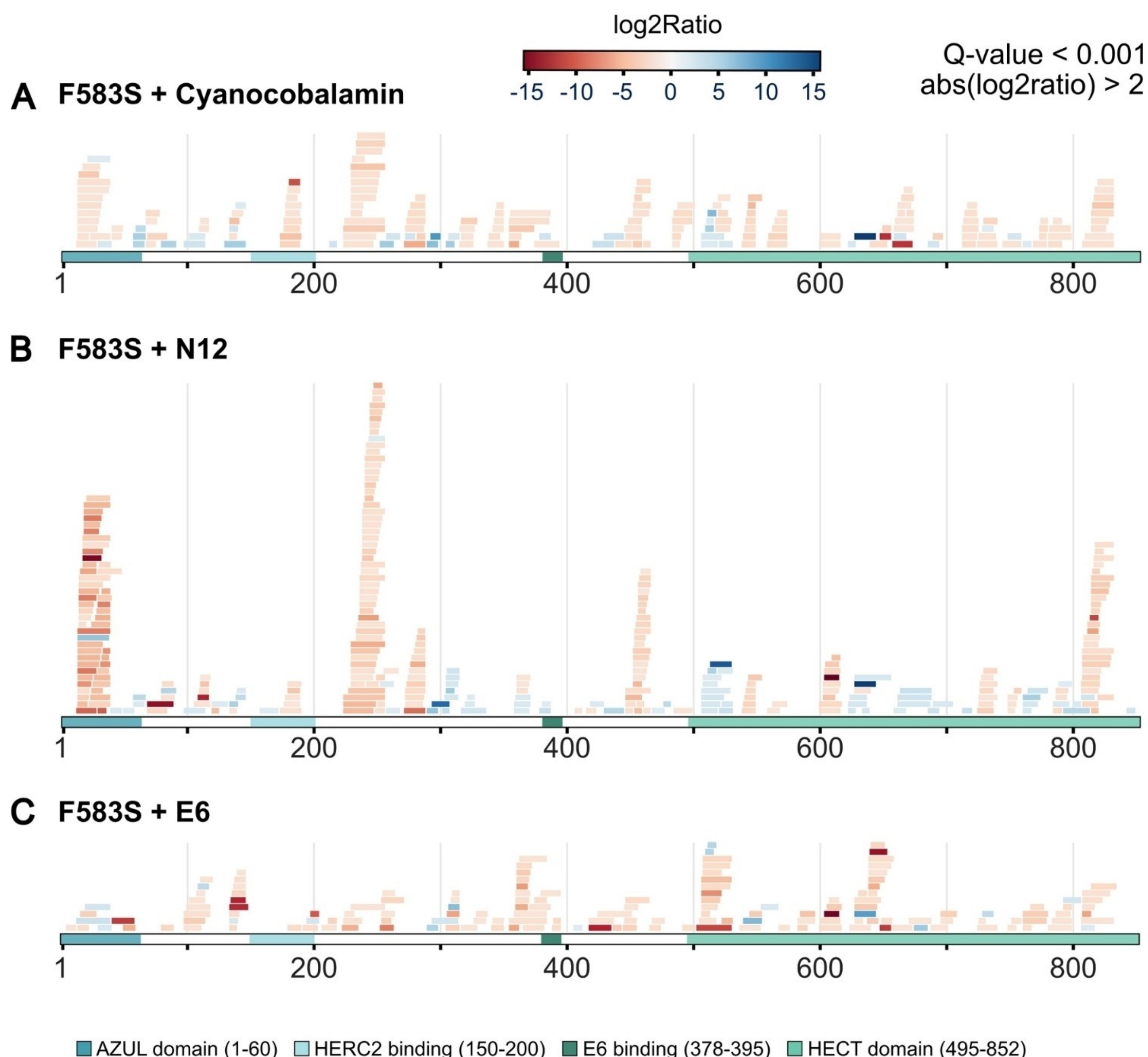

Figure 4. Small molecule activators and the HPV E6 oncoprotein affect different regions of E6AP-F583S. Shown are significantly altered peptide quantities in E6AP-F583S using LiP-MS in the presence of A) cyanocobalamin, B) the alloxazine derivative N12, and C) GST-16 E6. Fold changes of peptide quantities were determined by unpaired two-sample t-tests and considered significant with q-value <0.001 and log2ratio >2 (indicated in blue) or <-2 (indicated in red). For all comparisons, E6AP-F583S only was used as reference. Note that cyanocobalamin appears to have a more significant protective effect on E6AP-F583S than on wt E6AP (compare patterns in Figure 4A and Figure S5), suggesting that it may bind more stably to E6AP-F583S than to wt E6AP. For additional information, see Supporting Information.

helix is involved in binding of cyanocobalamin or alloxazine derivatives, it shows that the respective region is crucial for the E3 activity of E6AP.

In conclusion, we show that cobalamins can interact with certain AS-derived E6AP variants and wt E6AP and stimulate their E3 activity, indicating that besides their canonical function as coenzymes, cobalamins can exert additional functions. Although we have not yet been able to provide evidence that cobalamins can act on endogenous E6AP in cells, which is at least in part due to the fact that a reliable assay to measure the E3 activity of endogenous E6AP is not available, our data suggest that supplementation of the diet with cobalamins, which can cross the blood-brain barrier,[17] may be favorable for AS individuals. Firstly, in addition to rescuing the E3 activity of distinct AS-associated E6AP variants, residual levels of wt E6AP may be expressed from the paternal allele,[4a,b] the E3 activity of which can be stimulated by cobalamins. Secondly, current approaches in the treatment of AS aim at inducing the expression of the paternal *UBE3A* allele.[18] Yet, it remains unclear, if E6AP levels can be restored to those normally observed in neuronal cells. Since cobalamins can stimulate the E3 activity of wt E6AP and should have negligible, if any side effects, they may prove beneficial in combinatorial treatment regimens.

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Supporting Information

This communication is accompanied by a Supporting Information. The authors have cited additional references within the Supporting Information.[8,9,12,14a,19–25]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: high-throughput screen · UBE3A/E6AP · mass spectrometry · ubiquitination · enzymes

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