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WWP1 Gain-of-Function Inactivation of PTEN in Cancer Predisposition

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Abstract

BACKGROUND—Patients with *PTEN* hamartoma tumor syndrome (PHTS) have germline mutations in the tumor-suppressor gene encoding phosphatase and tensin homologue (*PTEN*). Such mutations have been associated with a hereditary predisposition to multiple types of cancer, including the Cowden syndrome. However, a majority of patients who have PHTS-related phenotypes have tested negative for *PTEN* mutations. In a previous study, we found that the E3 ubiquitin ligase WWP1 negatively regulates the function of PTEN.

METHODS—In a prospective cohort study conducted from 2005 through 2015, we enrolled 431 patients with wild-type *PTEN* who met at least the relaxed diagnostic criteria of the International Cowden Consortium. Patients were scanned for *WWP1* germline variants. We used the Cancer Genome Atlas (TCGA) data set as representative of apparently sporadic cancers and the Exome Aggregation Consortium data set excluding TCGA (non-TCGA ExAC) and the noncancer Genome Aggregation Database (gnomAD) as representative of population controls without a reported cancer diagnosis. We established both in vitro and murine in vivo models to functionally characterize representative *WWP1* variants.

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RESULTS—The existence of germline *WWP1* variants was first established in a family with wild-type *PTEN* who had oligopolyposis and early-onset colon cancers. A validation series indicated that *WWP1* germline variants occurred in 5 of 126 unrelated patients (4%) with oligopolyposis as a predominant phenotype. Germline *WWP1* variants, particularly the *WWP1* K740N and N745S alleles, were enriched in patients who did not have PHTS but had prevalent sporadic cancers, including *PTEN*-related cancer types in TCGA (odds ratio, 1.5; 95% confidence interval, 1.1 to 2.1; P = 0.01). The prioritized *WWP1* variants resulted in gain-of-function effects, which led to aberrant enzymatic activation with consequent PTEN inactivation, thereby triggering hyperactive growth-promoting PI3K signaling in cellular and murine models.

CONCLUSIONS—In this study involving patients with disorders resulting in a predisposition to the development of multiple malignant neoplasms without *PTEN* germline mutations, we confirmed the function of *WWP1* as a cancer-susceptibility gene through direct aberrant regulation of the PTEN–PI3K signaling axis. (Funded by the National Institutes of Health and others.)

HEREDITARY CANCER SYNDROMES, which account for approximately 10% of all cancers, serve as powerful models for the practice of precision medicine.[1] The identification of genes that are associated with seemingly rare cancer-predisposition syndromes often provides insights regarding aspects of normal development and pathways that can be targeted for the treatment of the more common sporadic forms of human cancers. One of these genes is *PTEN* (Online Mendelian Inheritance in Man database number, 601728), which encodes a dual-specificity lipid and protein phosphatase. This form of phosphatase has a classic tumor-suppressive function that is attributed largely to its ability to dampen the growth-promoting signaling cascade consisting of phosphatidylinositol 3-kinase (PI3K), AKT (also called protein kinase B), and mechanistic (previously called mammalian) target of rapamycin (mTOR).[2,3]

Regardless of the clinical diagnosis, patients who have germline *PTEN* mutations are described as having the *PTEN* hamartoma tumor syndrome (PHTS), which includes entities such as the Cowden syndrome, the Bannayan–Riley–Ruvalcaba syndrome (BRRS), the Proteus syndrome, and Proteus-like syndromes.[4] Clinically, the identification of a germline *PTEN* mutation not only establishes a PHTS molecular diagnosis but also informs cancer risk assessment and *PTEN*-specific medical management of mutation-positive probands and family members.[5,6] However, the fact that *PTEN* is mutated in only a subgroup of patients (approximately 25%)[7] poses a clinical challenge and yet offers a working hypothesis that wild-type *PTEN*-related overgrowth and neoplasia syndromes have other causes.

WWP1 is an E3 ubiquitin ligase enzyme that has been shown to be overexpressed or amplified in multiple types of cancer.[8–13] We recently found that WWP1 activation in animal and in vitro models inhibited PTEN function, which led to protumorigenic phenotypes.[14] In the study described here, we sought to determine and characterize whether human *WWP1* could be a broad cancer predisposition gene in neoplasia syndromes without *PTEN* mutations.

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Methods

STUDY PATIENTS AND OVERSIGHT

From 2005 through 2015, we prospectively enrolled patients with PHTS-related neoplasias from 66 sites, including academic medical centers, cancer centers, and community clinics associated with the International Cowden Consortium worldwide.[5,6] The study, which was sponsored by the National Institutes of Health and other granting agencies (including foundations), was approved by the institutional review board at the Cleveland Clinic. All the patients provided written informed consent.

Eligible patients met at least the relaxed operational diagnostic criteria of the International Cowden Consortium.[15] Relaxed criteria are defined as full criteria minus one factor, with such patients being described as having a Cowden-like syndrome. (Details regarding these criteria are provided in the Methods section in Supplementary Appendix 1, available with the full text of this article at NEJM.org.) The Cowden syndrome is characterized by an elevated lifetime risk of a specific set of cancers, which are collectively called Cowden component cancers.[5] All the patients underwent analysis for the presence of mutations and deletions in *PTEN* and *SDHx* (including *SDHB*, *SDHC*, and *SDHD*, which encode succinate dehydrogenase).[6] Only patients without pathogenic germline mutations in *PTEN* or *SDHx* were included in the study (Table S1 in Supplementary Appendix 1).

GENETIC STUDIES

For the discovery series of patients, we performed whole-exome sequencing of peripheral-blood DNA obtained from 83 probands, as described in detail in Supplementary Appendix 1. We performed Sanger sequencing using region-specific mutational analysis based on polymerase-chain-reaction assays to validate variants that had been prioritized through whole-exome sequencing. For the extended validation series of patients, we performed high-resolution melting analysis to scan for *WWP1* variants in an additional 348 patients. Suspected variants were validated with the use of Sanger sequencing. To compare frequencies among samples obtained from patients with various disorders and apparently healthy populations, we used the Cancer Genome Atlas (TCGA) data set as representative of apparently sporadic cancers and the Exome Aggregation Consortium data set excluding TCGA (non-TCGA ExAC) and the noncancer Genome Aggregation Database (gnomAD) as representative population controls without a reported cancer diagnosis.

IN VITRO FUNCTIONAL STUDIES

We performed in vitro assays in human-derived, commercially available cell lines, including 293T (embryonic kidney) cells, HCT116 (colorectal carcinoma) cells, and DLD-1 (colorectal adenocarcinoma) cells. We also used CRISPR (clustered regularly interspaced short palindromic repeats) techniques to generate knock-in *WWP1* wild-type and mutant mice and derived mouse embryonic fibroblast cells. Stable cell lines were generated either through lentiviral transduction or through CRISPR technology. We performed Western blot analysis (denaturing and native), immunoprecipitation, in situ ubiquitination assays, and soft agar colony-formation assays on wild-type and mutant cells.[14]

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IN VIVO XENOGRAFT MURINE MODELING

For assaying tumor growth in the xenograft model, 7-week-old male *FOXN* nude mice housed in specific pathogen-free environments were injected subcutaneously with stable HCT116 or DLD-1 derivatives. The care and treatment of animals were approved by the animal research committee at Beth Israel Deaconess Medical Center. Detailed protocols regarding the in vitro and in vivo studies are provided in Supplementary Appendix 1.

STATISTICAL ANALYSIS

For the genetic analysis, we used OpenEpi software (Open Source Epidemiologic Statistics for Public Health) to calculate odds ratios for the presence of *WWP1* variants (http://openepi.com/Menu/OE_Menu.htm). For analyses comparing various population groups, we used two-by-two tables to calculate odds ratios. We calculated 95% confidence intervals and corresponding P values using the mid-P exact test. An odds ratio of more than 1.0 implied that the incidence was greater in the population of interest than in the standard population. A P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

IDENTIFICATION OF *WWP1* AS A CANDIDATE GENE IN *PTEN* WILD-TYPE PROBANDS

The hypothesis that *PTEN* is mutated in only a subgroup of patients with PHTS-related neoplasias[7] points toward other unidentified causes of cancer predisposition in patients with wild-type *PTEN*. To address this hypothesis, we evaluated WWP1, a HECT-type ubiquitin E3 ligase (one of three broad types of ubiquitin E3 ligases; HECT stands for homologous to the E6-AP carboxy terminus) that has recently been shown to reduce PTEN tumor-suppressive function through polyubiquitination-mediated inhibition of PTEN dimerization and membrane recruitment.[14] If our hypothesis was correct, then the existence of germline *WWP1* variants in patients with wild-type *PTEN* would imply impaired PTEN function, independent of *PTEN* mutational status.

First, we investigated whole-exome sequencing data from 83 probands with wild-type *PTEN* who met at least the relaxed operational diagnostic criteria of the International Cowden Consortium (Table S1).[15] After performing variant filtration, quality control, and Sanger sequencing validation, we initially identified one proband with a Cowden-like syndrome who had a germline heterozygous *WWP1* c.2220G→C, p.K740N variant occurring at a highly evolutionarily conserved amino acid residue (Fig. 1A and Fig. S1). Patients with a Cowden-like syndrome do not meet the strict operational diagnostic criteria (i.e., minus one criterion); only 5% of these patients have germline *PTEN* mutations.[16] The proband belonged to a family with a notable history of early-onset colon cancer and the occurrence of gastrointestinal oligopolyposis (Fig. 1B). The same germline *WWP1* variant was found in two other affected family members (Family Members II-4 and IV-6) and was absent in three unaffected family members (Family Members II-5, IV-4, and IV-8), which indicated phenotype-dependent segregation (Figs. S2 and S3).

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VALIDATION SERIES

In patients with *PTEN* mutations who have undergone colonoscopy, more than 90% have colorectal polyps that are typically found in a mix of histologic subtypes.[17] Patients with PHTS with colorectal cancer tend to have either preexisting or coexisting colonic mixed polyposis. Gastrointestinal hamartomatous polyposis is also one of the classic manifestations of BRRS.[18] We therefore expanded our analyses to scan for *WWP1* germline variants in 126 patients with oligopolyposis and in 123 patients with BRRS who had wild-type *PTEN*. We identified 5 more unrelated patients with oligopolyposis and underlying *WWP1* germline variants (Fig. 1C, Fig. S4, and Table S2). Lymphoblastoid cell lines derived from patients with *WWP1* variants showed normal PTEN protein levels that were similar to levels in patients with wild-type *PTEN* (Fig. 1D). This finding was consistent with a mechanism of action for WWP1 that was independent of PTEN levels.[14] We did not identify additional *WWP1* variants in patients with BRRS or in a series of patients who did not meet the selection criteria of polyposis or BRRS but who had a high PHTS-related phenotypic burden. Overall, we identified germline heterozygous *WWP1* variants in 5 of 126 patients (4%) with a Cowden-like syndrome with wild-type *PTEN* who had gastrointestinal oligopolyposis as a predominant phenotype. Details regarding these analyses are provided in Supplementary Appendix 1.

APPARENTLY SPORADIC CANCERS

WWP1 is a proto-oncogene that has been shown to be somatically overexpressed or amplified in multiple cancer types.[8–13] To determine whether *WWP1* germline variants are overrepresented in the common apparently sporadic cancers, we analyzed the spectrum and frequencies of germline variants in 33 cancer types from TCGA, representing 10,389 cases (Table S3).[19] We found predicted deleterious germline *WWP1* variants in 28 of the 33 TCGA cancer types (Fig. 2A and 2B and Table S4 in Supplementary Appendix 2, which also includes Tables S5 through S9). The two most prevalent variants (c.2234A→G, p.N745S and c.2220G→C, p.K740N), which occurred in 22 of the 33 cancer types, accounted for 59 of 109 (54%) identified germline *WWP1* variants (Fig. 2B and Table S4). These two variants occur in the catalytic C-terminal HECT domain of WWP1 and were also identified in two patients with Cowden-like syndrome who had oligopolyposis (Fig. 1C).

Our findings indicate that rare germline *WWP1* variants (minor allele frequency [MAF], 0.1%) exist in patients with a Cowden-like syndrome who have oligopolyposis and in those with common sporadic cancers. This finding prompted us to investigate the frequency of *WWP1* variants in patients without a reported cancer diagnosis. To this end, we first analyzed the non-TCGA ExAC data set[20] representing 53,105 population controls without prevalent cancer. Implementing filtration criteria that were identical to those used in the analysis of TCGA patients, we identified predicted deleterious *WWP1* variants (MAF, 0.1%) in 428 of 106,210 alleles (Table S5 in Supplementary Appendix 2). In comparison, TCGA patients with apparently sporadic cancers showed deleterious *WWP1* variants in 109 of 20,778 alleles (Table S4). These data indicate a significantly higher frequency of deleterious *WWP1* variants in a cancer-affected population (TCGA) than in population controls without cancer (non-TCGA ExAC) (odds ratio, 1.3; 95% confidence interval [CI], 1.1 to 1.6; P = 0.02).

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Although we observed the *WWP1* variants K740N and N745S in non-TCGA ExAC, these variants remained overrepresented in TCGA patients with cancer (odds ratio, 1.6; 95% CI, 1.2 to 2.1; P = 0.003). We further substantiated these observations in the subgroup of patients with apparently sporadic Cowden syndrome component cancers, in whom we noted a significantly higher frequency of *WWP1* variants than that in the ExAC population controls (odds ratio, 1.5; 95% CI, 1.1 to 2.1; P = 0.01), particularly *WWP1* K740N and N745S (odds ratio for the presence of either allele, 1.9; 95% CI, 1.2 to 2.8; P = 0.006). Similarly, an analysis of the noncancer subgroup (233,473 alleles) in the expanded gnomAD database revealed deleterious *WWP1* variants (MAF, 0.1%) in 637 alleles (Table S6 in Supplementary Appendix 2). Thus, similar to the patients in the ExAC non-TCGA listings, patients with cancer in the TCGA database had significantly higher frequencies of *WWP1* variants than those without reported cancer in the gnomAD database (odds ratio, 1.9; 95% CI, 1.6 to 2.4; $P<1\times10^{-7}$). This observation remained consistent for patients in TCGA with Cowden component cancers (odds ratio, 2.3; 95% CI, 1.7 to 3.0; $P = 3\times10^{-6}$).

COMPARISON WITH CLASSIC CANCER-PREDISPOSITION GENES

Next, we compared germline allele frequencies of *WWP1* with those of genes that have been classically known to be associated with polyposis or a heredity predisposition to colon cancer, including *SMAD4*, *BMPR1A*, *MSH2*, *PMS2*, and others (Fig. 2C and Table S7 in Supplementary Appendix 2). An analysis of the colon adenocarcinoma subgroup in the TCGA database on the basis of its relevance to the predominant oligopolyposis phenotype revealed a similar and significant frequency of germline variants among *WWP1* and the representative genes for polyposis and a genetic predisposition for colon cancer. Notably, even though *PTEN* is known to be associated with colon-cancer susceptibility, we did not identify germline *PTEN* mutations in this database. We then compared germline allele frequencies of *WWP1* with those of classic cancer-susceptibility genes, including *PTEN*, *TP53*, *BMPR1A*, and *STK11*, across all 33 TCGA cancer types. We identified significantly higher frequencies of *WWP1* variants in multiple cancer types, including two Cowden component cancers (colon adenocarcinoma and thyroid cancer), than in non-TCGA ExAC population controls[5] (Fig. 2D and Tables S8 and S9 in Supplementary Appendix 2). As illustrative positive controls, we similarly identified higher frequencies of variants in other cancer-predisposition genes, including germline mutations in *PTEN* that have been associated with breast cancer and uterine cancer and in *TP53* mutations that have been associated with adrenocortical carcinoma and sarcoma (Fig. 2D). Overall, these observations support a nonrandom enrichment of *WWP1* germline variants among patients who have oligopolyposis, a hereditary predisposition for cancer, or particular malignant neoplasias. An analysis of all types of cancer in the study patients revealed that the majority of predicted deleterious germline *WWP1* variants occurred within the HECT catalytic domain (odds ratio, 2.9; 95% CI, 1.7 to 5.1; P<0.001) (Fig. 3A), which led to our hypothesis that mutation-mediated catalytic hyperactivation of WWP1 could be the underlying mechanism for tumorigenesis.

FUNCTIONAL CHARACTERIZATION OF *WWP1* VARIANTS

To address our hypothesis, we first analyzed both colon and kidney cell lines as relevant to the observed human phenotypes. We first assessed whether representative germline variants

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affect WWP1 catalytic activity toward PTEN polyubiquitination, based on the identification of the HECT-type E3 ubiquitin ligase WWP1 as a physical interactor that functionally triggers atypical nondegradative K27-linked polyubiquitination of PTEN to suppress its dimerization, membrane recruitment, and tumor suppressive function.[14] We prioritized the three most frequently mutated *WWP1* variants: R86H (C2 domain) along with K740N and N745S (both in the HECT domain) (Table S4). As proof of principle, we prioritized *WWP1* R86H on the basis of frequency alone, even though the variant did not pass computational filters for deleteriousness. (Gain-of-function mutations are difficult to predict with computational filters, since functional effects can be understood only in the context of the particular biologic features of the gene.[21]) Overexpression of wild-type WWP1 in human embryonic kidney 293T (HEK293T) cells increased PTEN polyubiquitination, whereas the expression of individual WWP1 mutants (R86H, K740N, and N745S) further exacerbated this effect without affecting PTEN protein levels; these findings indicate that these mutants play a gain-of-function role in triggering PTEN polyubiquitination (Fig. S5A). The expression of WWP1 mutants suppressed PTEN dimerization or oligomerization in a process that was similar to wild-type WWP1 overexpression or amplification,[14] as evaluated in native gel analyses (Fig. S5B). This expression in turn triggered downstream AKT activation in both HCT116 and DLD-1 colon cancer cells with endogenous wild-type PTEN (Fig. 3B and Fig. S5C).

Next, to study the aberrant PTEN regulation by WWP1 mutants endogenously, we used CRISPR technology to generate endogenous knock-in $WWP1^{K740N/+}$ HCT116 cells, which reproduced the mutant heterozygous genotype observed in human patients (Fig. S6). In vivo ubiquitination assays revealed that endogenous K740N knock-in WWP1 mutant protein robustly elevated PTEN polyubiquitination, which in turn inhibited PTEN dimerization or oligomerization (Fig. 3C). In support of our hypothesis, *WWP1* K740N knock-in suppressed PTEN membrane localization and in turn promoted AKT activation, as evaluated by cell fractionation and Western blotting analyses (Fig. 3D). To further mimic physiologic conditions (i.e., the constitutional heterozygous mutant state), we also generated CRISPR knock-in K736N mutant mice (corresponding to the K740N variant in humans) and further isolated primary mouse embryonic fibroblasts. We specifically prioritized the *WWP1* K740N variant to generate the knock-in mice since it represents the variant that is segregated in our family with Cowden-like syndrome (Fig. 1B) and is one of the most frequently mutated *WWP1* variants in apparently sporadic cancers (Table S4). Western blot analyses of protein lysates derived from mouse embryonic fibroblasts with the $Wwp1^{K736N/+}$ genotype showed stronger activation of the PI3K–AKT–mTOR pathway than mouse embryonic fibroblasts with the wild-type $Wwp1^{+/+}$ genotype, as revealed by higher levels of phosphorylation of AKT and ribosomal protein S6 (Fig. S7).

WWP1, which belongs to the NEDD4 family of ubiquitin E3 ligases, contains a C2 domain and four WW domains, along with the catalytic HECT domain. Previous studies have shown that members of the NEDD4 family of E3 ubiquitin ligases are characterized by an autoinhibitory regulatory mechanism that is mediated by interactions between its different domains,[22] such as the interaction between the HECT domain and the WW2–WW3 linker region in WWP2 (another E3 ubiquitin ligase closely related to WWP1)[23] and interaction between the C2 domain and the HECT domain in SMURF2 or SMURF1.[24–26] Chen et al.

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found that deletion of the WW2–WW3 linker activated full-length WWP1.[23] Therefore, one possibility is that the K740N mutation within the HECT domain disrupts the autoinhibitory interaction of this domain with the WW2–WW3 linker, which results in uncontrolled activation and gain-of-function effects. We found that the wild-type WWP1 HECT domain interacts with the N-terminal region of WWP1 (including the C2–WW1–4 domains), whereas the K740N mutation within the HECT domain decreased its binding with the N-terminal domain (Fig. 4A and 4B). Moreover, the deletion of the WW2–WW3 linker or inclusion of the C2 domain alone robustly decreased the respective interactions with the HECT domain, which corroborates the essential role of the WW2–WW3 linker and the dispensable role of the C2 domain in mediating these interactions (Fig. 4A and 4B). Functionally, as compared with wild-type WWP1, deletion of the WW2–WW3 linker triggered WWP1 catalytic activity, as revealed by its autoubiquitination and AKT activation (Fig. S8A and S8B). Native gel analyses revealed that the K740N mutation of WWP1 suppressed its ability to dimerize and oligomerize (Fig. S8C). Hence, several lines of evidence suggest that the *WWP1* K740N mutation may promote a gain of catalytic activity by relieving the autoinhibitory physical interaction between its HECT domain and the WW2–WW3 linker region (Fig. 4C).

XENOGRAFT MURINE MODELING

To determine the influence of *WWP1* mutations on tumorigenesis and tumor growth in vivo, we next compared the oncogenic potential of wild-type *WWP1* with endogenous CRISPR knock-in $WWP1^{K740N/+}$ mutant cells. In support of our working model, the endogenous WWP1 K740N knock-in mutation, which strongly increased PTEN polyubiquitination (Fig. 3C), resulted in a higher level of anchorage-independent growth than wild-type WWP1 (Fig. 5A). Similarly, the overexpression of various germline WWP1 mutants, including R86H, K740N, and N745S, in DLD-1 colon cancer cells also markedly increased their tumorigenic potential in vitro and tumor growth in vivo in terms of both tumor weight and size, as evaluated in analyses involving soft agar and xenograft tumor methods, respectively (Fig. 5A and Fig. S9). In addition, tumors with the endogenous *WWP1* K740N variant ($WWP1^{K740N/+}$) grew much faster than those expressing wild-type WWP1 in a xenograft tumor model system, as revealed by analyses of tumor growth, weight, and size (Fig. 5B and 5C). Western blot analyses of tumors derived from cells of $WWP1^{+/+}$ or $WWP1^{K740N/+}$ genotypes showed the stronger oncogenic activity of the WWP1 K740N mutant in triggering hyperactivation of the PI3K–AKT–mTOR oncogenic pathway (Fig. 5C).

DISCUSSION

Heritable mutations in multiple genes for both adenomatous and hamartomatous polyposis have been identified over the years. However, the genetic basis for predisposition to oligopolyposis is elusive. We found that germline *WWP1* variants occur in patients with wild-type *PTEN* with characteristic PHTS-like phenotypes, particularly oligopolyposis. Patients with *WWP1* germline mutations include those with Cowden-like syndrome (Table S1), with the shared clinical diagnosis of at least five gastrointestinal polyps, including at least one hamartomatous polyp or a polyp that is hyperplastic or serrated. In addition, all the patients were found to have wild-type genes that are known to be associated with a polyposis

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syndrome (*PTEN*, *BMPR1A*, *SMAD4*, *ENG*, *APC*, and *STK11*).[17,27] Thus, mutations in *WWP1* may represent a clear genetic cause of oligopolyposis, in which we found a mechanistic pathogenesis for intestinal neoplasia and cancer.

We further found that germline *WWP1* variants were also markedly enriched in patients affected by prevalent, apparently sporadic human cancers. Notable enrichment of *WWP1* variants was observed in *PTEN*-related Cowden syndrome component cancers,[5] particularly colorectal adenocarcinoma and thyroid cancer. These data suggest that *WWP1* may represent a broad cancer predisposition gene that warrants further investigation in *PTEN*-independent contexts. Although further data are required before recommending routine *WWP1* genetic testing in the hereditary cancer clinic, the most prevalent and functionally characterized *WWP1* K740N and N745S mutations may be considered in patients with wild-type *PTEN* who have a Cowden-like syndrome and an unexplained personal or family history of oligopolyposis or early-onset colorectal cancers.

We found that representative *WWP1* variants provide mechanistic gain of function by relieving the autoinhibitory interaction between their WW2–WW3 linker and HECT domains. This process results in aberrant enzymatic activation of the WWP1 E3 ligase, with consequent ubiquitination and inactivation of PTEN to trigger hyperactivation of PI3K signaling. Although we found both in vitro and in vivo evidence of the deleterious effects of the three most frequently mutated prioritized variants (R86H, K740N, and N745S), the functional significance and associated mechanisms of the remaining variants have yet to be determined. Thus, we identified *WWP1* as a proto-oncogenic neoplasia-susceptibility gene through aberrant regulation of PTEN–PI3K signaling, independent of germline *PTEN* mutational status. These findings have important implications for cancer prevention and therapy, since WWP1 and the pathway it regulates are potential therapeutic targets,[14] whereas targeting PTEN has not been feasible in clinical practice to date.[28]

Before mechanism resolution, WWP1 had been shown to be overexpressed or amplified in multiple tumor types, which suggested that it had a role as an oncogene.[8–13] It was only recently that WWP1 was shown to be a switch that could be targeted to reactivate PTEN.[14] WWP1 depletion resulted in reduced oncogenic PI3K–AKT signaling in mouse embryonic fibroblast cells with *Pten* mutations or heterozygous deletions; the presence of such mutations causes PHTS.[4] Indole-3-carbinol, a compound found in cruciferous vegetables, was found to be a natural and potent WWP1 inhibitor.[14] Thus, our findings suggest that patients with wild-type *PTEN* with germline *WWP1* variants may also benefit from the modulation of the WWP1–PTEN axis as a preventative or therapeutic measure. Clinical trials are anticipated to determine the effective dose and efficacy in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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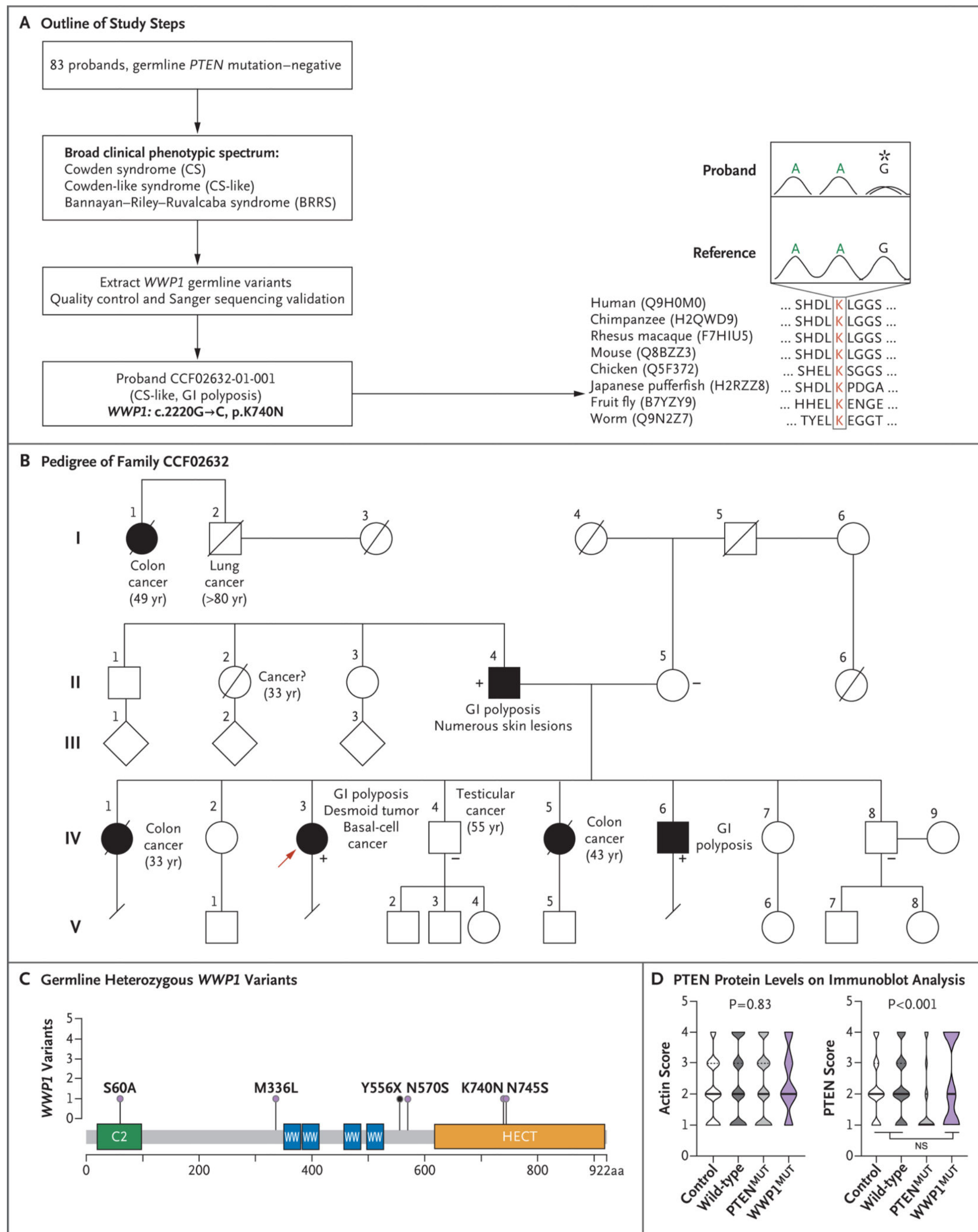

Figure 1. Identification of Germline Heterozygous *WWP1* Variants in Cowden-like Syndrome.

Panel A provides an outline of the steps that were performed in the *WWP1* discovery study. The missense heterozygous *WWP1* variant shows high evolutionary conservation of the affected amino acid residue across seven different species besides human. UniProt accession codes are presented in parenthesis for each species, and the affected amino acid is shown in red in the boxed area. Above the sequence alignments is the Sanger sequencing chromatogram from proband CCF02632–01-001; the asterisk indicates the location of the mutation. GI denotes gastrointestinal, and K lysine. Panel B shows the pedigree of the

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CCF02632 family with the presence of wild-type *WWP1* (−) or the *WWP1* K740N variant (+). Male family members are indicated by squares, female family members by circles, affected members by solid figures, unaffected members by open figures, and deceased members by a slash. The proband (IV-3) for whom whole-exome sequencing was performed is indicated by a red arrow. Roman numerals on the left represent the generation number, and Arabic numerals represent consecutively numbered family members within each generation. The age at the time of the cancer diagnosis, when known, is indicated in parentheses. Panel C shows germline heterozygous *WWP1* variants that were identified in five unrelated probands with Cowden-like syndrome; K740N denotes the variant identified in the CCF02632 family. The illustration represents WWP1 protein functional domains. Arabic numerals correspond to amino acid numbers. Variants are depicted in the lollipop plot overlying the WWP1 protein structure. The black lollipop indicates a truncating variant. The frequency of each variant correlates with the height of the vertical line representing each lollipop. C2 denotes C2-domain, HECT homologous to the E6-AP carboxyl terminus domain, and WW WWP repeating motif. Panel D shows violin plots indicating PTEN protein levels on immunoblot analysis. Protein lysates were extracted from lymphoblastoid cell lines derived from 26 population controls, from patients with oligopolyposis (121 with wild-type *PTEN* and *WWP1* and 5 patients with *WWP1* mutations), and from 107 patients with *PTEN* mutations. Protein levels are divided across quartiles, with 1 indicating the lowest level and 4 indicating the highest. Solid black horizontal bars indicate median protein scores; dashed bars indicate quartiles. Actin was used as a loading control. P values are derived from Kruskal–Wallis one-way testing of analysis of variance comparing all four groups. For the PTEN score (plot at right), unpaired two-tailed t-tests indicate a significant difference only for mutant PTEN as compared with the other three groups. NS denotes not significant. In contrast to PTEN protein levels in cells from germline *PTEN* mutation carriers, these levels were similar among the patients with *WWP1* variants, those with oligopolyposis who had wild-type *PTEN* or *WWP1*, and controls.

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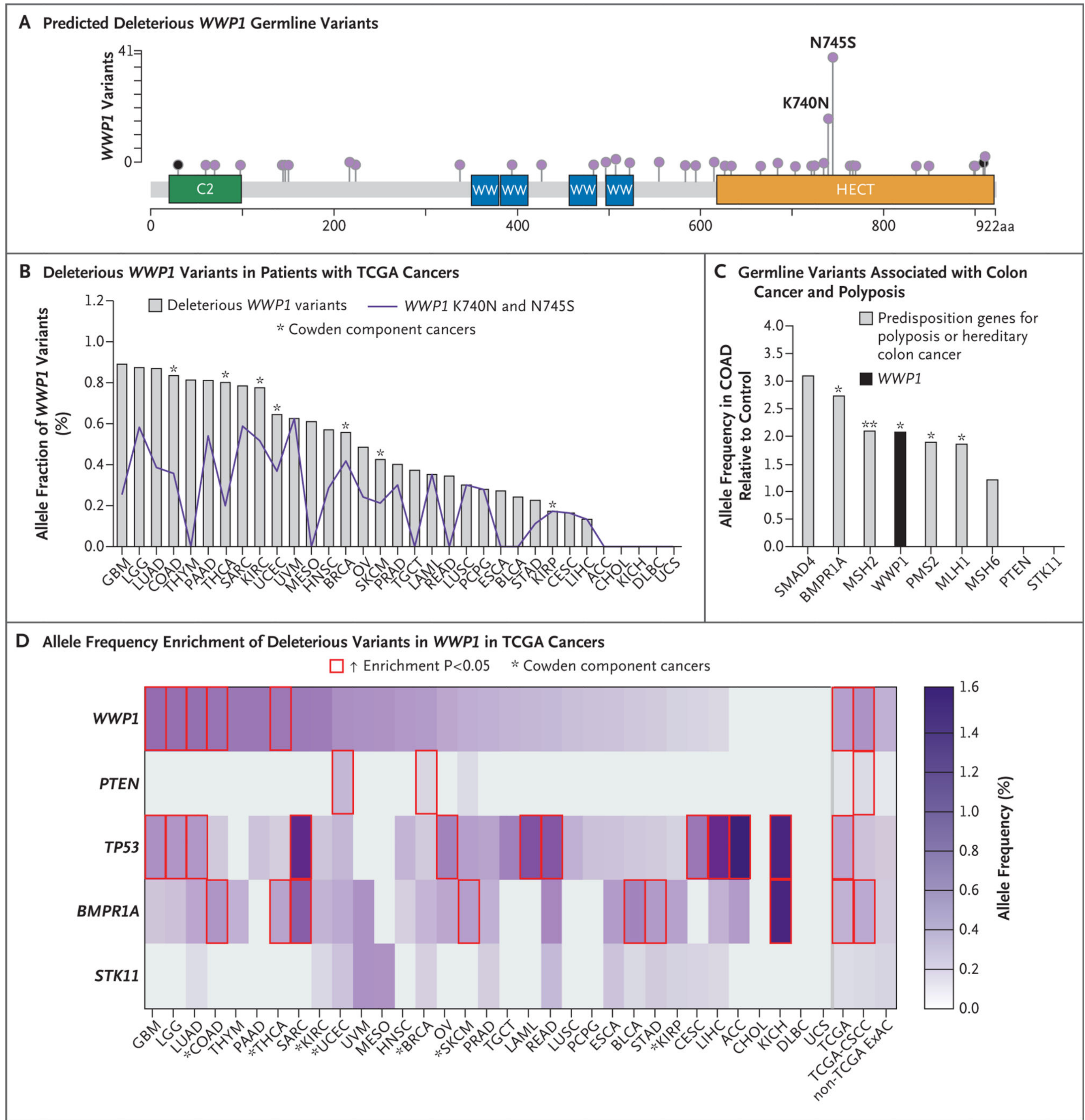

Figure 2. Enrichment of Germline *WWP1* Variants in 33 Cancer Types from the Cancer Genome Atlas (TCGA).

Panel A shows the predicted deleterious germline *WWP1* variants that have been identified in patients with 33 TCGA cancer types. Variants are depicted in the lollipop plot overlying the WWP1 protein structure. Black lollipops indicate truncating variants. The frequency of the variant correlates with the height of the vertical line representing each lollipop. Panel B shows the percentage of deleterious *WWP1* variants identified in patients with TCGA cancers. The purple line depicts the frequency of *WWP1* K740N and N745S variants. Asterisks indicate Cowden syndrome component cancers represented in TCGA. Panel C

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shows germline variants in known predisposition genes for colon cancer and polyposis identified in the TCGA colorectal adenocarcinoma (COAD) data set. Allele frequencies are normalized to background allele frequencies in the Exome Aggregation Consortium (ExAC) data set excluding TCGA cancers. One asterisk indicates P<0.05 and two asterisks P<0.01 for the comparison between the two databases, as calculated by one-tailed mid-P exact testing. Panel D shows a heat map of allele frequency enrichment of deleterious variants in *WWP1* across all 33 TCGA cancer types, as compared with known cancer predisposition genes. The red outlines indicate that the cancer type has significant enrichment (P<0.05 by one-tailed mid-P exact testing) for patients with deleterious germline variants of a particular gene, as compared with non-TCGA ExAC population controls without cancer. The last three columns represent collective summaries for each gene. CSCC denotes Cowden syndrome component cancers.

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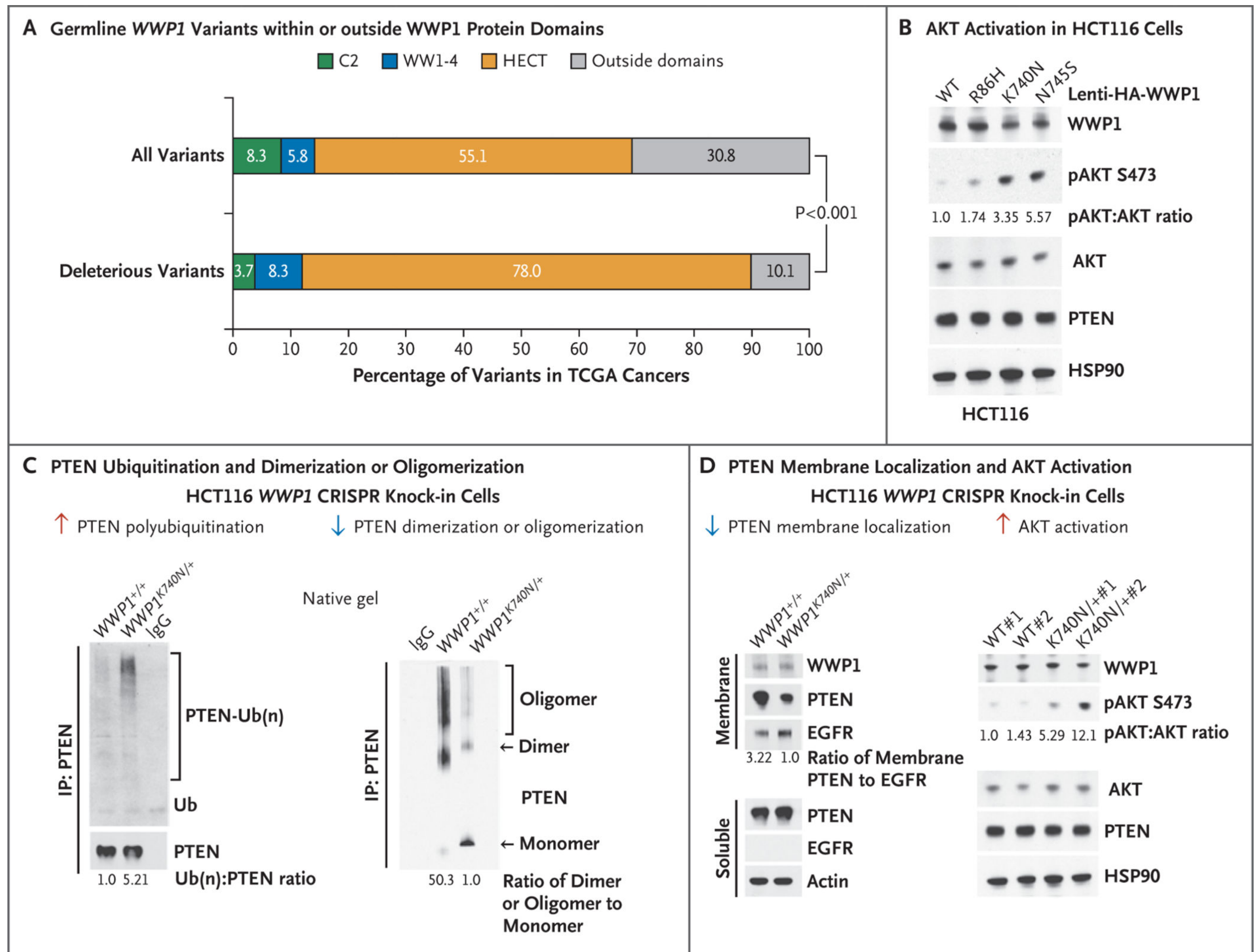

Figure 3. Increase in PTEN Polyubiquitination, Membrane Dissociation, and Subsequent AKT Activation with *WWP1* Gain-of-Function Mutations.

Panel A shows the distribution of germline *WWP1* variants within or outside WWP1 protein domains. Amino acid boundaries of each structural domain were extracted from UniProt (WWP1_HUMAN, Q9H0M0) as follows: C2 (5–98), WW1 (349–382), WW2 (381–414), WW3 (456–489), WW4 (496–529), and HECT (588–922). Panel B shows the activation of AKT (also called protein kinase B) in colorectal carcinoma cells (HCT116), with stable expression of the indicated constructs. Total lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then probed with the indicated antibodies. Lenti-HA denotes lentiviral HA-tagged plasmids. Panel C shows an analysis of HCT116 cells with $WWP1^{+/+}$ or CRISPR knock-in $WWP1^{K740N/+}$ with respect to endogenous PTEN ubiquitination (blot at left) and dimerization and oligomerization (blot at right) by immunoprecipitation (IP) of PTEN, followed by Western blotting with the use of the indicated antibodies. Ub(n) denotes the number of ubiquitin chains. Panel D shows Western blot analysis of membrane and soluble fractions isolated from HCT116 cells with $WWP1^{+/+}$ and $WWP1^{K740N/+}$ with respect to PTEN membrane localization (blot at left) and AKT activation (blot at right). In the blot at left, epidermal growth factor receptor (EGFR)

served as a membrane marker and actin as the internal control. In the blot at right, total lysates were resolved by SDS-PAGE and then probed with indicated antibodies.

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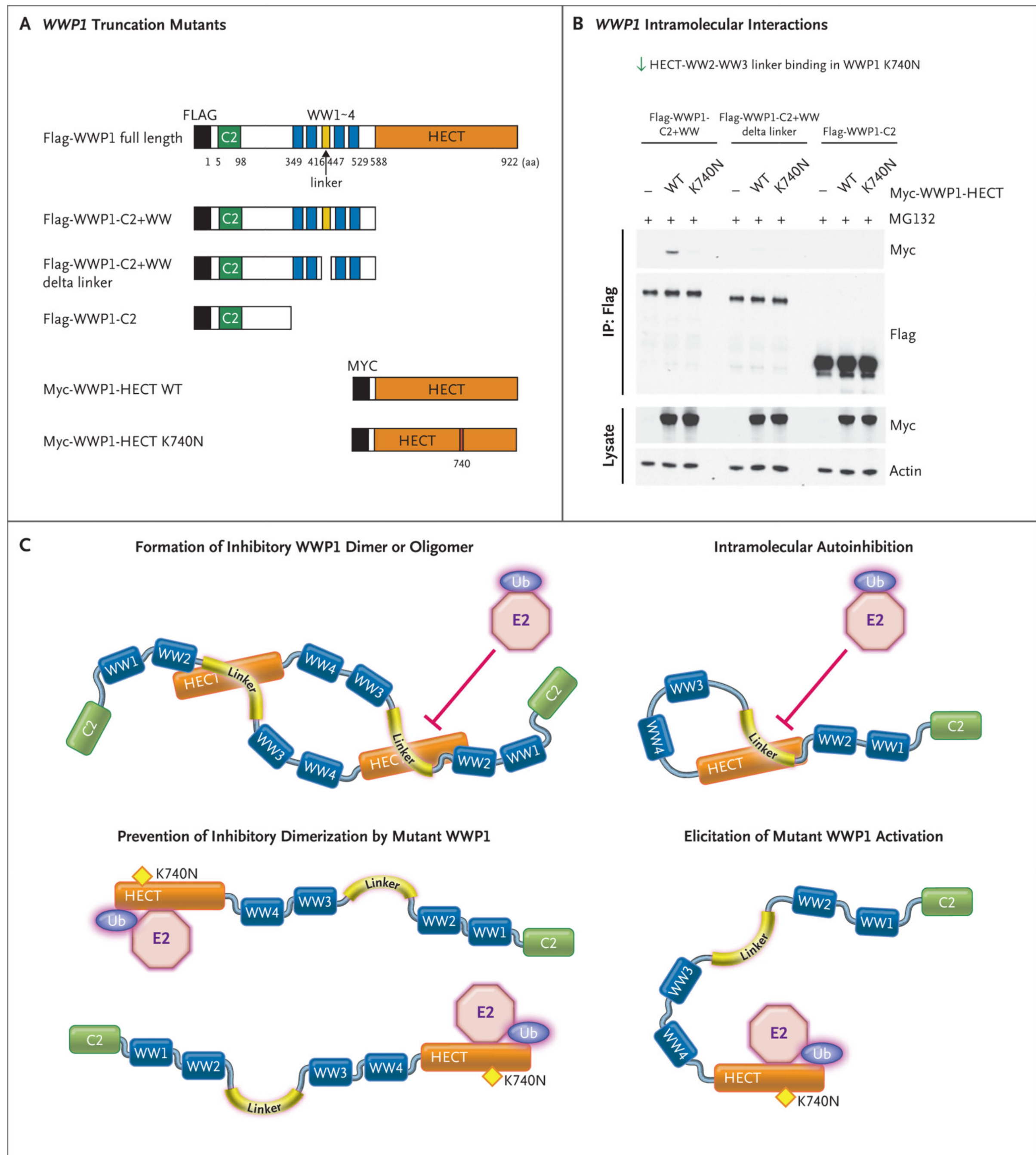

Figure 4. Promotion of WWP1 Catalytic Activity by *WWP1* K740N through Disruption of Interaction between HECT and WW2–WW3 Linker Domains.

Panel A shows the truncation mutants of WWP1 with or without the K740N mutation within the HECT domain. Both Flag and Myc are short peptides used to tag proteins. Panel B shows an immunoprecipitation analysis of the interaction between multiple Flag-WWP1 truncation mutants and individual Myc-tagged HECT domains with or without the K740N mutation. Immunoprecipitation (IP) of Flag-WWP1 truncation mutants was performed with Flag antibody, followed by probing with Myc-tag antibody to detect the differential interaction between the HECT and WW2–WW3/C2 domains with or without the K740N

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mutation. Panel C shows a model of how the WWP1 E3 ligase activity is activated by the K740N variant by the disruption of WWP1 autoinhibition mediated by either homodimer or intramolecular processes. Ubiquitin conjugation enzyme, also known as E2, performs the second step in the ubiquitination process to coordinate with ubiquitin ligase for the transfer of ubiquitin moieties to the substrates. In turn, the inhibition of WWP1 activity is mediated by the interaction of its HECT domain with the WW2–WW3 linker domain.

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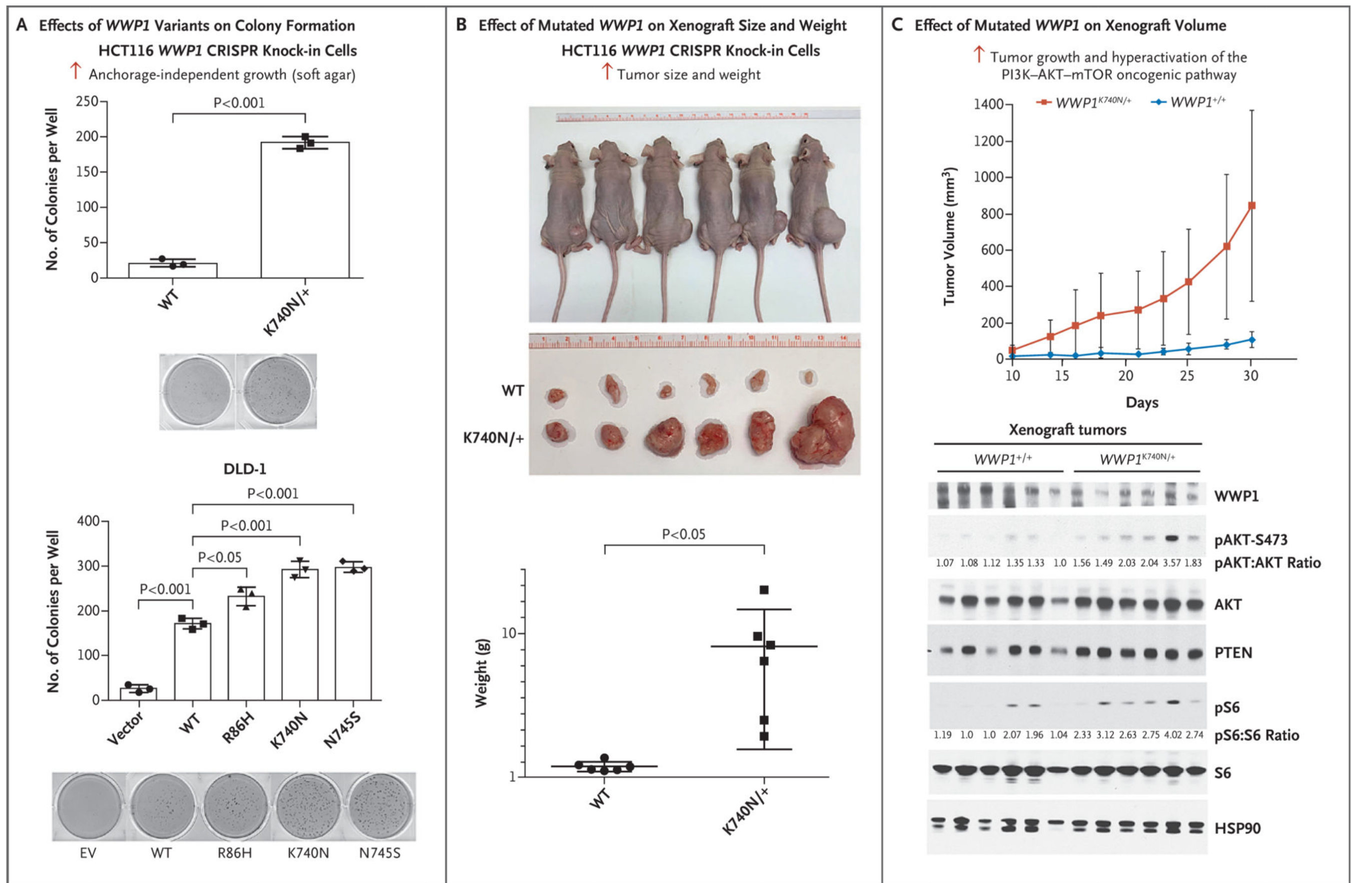

Figure 5. Characterization of Mutated *WWP1* in Vitro and in Vivo.

Panel A shows the effects of *WWP1* germline variants on colony-forming ability in soft agar in HCT116 cells (top graph) and in a colorectal adenocarcinoma cell line (DLD-1) (bottom graph). The numbers of colonies are quantified and presented as means of triplicate experiments. Panel B shows tumor xenograft assays performed by subcutaneously implanting HCT116 cells with either $WWP1^{+/+}$ or $WWP1^{K740N/+}$ genotypes in a group of six mice. Wild-type tumors were implanted on the left side of the mice, and tumors that stably expressed the K740N variant were implanted on the right side. The mean weight of the resulting tumors was much higher in mice implanted with the K740N genotype. Panel C shows mean tumor volumes as a function of the time in days in the same group of mice. After the mice were euthanized, the tumors were dissected and were analyzed by means of Western blotting with the use of the indicated antibodies. In all three panels, I bars indicate standard deviations.