

# Two Modes of Degradation of the Tramtrack Transcription Factors by Siah Homologues\*

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The Siah proteins, mammalian homologues of the *Drosophila* Sina protein, function as ubiquitin-protein isopeptide ligase enzymes to target a wide range of cellular proteins for degradation. We report here a novel *Drosophila* protein that is homologous to Sina, named Sina-Homologue (SinaH). We show that it can direct the degradation of the transcriptional repressor Tramtrack (Ttk) using two different mechanisms. One is similar to Sina and requires the adaptor Phyllopod, and the other is a novel mechanism of recognition. This novel mode of targeting for degradation is specific for the 69-kDa Ttk isoform, Ttk69. Ttk69 contains a region that is required for binding of SinaH and for SinaH-directed degradation. This region contains an AXVXP motif, which is the consensus sequence found in Siah substrate proteins. These results suggest that degradation directed by SinaH differs from that directed by Sina and is more similar to that found in vertebrates. We speculate that SinaH may be involved in regulating the levels of developmentally important transcription factors.

Proteins are tagged for selective destruction in the proteasome by covalent polyubiquitination, a process that requires the concerted action of E1,<sup>2</sup> E2, and E3 enzymes. The E1 activating enzyme forms a high energy thioester bond with ubiquitin and transfers this to one of several E2 conjugating enzymes. E3 ligases interact directly with protein substrates and assist the transfer of ubiquitin from the E2 enzyme to  $\epsilon$ -amino groups of lysine residues on the target. This series of events results in a tightly regulated polyubiquitination of the target proteins (1). E3 ubiquitin ligases are often multisubunit complexes, in which one essential subunit is a protein containing either a HECT domain or a RING finger (2).

The *Drosophila* Sina protein contains a RING finger and can act as a component of an E3 ubiquitin ligase. It is required for the formation of R7 photoreceptors during development of the eye, as well as for the proper development of other sensory organs (3). Humans have two highly conserved proteins, Siah1 and Siah2 (77 and 68% identity to Sina, respectively), which also possess E3 ligase activity (4). We report here a novel *Drosophila*

protein that is homologous (46% identical) to Sina, named Sina-Homologue (SinaH), and we show that it can trigger degradation of the transcriptional repressor Tramtrack, which is also a target of Sina. Alternative splicing of the *tramtrack* gene gives rise to two proteins, Tramtrack 69 (Ttk69) and Tramtrack 88 (Ttk88). These isoforms share an N-terminal Bric-à-Brac-Tramtrack-Broad (BTB) domain but contain different C-terminal zinc fingers (5). We show that SinaH recognizes Ttk69 in a unique manner, different from Sina. The novel mechanism used by SinaH is specific for the Ttk69 isoform and is distinct from that used to recognize Ttk88.

Tramtrack proteins have been demonstrated to be important transcriptional repressors of neuronal development (6–10). They function through direct DNA binding to developmentally important target genes (5, 11, 12) and exert their repressive effects via the recruitment of a variety of co-repressors that serve to transcriptionally silence their targets (13–15). One method of control of the activity of Tramtrack proteins is at the level of protein stability, and this mechanism requires not only Sina but also the adaptor protein Phyllopod (Phyl) (16, 17) and the F-box protein Ebi (18, 19). In the *Drosophila* nervous system and the eye, the activity of these factors is controlled by signaling pathways (20, 21), which allows the level of Tramtrack proteins, and subsequent cell fate decisions, to be tightly controlled throughout development.

In the peripheral nervous system, Tramtrack proteins are expressed exclusively in non-neuronal cells, where they act to repress neural identity (7). Similarly, in third instar eye discs Tramtrack proteins are expressed in the cone cells, not in the neuronal photoreceptor cells. If either Tramtrack protein is overexpressed in the eye, photoreceptor development is inhibited (16, 17). Conversely, Ttk88 mutant flies have rough eyes because of the differentiation of extra photoreceptors, particularly R7 cells, in many of the ommatidia (6). The Tramtrack proteins therefore normally restrict the ability of cells to differentiate as R7 photoreceptors. This is overcome in the R7 cells by a Ras/mitogen-activated protein kinase (MAPK) kinase signal from the R8 photoreceptor, which causes an elevation in *phyl* transcription and hence Tramtrack proteolysis in the R7 (20, 21). Mutations in *phyl*, *ebi*, and *sina* genes all cause the transformation of R7 cells into cone cells (3, 18, 20), and genetic experiments suggest a model in which Sina, Phyl, and Ebi all act to degrade Tramtrack proteins in R7 cells (16, 17, 19). These proteins also antagonize Tramtrack activity to allow the specification of sensory organ precursor cells and subsequent neuronal lineages (22).

In *Drosophila* embryonic S2 cells, the expression of Phyl is sufficient to induce degradation of Ttk88 (17), suggesting that

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<sup>2</sup> The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; BTB, Bric-à-Brac-Tramtrack-Broad; HA, hemagglutinin; HEK, human embryonic kidney.

endogenous levels of the other components are sufficient for degradation in these cells. *In vitro*, Sina, Phyl, and Ebi are all required to degrade Ttk88 (19). Physical interactions between these components have been mapped. Both Sina and Ebi interact very weakly with Ttk88 (19, 23). Phyl binds strongly to Ttk88, and this is dependent on the BTB domain, which is common to both Ttk isoforms. Sina and Phyl also interact strongly, suggesting a model whereby Phyl acts as an adaptor protein to bring the substrate Ttk and the RING finger ligase Sina together (23). Sina can then recruit the E2 conjugating enzyme UBCD1 to cause polyubiquitination and degradation (16). The role of Ebi here is unknown, but it may stabilize the complex (23).

The mammalian homologues of Sina, the Siah proteins, target a large variety of proteins for degradation, for example NcoR (24), Bag1 (25, 26), BOB1/OBF1 (27, 28), and prolyl hydroxylase domain proteins (29). Human Siah1 is implicated in a novel pathway of  $\beta$ -catenin degradation, which involves a complex containing Siah1, Siah-interacting protein, the adaptor protein Skp1, and Ebi, which binds to  $\beta$ -catenin directly (30). A consensus motif found in Siah-binding proteins has been identified (PXAXVXP) (31), and more than half of the known Siah-binding proteins, including the adaptor Phyl, contain this motif. The crystal structure of Siah1 binding this peptide sequence has been solved (32), and often proteins that contain this motif are the direct substrate for degradation by Siah proteins.

In this study we have shown that SinaH uses two modes of recognition to direct the degradation of the substrate Ttk69. One requires the BTB domain of Ttk69 and the adaptor Phyl, suggesting a recognition mode similar to Sina. The other requires a specific sequence in Ttk69 that contains AXVXP and GXVXP motifs and suggests that the Sina homologue, in contrast to Sina, may act in a similar manner to the mammalian homologues.

## EXPERIMENTAL PROCEDURES

**Plasmids**—SinaH cDNA was amplified from the expressed sequence tag clone GH28479 using primers 5'-GCGAATTCGGTACCAAGATGTCTGTTCGCAACTCACG-3' and 5'-CGCTGCGGCCGCTACAGATCCTCCTCGGAGATCAGCTTCTGCTCACTAGTGTGGTACGCTCCTCCAC-3'. This added EcoRI and KpnI sites as well as a Kozak consensus translation site at the 5' end and incorporated a SpeI site, Myc epitope tag, stop codon, and NotI site at the 3' end. Following PCR, the amplified DNA was digested with EcoRI and NotI and cloned into the pRMHA3 S2 expression vector under the control of a metallothionein promoter. Full-length Ttk69-HA, Ttk88-HA, and Pointed-P1-HA cDNAs were cloned into pRMHA3. The pRMHA3-5'polyoma-Sina and pRMHA3-5'HA-Phyl constructs were kind gifts of G. Rubin. Full-length Sina and SinaH cDNAs were subcloned into pRMHA3 to include a FLAG epitope tag. RING finger mutants Sina-C120A and SinaH-C87A were created using the QuikChange mutagenesis kit (Stratagene). Ttk69 deletion mutants were made by PCR using primers that incorporated an EcoRI site and an initiation codon (ATG) at the 5' end and an HA epitope tag and XbaI site at the 3' end. Fragments were cloned into the pIZT constitutive S2 expression vector. Ttk69 $\Delta$ VXP (deletion of amino acids 415–436) and Ttk88 $\Delta$ BTB (deletion of the first 98 amino acids) were

also generated by PCR to include an HA epitope tag and were cloned into pRMHA3. Full-length Ttk69-HA (using EcoRI and XbaI), Ttk88-HA (using EcoRI and NotI), Pointed-P1-HA (using EcoRI and XbaI), Sina-FLAG (using EcoRI and NotI), SinaH-FLAG (using EcoRI and NotI), and Phyl-HA (using BamHI and XhoI) were subcloned into pcDNA3.1 for expression in HEK-293 cells. Full-length Ttk69-HA, Ttk69 $\Delta$ BTB-HA (deletion of the first 131 amino acids), and Ttk69 $\Delta$ VXP-HA were subcloned into the pT7 $\beta$ Link vector (R. Treisman) using EcoRI and XbaI sites. Full-length Sina (using EcoRI and XhoI), SinaH (using BamHI and EcoRI), and Phyl (using BamHI and XhoI) cDNAs were cloned into the pT7 $\beta$ Link vector using primers that incorporated a Myc epitope tag. Phyl was also cloned using a primer that incorporated an HA epitope tag.

**S2 Cell Degradation Assay**—S2 cells were grown in Schneider's medium (Invitrogen) containing 10% fetal calf serum.  $0.5 \times 10^6$  cells/well were plated in a 24-well plate and transfected with 1.25  $\mu$ g of DNA using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. The cells were left for 24 h, and the medium was replaced with medium plus 0.7 mM CuSO<sub>4</sub> to induce expression from the metallothionein promoter. After induction for 18 h the cells were harvested, washed in phosphate-buffered saline, boiled in 100  $\mu$ l of 3 $\times$  SDS sample buffer, and analyzed by SDS-PAGE and Western blotting. The degradation assays contained 0.25  $\mu$ g of pRMHA3-Ttk69-HA, Ttk88-HA, or Ttk deletion mutants; 0.25  $\mu$ g of pRMHA3-PntP1-HA; 0.25  $\mu$ g of pRMHA-Phyl-HA; and 0.0375  $\mu$ g of pRMHA3-Sina-FLAG or pRMHA3-SinaH-FLAG. Each transfection was made up to 1.25  $\mu$ g of DNA using empty pRMHA3 vector.

**Quantitative Reverse Transcription-PCR**— $2 \times 10^6$  cells/well were plated in a 6-well plate and transfected using FuGENE 6 with 1  $\mu$ g of pRMHA3-Ttk69-HA and with either 0.15  $\mu$ g of pRMHA3 or 0.15  $\mu$ g of pRMHA3-SinaH. Cells were induced, and mRNA was isolated using a Dynabeads mRNA DIRECT kit (Invitrogen) according to the manufacturer's instructions. cDNA was prepared using SuperRT (HT Biotechnology), and quantitative PCR was performed with an Applied Biosystems 7900HT reverse transcription-PCR system using actin mRNA for normalization and Ttk69-HA-specific primers. Results are averages of duplicate or quadruplicate data points on plates using three independent transfections.

**HEK 293 Proteasome Assay**—HEK 293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum.  $1 \times 10^5$  cells/well were plated in a 24-well plate and left overnight. 1.25  $\mu$ g of DNA (as S2 cell degradation assay) was transfected for 4 h using FuGENE 6 according to the manufacturer's instructions. After 16 h, 50  $\mu$ M MG132 in Me<sub>2</sub>SO (or Me<sub>2</sub>SO alone) was added to the cells for 3.5 h. The cells were then harvested, washed in phosphate-buffered saline, boiled in 100  $\mu$ l of 3 $\times$  SDS sample buffer, and analyzed by SDS-PAGE and Western blotting.

**Radiolabeled Immunoprecipitation Experiments**—<sup>35</sup>S-Labeled proteins were synthesized *in vitro* using the TNT quick coupled transcription/translation system (Promega). For each immunoprecipitation experiment, pT7 $\beta$ Link constructs were cotranslated, and 30  $\mu$ l of the TNT mixture was mixed with 70  $\mu$ l of radioimmune precipitation assay buffer (150 mM NaCl, 1%



## Degradation of Tramtrack by Sina-Homologue

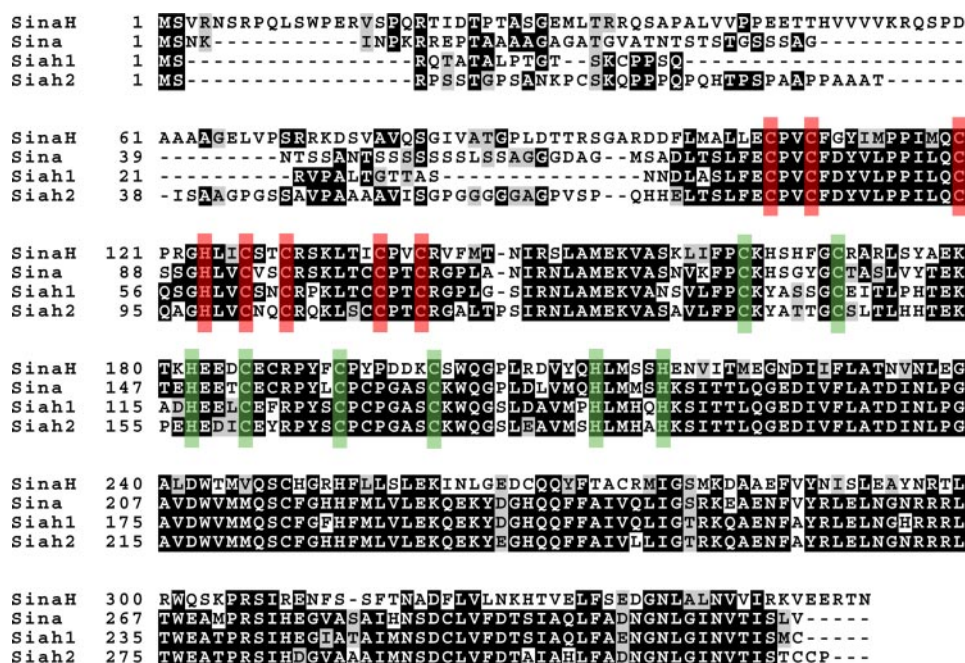


FIGURE 1. Sina-Homologue is a novel protein highly related to Sina. Shown is an amino acid alignment of SinaH with the *Drosophila* Sina protein and the two human homologues, Siah1 and Siah2. The conserved cysteines and histidines of the RING finger (red) and the zinc finger (green) are shown.

Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 8.0) and 50  $\mu$ l of protein A-Sepharose beads (GE Healthcare) and rotated at 4  $^{\circ}$ C for 1 h to preclear. The beads were pelleted, and the supernatant was mixed with 50  $\mu$ g of anti-HA-agarose conjugate (Santa Cruz Biotechnology) and rotated at 4  $^{\circ}$ C for 2 h. The beads were washed five times with radioimmune precipitation assay buffer and boiled with 20  $\mu$ l of 4 $\times$  SDS sample buffer. 10% of the TNT input mixture and all of the pulldown sample were analyzed by SDS-PAGE. After fixing, the gel was dried for 45 min at 80  $^{\circ}$ C and exposed overnight to a storage phosphor screen before being scanned using a Typhoon 8600 variable mode imager (Molecular Dynamics).

**Co-immunoprecipitation Experiments in S2 Cells**— $2 \times 10^6$  S2 cells/well were plated in a 6-well plate and transfected using FuGENE 6 with 2.5  $\mu$ g of either pRMHA3-Sina-C120A-FLAG or pRMHA3-SinaH-C87A-FLAG and 2.5  $\mu$ g of pRMHA3-Ttk69-HA (full-length or deletion constructs) or pRMHA3-HA-Phyl. Cells were induced and collected as for S2 cell assays. Cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 450 mM NaCl, 0.2% Nonidet P-40, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, and Complete protease inhibitor (Roche Applied Science)) for 15 min at 4  $^{\circ}$ C and then centrifuged at  $20,000 \times g$  for 15 min at 4  $^{\circ}$ C. The supernatant was added to 25  $\mu$ l of a 50% slurry of anti-FLAG M2-agarose (Sigma) and incubated for 2 h at 4  $^{\circ}$ C with mixing. The agarose was washed four times with immunoprecipitation buffer and eluted from the beads using 1 mg/ml FLAG peptide (Sigma). 10% of input and the total eluted sample were analyzed by SDS-PAGE and Western blotting.

**Antibodies**—Anti-HA antibody (Roche Applied Science) was used at 1:2000; anti-FLAG antibody M2 (Sigma) was used at

1:2000, and a loading control anti-histone H3 antibody (Abcam) was used at 1:2000.

## RESULTS

**CG13030 Encodes a Novel Protein Homologous to Sina**—A BLAST search of the *Drosophila* genome using the Sina protein sequence revealed a gene (CG13030) that is homologous (41% identical amino acids) to Sina (Fig. 1). Because of this high degree of similarity it was named Sina-Homologue (SinaH). SinaH is also homologous to the human Siah proteins, showing 44% identity to Siah1 and 40% identity to Siah2. As with other Siah proteins, the N terminus of SinaH was more divergent compared with the C terminus, and all key residues in the RING and zinc fingers were conserved.

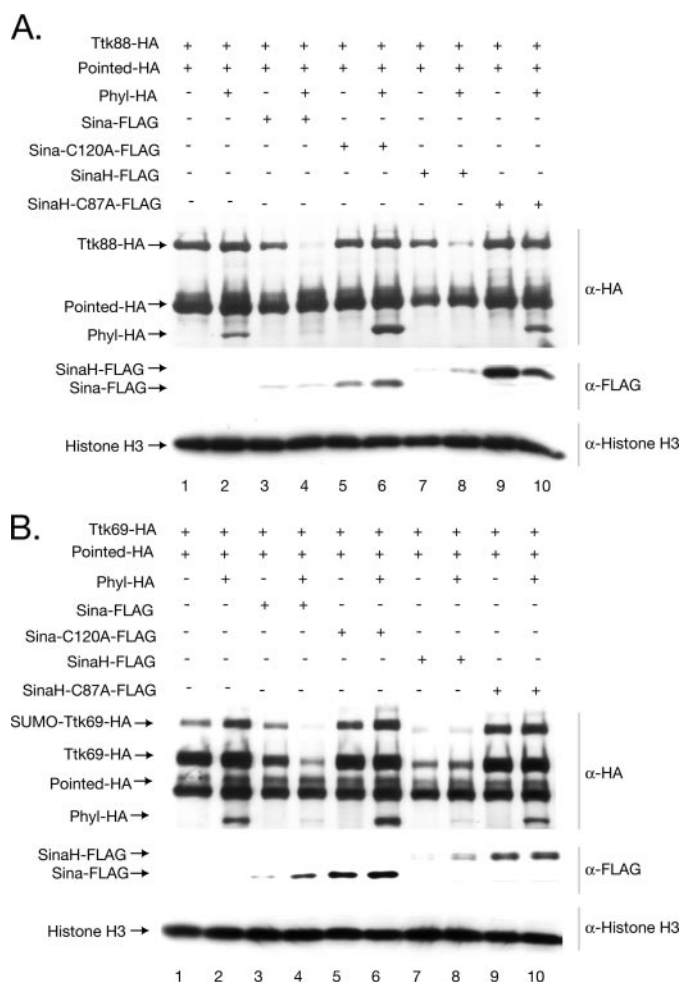
**Both Sina and SinaH Require Phyl to Direct the Degradation of Ttk88**—It has previously been shown that

Ttk88 degradation in S2 cells is induced by the addition of Phyl (17). We established a similar assay using S2 cells in which HA-tagged Ttk88, on an inducible plasmid, was transfected into cells, and the levels of protein were detected by probing Western blots of cell extract. In our assay, the addition of Phyl (Fig. 2A, lane 2) did not alter the level of Ttk88. The addition of Sina (Fig. 2A, lane 3) did cause a slight lowering in the amount of Ttk88. However, the transfection of both Sina and Phyl together caused Ttk88 to be considerably degraded (Fig. 2A, lane 4). As a control, an unrelated nuclear protein, HA-tagged Pointed, was also cotransfected, and under the conditions we used for the assay, the levels of Pointed remained relatively constant. This assay (and the subsequent assays) were carried out several times ( $>10$ ), and despite some variation in levels of Pointed and Ttk88, the effect was highly consistent between each repeat: Ttk88 was degraded only upon the addition of both Sina and Phyl.

We created a mutant Sina construct (Sina-C120-FLAG), shown in other E3 ligases to eliminate activity, in which the third cysteine residue in the RING finger was mutated to alanine. This mutant Sina was unable to direct the degradation of Ttk88 even upon the addition of Phyl (Fig. 2A, lane 6).

We then tested Sina-Homologue in the same assay, and the result was strikingly similar to that seen with Sina. Sina-Homologue was able to degrade Ttk88 when Phyl was added (Fig. 2A, lane 8), and a comparable mutation in the RING finger of SinaH (SinaH-C87A-FLAG) abolished this degradation (Fig. 2A, lane 10).

Both the wild-type and mutant Sina and SinaH contained a FLAG tag, and the protein levels of both Sina and SinaH used in the assay were detected with an antibody against the epitope. Previously it has been noted that Siah proteins are able to direct their

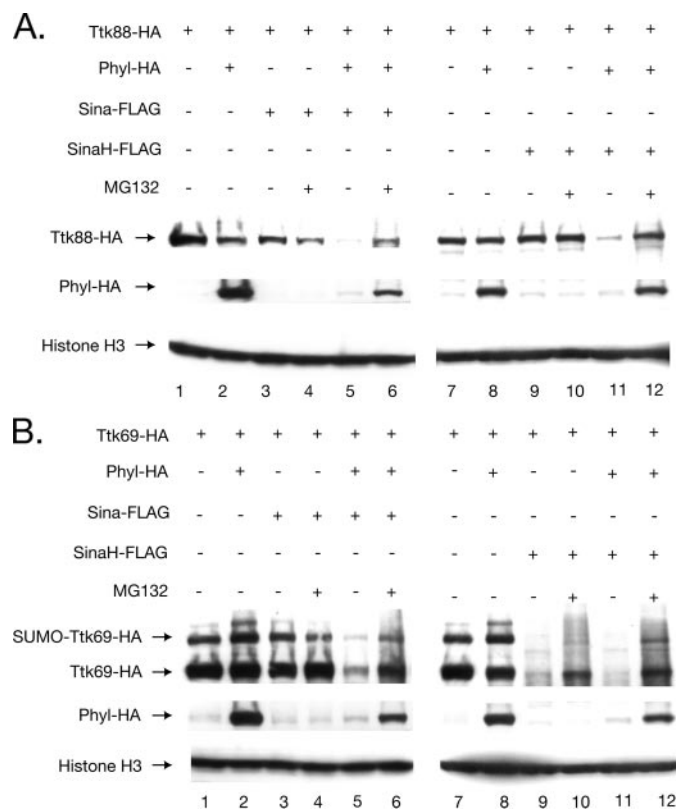


**FIGURE 2. Sina and SinaH can direct the degradation of Ttk88 and Ttk69.** A, S2 cells were transfected with the expression vector (pRMHA3) containing Ttk88-HA (0.25  $\mu$ g) and Pointed-P1-HA (0.25  $\mu$ g) and with combinations of Phyl-HA (0.25  $\mu$ g) and Sina-FLAG, SinaH-FLAG, mutant Sina-C120A-FLAG, or SinaH-C87A-FLAG (0.0375  $\mu$ g). 18 h after induction with CuSO<sub>4</sub>, cell extracts were analyzed for protein levels on a Western blot probed with either anti-HA or anti-FLAG antibody. The lower part of the blot was probed with anti-histone H3 antibody as a loading control. B, same as A, except Ttk69-HA instead of Ttk88-HA was transfected. Ttk69 is SUMOylated, and this larger Ttk69 species is labeled.

own degradation by association with E2 enzymes, causing autoubiquitination (4). The low protein levels of wild-type Sina-FLAG (Fig. 2A, lanes 4 and 5) and SinaH-FLAG (lanes 7 and 8) are consistent with such an effect, along with an increase in protein levels when the RING finger is mutated: Sina-C120A-FLAG (Fig. 2A, lanes 6 and 7) and SinaH-C87A-FLAG (lanes 9 and 10). As well as autoubiquitination of the RING domain-containing protein, other members of the E3 complexes are also targeted for degradation (33), and in this assay Phyl (HA-tagged) is reduced in level when both wild-type Sina and SinaH are added, but this effect is abolished upon mutation of the RING fingers.

These results show that in our S2 assay, Sina and Phyl cooperate in directing the degradation of Ttk88. Moreover, the novel SinaH protein can act in a similar manner to Sina and also direct the degradation of Ttk88 in combination with Phyl.

**Sina-Homologue Directs the Degradation of Ttk69 in a Different Manner from Sina**—We then tested the other isoform of Tramtrack, Ttk69, in the S2 degradation assay. Again, the addition of Phyl or Sina alone did not cause any lowering of Ttk69



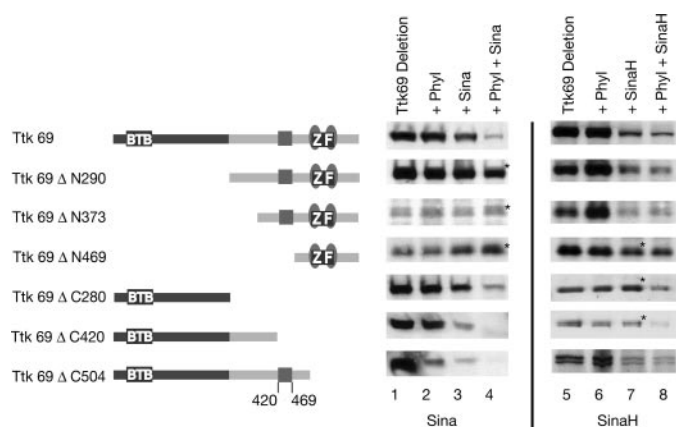
**FIGURE 3. Sina- and SinaH-directed degradation of Ttk88 and Ttk69 is proteasome-dependent.** A, HEK 293 cells were transfected with the expression vector (pcDNA3.1) containing Ttk88-HA (0.25  $\mu$ g) and with combinations of Phyl-HA (0.25  $\mu$ g) and Sina-FLAG (0.05  $\mu$ g) or SinaH-FLAG (0.25  $\mu$ g). Cells were treated with 50  $\mu$ M MG132 in Me<sub>2</sub>SO (+) or Me<sub>2</sub>SO alone (-) for 3.5 h. Cell extracts were analyzed for protein levels on a Western blot probed with anti-HA antibody, and the lower part of the blot was probed with anti-histone H3 antibody as a loading control. B, same as A, except Ttk69-HA instead of Ttk88-HA was transfected.

levels (Fig. 2B, lanes 2 and 3). However, the addition of Phyl and Sina together caused Ttk69 degradation (Fig. 2B, lane 4), and this was abrogated when the RING domain was mutated in Sina (lane 6). SUMO-modified Ttk69 behaved identically to unmodified Ttk69 in this assay, therefore suggesting that the SUMOylation had no detectable effect on protein stability.

Interestingly, the addition of SinaH alone to the assay (Fig. 2B, lane 7) was sufficient to cause the degradation of Ttk69 without the need for Phyl. This effect was not due to changes in the mRNA abundance as determined by quantitative reverse transcription-PCR (-fold change of Ttk69-HA mRNA in SinaH-transfected cells equals 1.00 with an S.D. of 0.27). The addition of SinaH and Phyl together did not produce a further decrease in the amount of Ttk69, which was different from the Phyl dependence seen in Sina-directed degradation. This suggested that SinaH could be recognizing or acting on the substrate Ttk69 in a different manner from Sina and that the two isoforms of Tramtrack could be degraded differently.

**Ttk88 and Ttk69 Degradation Is Proteasome-dependent**—Proteasome inhibitors (e.g. MG132) were not effective in our S2 cells, and therefore to investigate if the reduction in levels of Ttk88 and Ttk69 seen in the above assays was due to proteasome-dependent degradation, we set up similar assays in MG132-responsive HEK 293 cells. Fig. 3A shows that, as in S2 cells, Sina and Phyl are both required for Ttk88 degradation (lane 5), and





**FIGURE 4. Sina- and Phyl-dependent degradation of Ttk69 requires the BTB domain, but SinaH-dependent degradation of Ttk69 requires a different region.** Various HA-tagged fragments of Ttk69 were transfected into S2 cells and are shown here schematically. The dark gray region corresponds to the common region between Ttk69 and Ttk88, and the N-terminal BTB domain is shown. The light gray region is unique to Ttk69, and the zinc fingers (ZF) are marked at the C terminus. The anti-HA antibody-probed Western blot of the cell extracts, 18 h after induction, shows the levels of each of the fragments of Ttk69. *Lanes 1 and 5*, Ttk69-HA deletion constructs alone (0.25 μg); *lanes 2 and 6*, levels of Ttk69-HA fragments with the addition of Phyl (0.25 μg); *lane 3*, addition of Sina (0.075 μg); *lane 7*, addition of SinaH (0.01875 μg); *lanes 4 and 8*, Phyl and Sina or SinaH together. Recognition by Sina and Phyl requires the common region of Ttk69 and Ttk88 because if this is deleted, protein levels remain the same (\*). SinaH recognition is not dependent on Phyl and requires the region marked 420–469. Deletion of this region causes the Ttk69-HA protein levels to remain constant upon SinaH addition (\*).

this degradation can be inhibited by the addition of MG132 (*lane 6*). This was also the case for Sina- and Phyl-dependent Ttk69 degradation, shown in Fig. 3B (*lanes 5 and 6*).

SinaH-directed degradation was also proteasome-dependent. As in S2 cells, SinaH acted with Phyl to direct the degradation of Ttk88, and this was inhibited by MG132 (Fig. 3A, *lane 12*). Interestingly, the degradation of Ttk69 by SinaH was independent of Phyl (Fig. 3B, *lane 9*) and could be abolished by the addition of MG132 (*lane 10*), suggesting that this mode of degradation was also proteasome-dependent. The levels of Phyl-HA are indicated in Fig. 3, A and B, and both Sina- and SinaH-directed degradation of the adaptor, Phyl-HA, was inhibited by the addition of the proteasome inhibitor.

**Sina and Phyl Recognize the BTB Domain in Ttk69**—To identify the region of Ttk69 required for recognition by Sina/Phyl, degradation assays were performed on a set of deletion constructs of Ttk69 (Fig. 4). There was some variability in levels of the deletion constructs, but consistently (repeated four times) three constructs were resistant to degradation upon addition of Sina and Phyl (Fig. 4, *lane 4*, \*). These lacked the N-terminal part of the protein (Ttk69ΔN290, Ttk69ΔN373, and Ttk69ΔN469), showing that this region is important for recognition. The N-terminal region is common to both Ttk isoforms and contains the BTB domain. This domain is where Phyl binds to Ttk (17, 23) to bring the E3 ligase Sina to its substrate. These results give a functional confirmation of this model and explain why both isoforms of Ttk are similarly susceptible to Sina and Phyl.

**SinaH Requires a 50-Amino Acid Region Unique to Ttk69 for Recognition**—To investigate if SinaH acted on Ttk69 differently from Sina, we tested the same set of Ttk69 deletion constructs.

Consistently three constructs were stable in this assay: Ttk69ΔN469, Ttk69ΔC280, and Ttk69ΔC420 (Fig. 4, *lane 7*, \*). However, these constructs were different from the resistant fragments in the Sina experiment. Using this assay, the region necessary for SinaH recognition was mapped between amino acids 420 and 469 (marked on Fig. 4) of Ttk69.

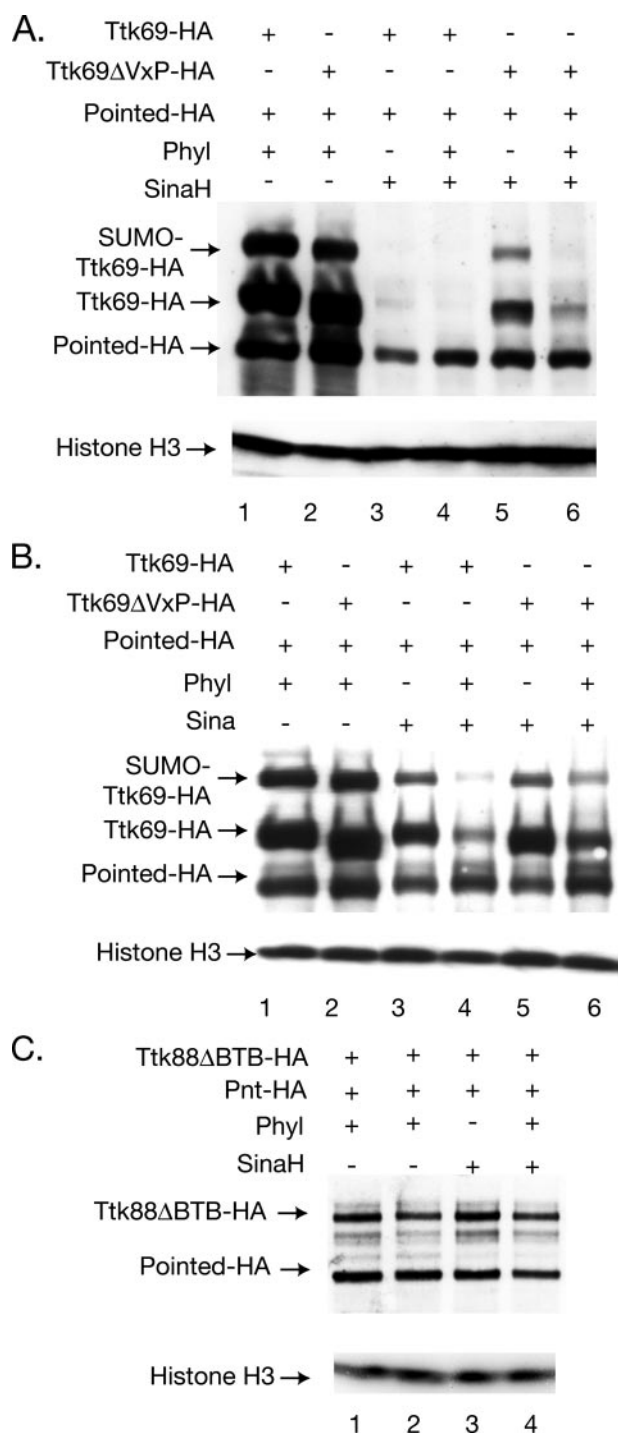
In this experiment, Phyl and SinaH were also transfected together (Fig. 4, *lane 8*). The addition of Phyl did not cause any further reduction in protein levels of any of the SinaH-susceptible constructs. However, of the three constructs resistant to SinaH-mediated degradation, the two that contained the BTB domain (Ttk69ΔC280 and Ttk69ΔC420) were degraded upon addition of Phyl, but Ttk69ΔN469 remained resistant to degradation even when Phyl was introduced.

Two AXVXP and GXVXP motifs, the consensus motif found in mammalian Siah-binding proteins, are located around region 420–469 of Ttk69 that was required for SinaH-mediated degradation. A mutant form of Ttk69 (Ttk69ΔVXP-HA) was made and tested in the assays (Fig. 5A). As predicted, removal of these motifs made Ttk69ΔVXP significantly less susceptible to SinaH-mediated degradation compared with wild-type Ttk69 (Fig. 5A, compare *lane 3* (wild-type) with *lane 5* (mutant)). Interestingly, the addition of Phyl caused a reduction in protein levels of Ttk69ΔVXP, an effect that was not visible for wild-type Ttk69 (Fig. 5A, *lane 6*) but was similar to the effect seen for the deletion constructs Ttk69ΔC280 and Ttk69ΔC420. The Ttk69ΔVXP, Ttk69ΔC280, and Ttk69ΔC420 mutants all contain the BTB domain but remove the VXP motif. These experiments show that without the VXP motif, SinaH acts similarly to Sina, causing reduction of Ttk69 levels in association with Phyl via the BTB domain. Fig. 5B shows that both wild-type Ttk69 and mutant Ttk69ΔVXP behave identically upon addition of Sina and Phyl.

These results demonstrate two distinct modes of Ttk69 recognition and show that SinaH acts through a novel region of the substrate protein Ttk69. As a further test of the principle, a mutant of Ttk88 lacking the BTB domain was created (Ttk88ΔBTB-HA). This Tramtrack protein does not contain VXP motifs (they are specific for Ttk69), nor does it contain the BTB domain. Without either of these interaction motifs, Ttk88ΔBTB should be completely resistant to SinaH-mediated degradation. Indeed, Fig. 5C shows that, upon transfection of SinaH DNA, Ttk88ΔBTB remained at a constant level.

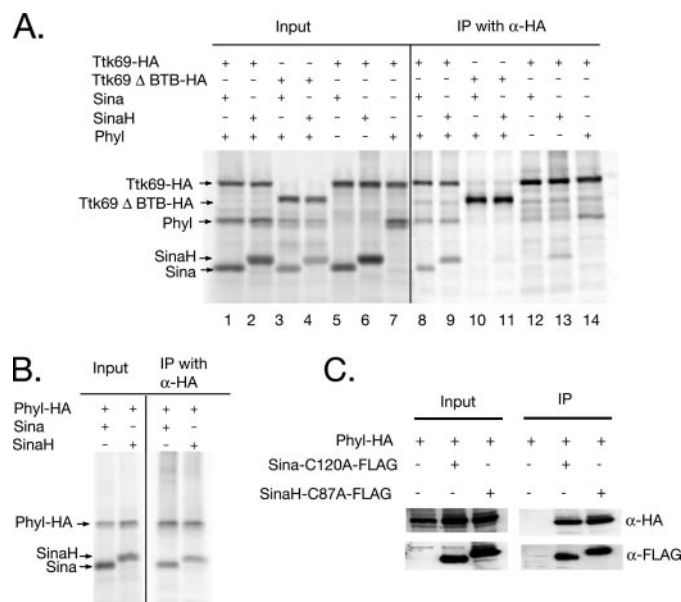
**Phyl Acts as an Adaptor between Ttk69 and Both Sina and SinaH**—We carried out protein interaction studies to investigate if SinaH could bind to Ttk69. We produced [<sup>35</sup>S]methionine-labeled *in vitro* translated proteins, which contained an HA-tagged protein and other untagged test proteins. Using anti-HA-agarose we immunoprecipitated the HA-tagged protein. Any proteins that could bind to the HA-tagged protein were also precipitated. This system did not contain the rest of the degradation machinery, allowing interactions with active proteins to be tested without the problem of degradation.

Previous work has shown that Phyl binds to the BTB domain in the common region of the Tramtrack isoforms and that Sina and Phyl bind each other strongly, thereby suggesting that Phyl is an adaptor, acting to bring Sina and Tramtrack together (23).



**FIGURE 5. Deletion of the VXP motifs in Ttk69 abolishes the novel mode of SinaH-directed degradation.** A, S2 cells were transfected with either wild-type Ttk69-HA (0.25  $\mu$ g) or mutant Ttk69 $\Delta$ VxP-HA (0.25  $\mu$ g) and Pointed-P1-HA (0.25  $\mu$ g), along with combinations of Phyl (0.25  $\mu$ g) and SinaH (0.0375  $\mu$ g). Blots were probed with anti-HA (top) and anti-histone H3 (bottom) antibodies. B, same as A, except Sina was transfected instead of SinaH. C, S2 cells were transfected with Ttk88 $\Delta$ BTB-HA (0.25  $\mu$ g) and Pointed (Pnt)-P1-HA (0.25  $\mu$ g), along with combinations of Phyl (0.375  $\mu$ g) and SinaH (0.0375  $\mu$ g).

In Fig. 6A we also show that HA-tagged Ttk69 bound Phyl but not Sina (lanes 14 and 12, respectively). When Sina and Phyl were both present, Ttk-HA bound to both (lane 8), and this interaction was dependent on the BTB domain (lane 10). Ttk69 was also able to bind to Phyl and SinaH when they were both



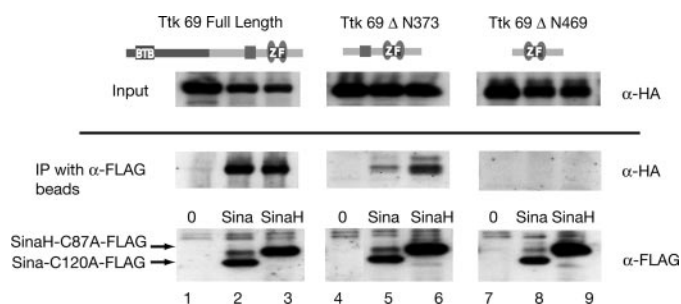
**FIGURE 6. Phyl acts as an adaptor binding the BTB domain of Ttk69 and also Sina and SinaH.** A, combinations of proteins were produced by *in vitro* transcription/translation and labeled with [ $^{35}$ S]methionine. Lanes 1–7 show 10% of the input. Lanes 8–14 show the material recovered from immunoprecipitations (IP) using anti-HA-agarose and therefore the proteins that can bind to the HA constructs (either full-length Ttk69 or Ttk69 $\Delta$ BTB). B, same as A, except here Phyl is HA-tagged. Both Sina and SinaH bind to Phyl *in vitro*. C, S2 cells were transfected with 2.5  $\mu$ g of pRMHA3-HA-Phyl and with 2.5  $\mu$ g of carrier pRMHA3, pRMHA3-Sina-C120A-FLAG, or pRMHA3-SinaH-C87A-FLAG. After induction for 18 h, cell lysate was incubated with anti-FLAG-agarose. Western blots of 10% of the input and the material eluted from the anti-FLAG beads were probed with both anti-FLAG and anti-HA antibodies. Anti-FLAG beads alone do not interact with Phyl-HA, but both Sina and SinaH can bind Phyl-HA *in vivo*.

present (lane 9), and this interaction was abolished when the BTB domain was deleted (lane 11). Given the similarity between Sina and SinaH it was not surprising that SinaH could also bind Phyl-HA very strongly both *in vitro* and *in vivo* (Fig. 6, B and C), and this indicates that SinaH has a similar mode of binding to Ttk69 via the adaptor Phyl.

**SinaH Binds to the VXP Motifs of Ttk69 *in Vivo***—Using our *in vitro* produced proteins, we saw that Ttk69 could also weakly interact with SinaH alone under certain conditions (Fig. 6A, lane 13). However, because of the small amounts of proteins produced *in vitro*, this binding was not very reproducible. It is known that Sina can bind to Ttk69 directly, but this interaction is weak and not sufficient to cause significant degradation under physiologically relevant conditions (23). We investigated whether there was a direct interaction between SinaH and Ttk69 but found no difference from the very weak Sina/Ttk69 binding (data not shown). We therefore decided to use a co-immunoprecipitation experiment in S2 cells, as an unknown adaptor might be necessary to allow strong binding of SinaH to the VXP motifs in Ttk69.

To perform co-immunoprecipitation experiments it was necessary to use the RING domain mutants of Sina and SinaH, shown in Fig. 2, to allow higher levels of Ttk69 proteins to be co-expressed. Sina-C120A-FLAG and SinaH-C87A-FLAG were transfected into S2 cells with full-length Ttk69, Ttk69 $\Delta$ N373, or Ttk69 $\Delta$ N469. Both Sina and SinaH interacted strongly with full-length Ttk69 (Fig. 7, lanes 2 and 3). Unlike the

## Degradation of Tramtrack by Sina-Homologue



**FIGURE 7. *In vivo* SinaH interacts with the VXP motif of Ttk69.** S2 cells were transfected with 2.5  $\mu$ g of carrier pRMHA3, pRMHA3-Sina-C120A-FLAG, or pRMHA3-SinaH-C87A-FLAG and with 2.5  $\mu$ g of full-length Ttk69-HA, Ttk69 $\Delta$ N373-HA, or Ttk69 $\Delta$ N469-HA. After induction for 18 h, cell lysate was incubated with anti-FLAG-agarose. Western blots of 10% of the input were probed with anti-HA antibody. Western blots showing the material eluted from the anti-FLAG beads were probed with both anti-FLAG and anti-HA antibodies. None of the Ttk69-HA fragments bound to anti-FLAG beads alone. Both Sina and SinaH bound full-length Ttk69-HA. Neither Sina nor SinaH bound Ttk69 $\Delta$ N469-HA. SinaH bound to Ttk69 $\Delta$ N373-HA, the fragment containing the 50-amino acid region required for degradation (gray box), better than Sina. *IP*, immunoprecipitation; *ZF*, zinc fingers.

*in vitro* transcription/translation system described above, there is endogenous Phyl present in these cells. Therefore, a likely explanation for this result is that Phyl is sufficient to act as an adaptor protein by binding to the BTB domain.

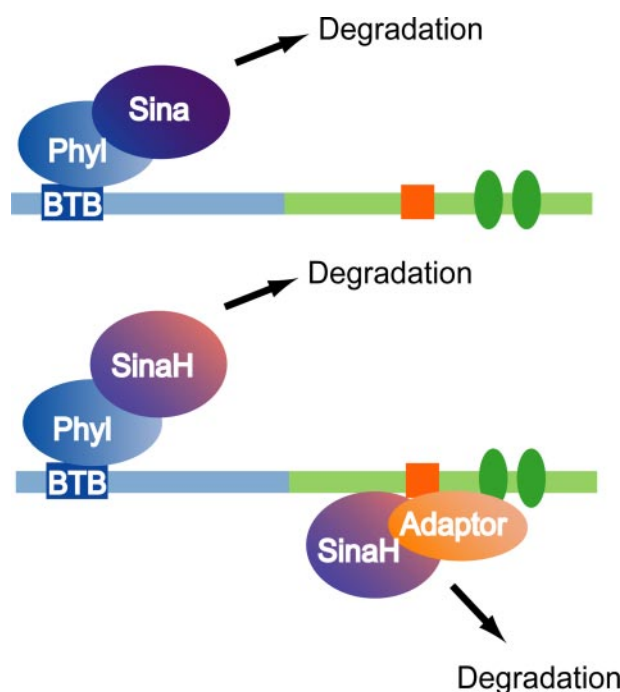
Ttk69 $\Delta$ N373 and Ttk69 $\Delta$ N469 both lacked the BTB domain, and therefore any interaction could not be via endogenous Phyl. Neither Sina nor SinaH bound to Ttk69 $\Delta$ N469 (Fig. 7, lanes 8 and 9), a fragment that was resistant to degradation in the previous assays. However, compared with Sina, SinaH bound to Ttk69 $\Delta$ N373 more strongly (Fig. 7, compare lane 5 with lane 6). Ttk69 $\Delta$ N373 contains the VXP motifs, and consistent with the previous data, levels of this fragment remained unaltered when Sina was transfected but were dramatically reduced upon addition of SinaH.

## DISCUSSION

We have shown that the novel RING finger-containing protein CG13030 or SinaH can direct the proteasome-dependent degradation of Tramtrack proteins in a similar way to the E3 ubiquitin ligase Sina. Mutation of the RING domain abolishes this ability. However, despite the high homology between these E3 ubiquitin ligases, we have shown that SinaH can direct the degradation of Ttk69 in a different manner from Sina and that SinaH recognizes Ttk69 by two different modes depending on the presence or absence of Phyl.

SinaH can direct the degradation of Ttk69 without the need for the adaptor Phyl. We have shown that a 50-amino acid region unique to the Ttk69 isoform (amino acids 420–469) is required for this effect. In a co-immunoprecipitation assay SinaH interacts with this region of Ttk69, and these results suggest a model by which SinaH recognizes Ttk69 either by interacting directly or, more likely, via an unknown adaptor molecule that interacts with region 420–469 in Ttk69 (Fig. 8).

Interestingly, SinaH can also bind to Phyl both *in vitro* and *in vivo*. Consistent with this result, degradation of Ttk88 and Ttk69 $\Delta$ VXP directed by SinaH is increased by the addition of Phyl. This implies a mechanism similar to the one shown previously for Sina and Phyl and suggests that SinaH could act



**FIGURE 8. Model showing two modes of degradation of Ttk69 by SinaH.** Mode 1, as for Sina, requires Phyl as an adaptor to bring SinaH close to the substrate, requiring the BTB domain of the substrate. Mode 2 requires either direct binding of SinaH to Ttk69 or via an adaptor protein, and this mode requires a different region of the substrate molecule.

redundantly with Sina in some circumstances (Fig. 8). Both Sina and SinaH interact equally well with full-length Ttk69 in a co-immunoprecipitation assay, most likely because of endogenous Phyl acting as an adaptor. Phyl interacts in the common region of the Ttk isoforms, so it seems likely that the SinaH/Phyl-targeting mechanism occurs for both Ttk69 and Ttk88. We did not see Phyl dependence in our assay of Ttk69 degradation by SinaH, which is probably because SinaH alone had already caused a substantial reduction in Ttk69 levels. However, for Ttk69 $\Delta$ VXP, which removes the direct mode of SinaH recognition, the SinaH/Phyl-dependent mode of recognition became apparent. SinaH activity toward Ttk69 $\Delta$ VXP is also comparable with its activity toward Ttk88, again consistent with the idea that SinaH recognizes Ttk69 using two different modes: one via the BTB domain, present in Ttk88 and Ttk69, and one via the VXP motifs found only in Ttk69.

We have shown that the mechanism that SinaH uses to degrade Ttk69 is distinct from that which it uses to degrade Ttk88. The Tramtrack isoforms have different developmental roles: removal of Ttk69 is embryonic lethal, whereas a Ttk88 null results in a variety of nonlethal developmental phenotypes (6). It could therefore be advantageous to have a mechanism for the selective destruction of the Ttk isoforms, and SinaH might be used specifically to turn over the Ttk69 isoform in a Sina/Phyl-independent manner. This could provide the organism with an added level of control over the activity of these developmentally crucial transcription factors. This might also suggest why another Sina-like protein is present in *Drosophila*.

We have also shown that SinaH can act in a way similar to Sina, requiring Phyl to recognize its substrate. However, it seems that the Phyl-independent recognition of Ttk69 is more



efficient than that mediated via the BTB domain. Unlike Sina and SinaH, Phyl is not conserved in higher eukaryotes, and therefore this Phyl-independent mechanism is more likely to be similar to that used by the Sina homologues in these other species. This suggestion is consistent with the presence of the two AXVXP and GXVXP motifs within the region of Ttk69 that mediates SinaH interaction. Given this biochemical similarity between the recognition methods used by SinaH and the human Siah protein, studies of the fly SinaH might offer a model to help clarify some of the different pathways in which the Siah proteins have been implicated (34).

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## **Two Modes of Degradation of the Tramtrack Transcription Factors by Siah Homologues**

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