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TWO MODES OF DEGRADATION OF THE TRAMTRACK TRANSCRIPTION FACTORS BY SIAH HOMOLOGUES

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Abstract

The Siah proteins, mammalian homologues of the *Drosophila* Sina protein, function as E3 ubiquitin 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 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, 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 ε-amino groups of lysine residues on the target. This series of events results in a tightly regulated polyubiquitination on 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 the catalytic component of a 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 respectively with Sina), 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 direct the degradation of the transcriptional repressor Tramtrack, 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 directs the degradation of Ttk69 in a unique manner, using a different mode of recognition compared with Sina. This novel mechanism for degradation by SinaH is specific for the Ttk69 isoform and is distinct from that with which it directs Ttk88 degradation.

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Tramtrack proteins have been demonstrated to be important transcriptional repressors of neuronal development (6-10). They function through direct DNA binding to critical 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 functioning of these factors lies downstream of signalling pathways (20,21), which allows the activity 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 due to 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/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 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 antagonise 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 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 where by 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 may stabilise the complex (23).

The mammalian homologues of Sina, the Siah proteins, target a large variety of proteins for degradation, for example NcoR (24), Bag 1 (25,26), BOB1/OBF1 (27,28), and prolyl-hydroylase domain proteins (29). Human Siah1 is implicated in a novel pathway of β-catenin degradation, which involves a complex containing Siah1, Siah interacting protein (SIP), the adaptor protein Skp1 and Ebi which binds to β-catenin directly (30). A consensus motif found in Siahbinding 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 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, which contains AxVxP and GxVxP motifs and suggests that Sina-Homologue, in contrast to Sina, may direct degradation in a similar manner to the mammalian homologues.

Experimental Procedures

Plasmids

SinaH cDNA was amplified from the EST clone GH28479 using primers 5′-GCGAATTCGGTACCAAGATGTCTGTTCGCAACTCACG - 3′ and 5′ - CGCTGCGGCCGCTACAGATCCTCCTCGGAGATCAGCTTCTGCTCACTAGTGTTGG TACGCTCCTCCAC-3′. This added EcoRI and KpnI sites as well as a Kozak consensus translation site at the 5′ end, and incorporates 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 pRMHA3 S2 expression vector under a metallothionein promoter. Full length Ttk69-HA, Ttk88-HA and Pointed-P1-HA cDNAs were cloned into pRMHA3. The pRMHA3 5′ polyoma-SINA (G. Rubin), pRMHA3-5′HA-Phyl (G. Rubin) and pRHMA3-Pygopus-HA (M. Bienz) constructs were kind gifts. Ring finger mutants Sina-C120A and SinaH-C87A were created using the Quikchange mutagenesis kit (Stratagene) and subcloned into pRHMA3 to include a FLAG-epitope tag. 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. Full length Ttk69-HA and Ttk69ΔBTB-HA (deletion of the first 131 amino acids) were cloned into the pT7βLink vector (R. Treisman) using EcoRI and XbaI sites. Full length Sina (using EcoRI and XhoI), SinaH (using BamHI and EcoRI) and Phyl (using BamH1 and Xho1) cDNAs were cloned into the pT7βLink vector using primers that incorporated a Myc-epitope tag. Phyl was also cloned using a primer that incorporated a HA-epitope tag.

S2 Cell Degradation Assay

S2 cells were grown in Schneider's Medium (Invitrogen) containing 10% fetal calf serum. 0.5×10^6 cells were plated per well in a 24 well plate, and transfected with 1.25 μg DNA using Fugene (Roche) according to manufacturer's instructions. The cells were left for 24 h, and the medium was replaced with medium plus 0.7 mM $CuSO_4$ to induce expression from the metallothionein promoter. After induction for 18 h the cells were harvested, washed in PBS, boiled in 200 μl 3 x SDS sample buffer, and analysed by SDS-PAGE and Western Blotting. The degradation assays contained 0.25 μg pRMHA3-Ttk69-HA or Ttk88-HA, 0.25 μg pRMHA3-Pygo-HA or PntP1-HA, 0.375 μg pRMHA-Phyl-HA and 0.375 μg pRMHA3-Sina or SinaH. The amount of Sina or SinaH that was transfected was lowered and 0.375 μg was normalised to allow comparison. Each transfection was made up to 1.25 μg DNA using empty pRMHA3 vector.

Radiolabelled Immunoprecipitation Experiments

^{35}S-labelled proteins were synthesised *in vitro* using the TnT Quick Coupled Transcription/ Translation System (Promega). For each immunoprecipitation experiment, pT7βLink constructs were cotranslated and 30 μl of the TnT mixture was mixed with 70 μl of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH8.0) and 50 μl protein-A Sepharose beads (Amersham Biosciences) and rotated at 4 °C for 1 h to preclear. The beads were pelleted and the supernatant mixed with 50 μg anti-HA agarose conjugate (Santa Cruz) and rotated at 4 °C for 2 h. The beads were washed 5 times with RIPA and boiled with 20 μl 4xSDS sample buffer. 10% of TnT input mixture and all of the bead sample were anaylsed by SDS-PAGE. After fixing, the gel was dried for 45 min at 80°C and exposed overnight to a storage phosphor screen (Molecular Dynamics) before being scanned using Typhoon 8600 variable mode imager (Molecular Dynamics).

Coimmunoprecipitation Experiments in S2 cells

2×10^6 cells were plated per well of a 6 well plate and were transfected using Fugene (Roche) with 2.5 μg of either pRMHA3-Sina-C120A-FLAG or pRMHA3-SinaH-C87A-FLAG and with 2.5 μg pRMHA3-Ttk69-HA full length or deletion constructs, or pRMHA3-HA-Phyl. Cells were induced and collected as for degradation assays. Cells were lysed in IP buffer (50 mM Tris-HCl pH 7.4, 450 mM NaCl, 0.2% NP40, 1 mM $MgCl_2$, 0.5 mM EDTA, Complete protease inhibitor (Roche)) for 15 min at 4 °C and then centrifuged at 20 000 x g for 15 min at 4 °C. The supernatant was added to 25 μ l of a 50 % slurry of M2 anti-FLAG agarose (Sigma) and incubated 2 h at 4 °C with mixing. The agarose was washed 4 times with IP buffer, and eluted from the beads using 1 mg/ml FLAG peptide (Sigma). 10% of input and total eluted sample were analysed by SDS-PAGE and Western Blot.

Antibodies

Anti-HA (Roche) was used at 1:2000, M2 Anti-Flag antibody (Sigma) was used at 1:1000 and a loading control anti-histone-H3 (Abcam) was used at 1:1000.

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). Due to this high degree of similarity it was named Sina-Homologue (SinaH). SinaH is also homologous to the human Siah proteins showing 44% identity with Siah 1 and 40% identity with Siah 2. 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.

Sina directed degradation of Ttk69 and Ttk88 is Phyl dependent

It has previously been shown that Ttk88 degradation in S2 cells is induced by the addition of Phyl (17). We established a similar degradation assay using S2 cells in which HA tagged Ttk88 or Ttk69, on an inducible plasmid, were transfected into cells, and the levels of protein detected by probing Western blots of cell extract. These experiments were carried out to verify that Ttk69, as well as Ttk88, was subject to degradation by the addition of Phyl. This seemed likely as Phyl binds the BTB domain, the region common to both Ttk isoforms.

Contrary to previous findings, in our assay, the transfection of Phyl alone did not cause the degradation of either Ttk88 or Ttk69 (Fig 2A and B, lane 3). However, the transfection of Sina alone caused the levels of both Ttk88 and Ttk69 to be reduced (Fig 2A and B, lane 4). When both Sina and Phyl were cotransfected into the cells, the levels of both Ttk isoforms were reduced further than when Sina was added alone (Fig 2A and B lane 5). We lowered the levels of Sina and under these conditions as well, the addition of Phyl caused a further degradation of both Ttk88 and Ttk69. The SUMO modified Ttk69 behaved identically to unmodified Ttk69 in this assay, therefore suggesting that the SUMOylation had no effect on degradation kinetics. As a control, an unrelated nuclear protein, Pygopus (Pygo) was also cotransfected. Pygo was not degraded by the addition of Phyl. However the levels of Pygo were reduced when high levels of Sina were transfected into the cells (Fig 2A and B, lanes 4 and 5). The degradation of this control suggested that at these high levels, Sina caused non-specific degradation of other overexpressed proteins, most probably by non-specific interaction and ubiquitination. At the lower levels of Sina, the amount of non-specific degradation was reduced, shown by the increasing levels of the control Pygo and both Ttk88 and Ttk69 (Fig 2A and B, lanes 8 and 9). Importantly, at all levels of Sina, the addition of Phyl did not reduce the level of the control Pygo. These results show that in our S2 assay, Sina and Phyl cooperate in directing degradation of both isoforms of Ttk.

Sina-Homologue can also direct the degradation of Ttk proteins

SinaH was transfected into S2 cells and we performed a set of degradation assays similar to those with Sina. When the equivalent amounts of Sina and SinaH DNA were used for the transfection, SinaH consistently caused substantially higher levels of degradation, suggesting that this protein was more active. This effect could also be due to a difference in expression levels of the two proteins, although this was difficult to test as the active forms of each protein degraded themselves at all levels of expression. However, this is unlikely as the same expression vectors were used for both proteins. The range of amounts of SinaH DNA transfected into cells was approximately 10 times lower than that for Sina. At these levels, the addition of SinaH caused some degradation of Ttk88 (Fig 3A, lane 3). The addition of Phyl caused the levels of Ttk88 to be reduced even further (Fig 3A, lane 4 and 6). The addition of Phyl alone had no effect on the degradation of Ttk88 (Fig 3A, lane 2). This result suggests that the novel SinaH protein can act in a similar manner to Sina, causing degradation of Ttk88 in combination with Phyl.

Figure 3B shows the degradation of Ttk69 by addition of SinaH. A nuclear protein, Pointed, was also transfected to control for non-specific background degradation. At this level of SinaH, Pointed was not susceptible to degradation compared with both Ttk69 and the SUMOylated form of Ttk69. This control serves to rule out non-specificity of SinaH in the degradation of not only Ttk69 but also Ttk88. Interestingly, Ttk69 also seemed to be more susceptible to degradation than the Ttk88 isoform (compare Fig 3A lanes 2 and 3 with 3B, lanes 4 and 5). Moreover, the addition of SinaH and Phyl together did not produce the marked decrease in the amount of Ttk69. A minor effect could be seen with the addition of Phyl, however this was different from the clear effect seen in other degradation assays. This suggested that SinaH might be directing the degradation the substrate Ttk69 in a different manner to Sina.

Sina and Phyl directed degradation of Ttk69 requires the BTB domain

In order to identify the region of Ttk69 required for Sina/Phyl directed degradation, assays were performed on a set of deletion constructs of Ttk69 using optimised levels of Sina DNA for transfection (Fig 4). Three constructs were not degraded upon addition of Sina and Phyl (see Fig 4, lane 4, marked with *). These lacked the N terminal part of the protein: Ttk69ΔN290, Ttk69ΔN373 and Ttk69ΔN469 showing that this region is important for degradation. 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 degraded in a similar manner in the presence of Sina and Phyl.

SinaH directed degradation of Ttk69 requires a 50 amino acid region unique to Ttk69

To investigate if SinaH directed the degradation of Ttk69 differently to Sina, we tested the same set of Ttk69 deletion constructs. We used low levels of SinaH, at which the control protein Pointed was not degraded, but full length Ttk69 was highly susceptible (Fig 3B, lane 7). Three constructs were resistant to degradation: Ttk69ΔN469, Ttk69ΔC280 and Ttk69ΔC420 (Fig 4, lane 7, marked with *) but these were different from the resistant fragments in the Sina experiment. In this experiment, Phyl and SinaH were also transfected together (Fig 4, lane 8), but addition of Phyl did not cause any further degradation. Using this assay, the region necessary for SinaH directed degradation was mapped between amino acids 420 and 469 (marked on Fig 4) of Ttk69. Together these results demonstrate two distinct modes of Ttk69 degradation and show that SinaH acts through a novel region of the Ttk69 substrate in order to direct its degradation.

Phyl acts as an adaptor between Ttk69 and both Sina and SinaH

We wanted to investigate if SinaH could bind to Ttk69, and if the same region on Ttk69 required for SinaH directed degradation also mediated this interaction. Protein interaction studies were therefore carried out. We produced ^{35}S methionine labelled *in vitro* translated proteins, which contained a 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 strongly to the BTB domain in the common region of the Tramtrack isoforms, and that Sina and Phyl bind strongly, thereby suggesting that Phyl is an adaptor acting to bring Sina and Tramtrack together (23). In Figure 5A 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). Ttk 69 was also able to bind to Phyl and SinaH when they were both 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* (Figure 5B and C) and this indicates that SinaH has a similar mode of binding to Ttk69 via the adaptor Phyl.

SinaH binds to Ttk69 in vivo at the region required for SinaH directed degradation

Interestingly, Ttk69 could also weakly interact with SinaH alone (Figure 5A lane 13). This binding was very weak compared with Phyl and Ttk69 binding, so it is possible that an unknown adaptor protein might be required. To investigate if Ttk69 interacts with SinaH *in vivo*, and to determine if such an interaction correlates with the region of Ttk69 that is required for SinaH directed degradation, we performed a coimmunoprecipitation experiment in S2 cells.

In S2 cells, Sina and SinaH both degrade Ttk69 and Ttk88 proteins and themselves. Therefore to perform coimmunoprecipitation experiments it was necessary to mutate the active site residues in Sina and SinaH to eliminate this activity. This was done by a mutation of the third cysteine residue in the RING finger to alanine (Sina C120A, SinaH C87A). These mutants were then tagged with the FLAG epitope and transfected into S2 cells with either full length Ttk69, Ttk69ΔN373 or Ttk69ΔN469. Both Sina and SinaH interacted strongly with full length Ttk69 (Fig 6, lanes 2 and 3). Unlike the *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.

Ttk69ΔN373 and Ttk69ΔN469 both lacked the BTB domain and therefore any interaction could not be via endogenous Phyl. Neither Sina nor SinaH bound to Ttk69ΔN469, a fragment that was resistant to degradation by both E3 ubiquitin ligases (Fig 6, lanes 8 and 9). However, compared with Sina, SinaH bound to Ttk69ΔN373 more strongly (Fig 6, lane 5 compared with lane 6). This is also consistent with the degradation data, as this fragment is resistant to Sina directed degradation, but contains the region required for SinaH directed degradation.

DISCUSSION

We have shown that the novel RING finger containing protein CG13030 or SinaH, can direct the degradation of Tramtrack and thus, in a similar way to Sina, act as a putative E3

ubiquitin ligase. However, despite the high homology between these E3 ligases, SinaH directs degradation of Ttk69 in a different manner to Sina. SinaH recognises 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 (420-469 amino acids) is required for this degradation. This region is unique to the Ttk69 isoform. In a coimmunoprecipitation assay SinaH interacts with this region of Ttk69, and *in vitro* there is a weak direct binding between Ttk69 and SinaH. These results suggest a model by which SinaH directs the degradation of Ttk69 either by interacting directly, or more probably, via an unknown adaptor molecule, that interacts with the 420-469 region in Ttk69 (Fig 7).

Interestingly, SinaH can also bind to Phyl both *in vitro* and *in vivo*. Consistent with this result, degradation of Ttk88 directed by SinaH is increased by the addition of Phyl. This implies a similar mechanism to Sina and Phyl directed degradation and suggests that SinaH could act redundantly with Sina in some circumstances (Fig 7). Both Sina and SinaH interact equally well with full length Ttk69 in a coimmunoprecipitation, most probably due to endogenous Phyl acting as an adaptor. Phyl interacts in the common region of the Ttk isoforms, so it seems likely that the SinaH/Phyl directed degradation occurs for both Ttk69 and Ttk88. We did not see Phyl-dependence in our assay of Ttk69 degradation by SinaH, which is probably due to the fact that SinaH directed degradation is more active. SinaH is more active towards Ttk69 in S2 cells, compared with its activity towards Ttk88 and other control proteins.

In developing the degradation assay we have found that in S2 cells, the amounts of DNA used to transfect are very critical, and that highly overexpressing either Sina or SinaH can cause non-specific degradation of unrelated proteins. We have found that specific degradation of both Ttk88 and Ttk69 requires both Phyl and a low level of Sina. Slightly different endogenous levels of Sina, Phyl and Ebi in our sub-strain of S2 cells might account for the difference between this and previous assays (17,19) in which only the addition of Phyl was necessary.

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 non-lethal 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 similar way to Sina, requiring Phyl in order to direct degradation. However it seems that the Phyl independent Ttk69 degradation 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 and degradation. Given this biochemical similarity between the degradation directed 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.

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The abbreviations used are

BTB	Bric-à-Brac-Tramtrack-Broad
SIP	Siah interacting protein

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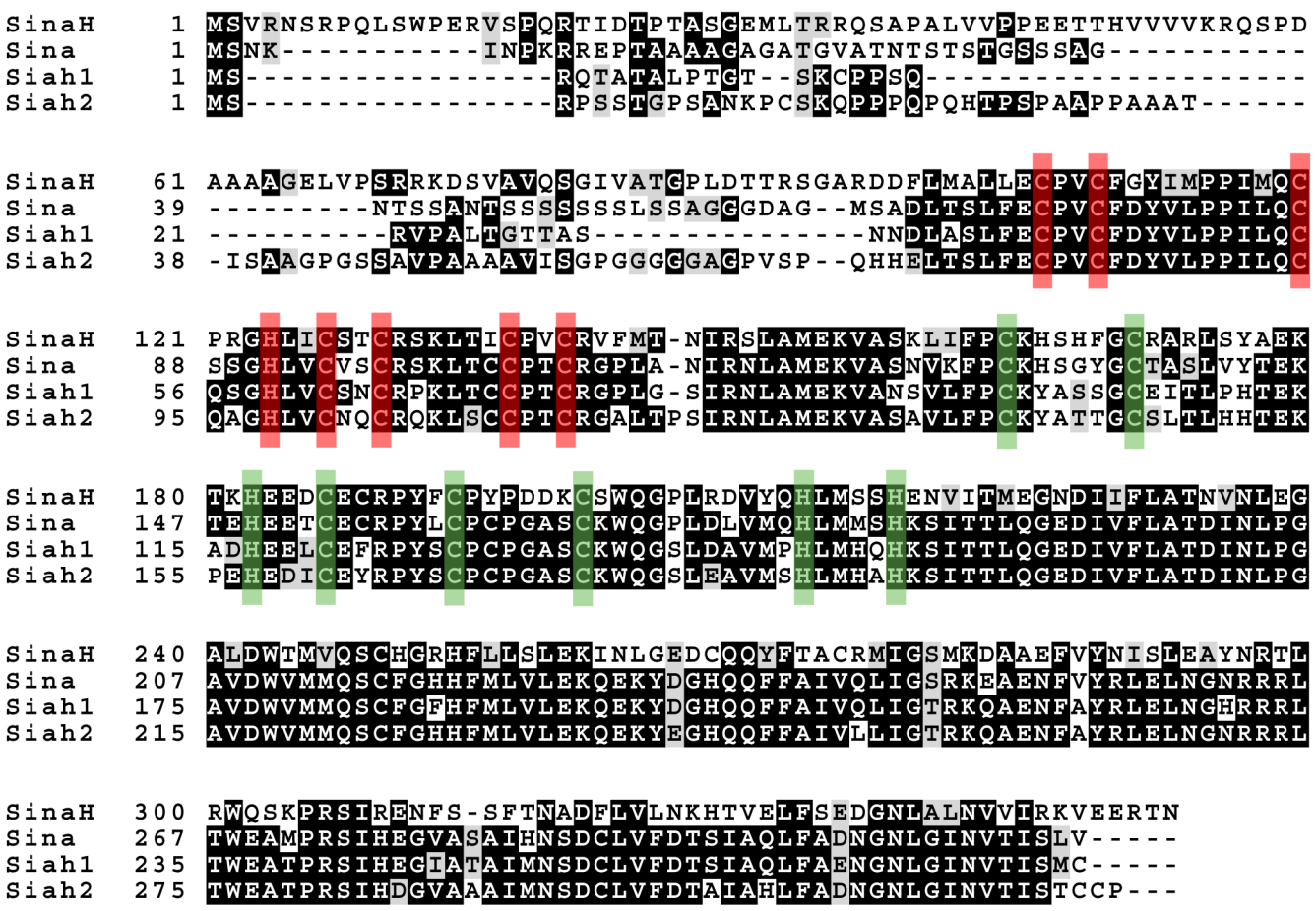

FIGURE 1. Sina-Homologue is a novel protein highly related to Sina

An amino acid alignment of SinaH with the *Drosophila* Sina protein and the two human homologous, Siah1 and Siah2. The conserved cysteines and histidines of the RING finger (red) and the zinc finger (green) are shown.

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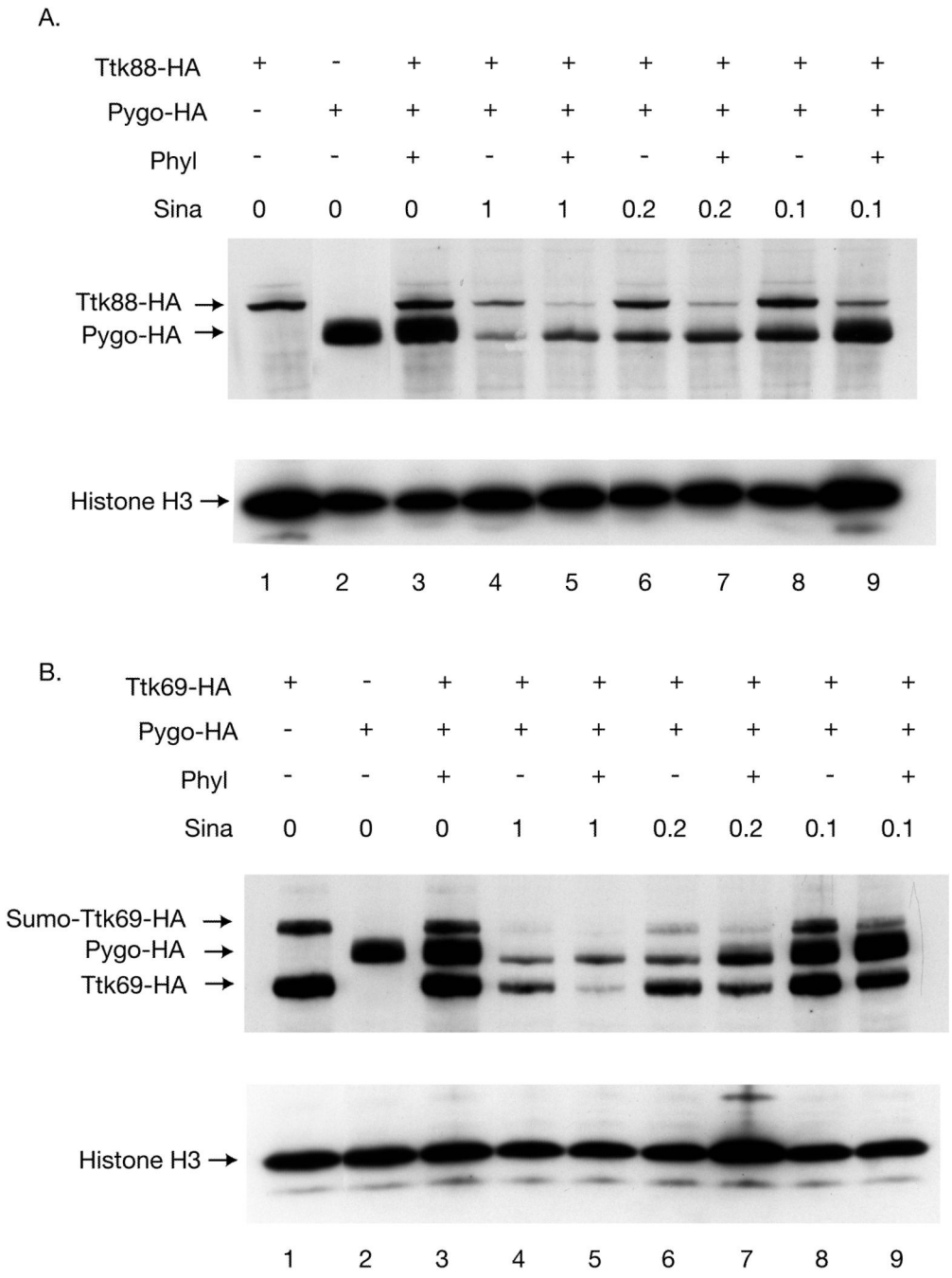

FIGURE 2. Ttk88 and Ttk69 degradation is dependent on Sina and Phyl

A. S2 cells were transfected with expression vector (pRMHA3) containing Ttk88-HA (0.25 μg) and Pygo-HA (0.25 μg) and with combinations of Phyl (0.375 μg) and Sina (various amounts). The highest amount of Sina added was normalised to 1 (which corresponds to 0.375 μg), and the subsequent transfections used lower amounts of DNA. 18 h after induction with $CuSO_4$, cell extracts were analysed for levels of Ttk88-HA and Pygo-HA by Western blot probed with HA antibody. The lower part of the blot was probed with anti-Histone H3 as a loading control. B. As *A*, except to shown degradation of Ttk69-HA. Ttk69 is SUMOylated and this larger Ttk69 species is labelled.

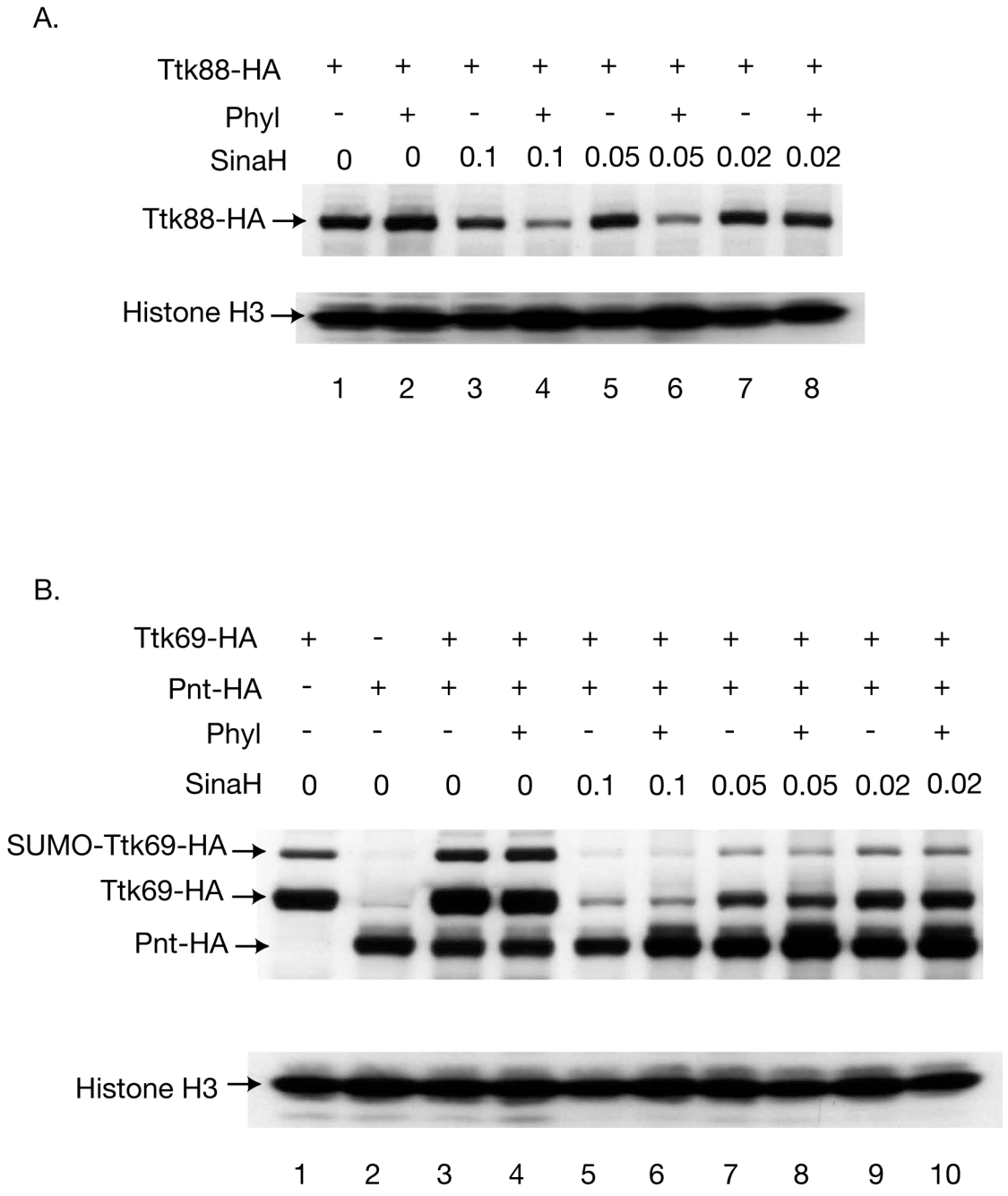

FIGURE 3. SinaH can degrade both Ttk88 and Ttk69

A. S2 cells were transfected with expression vector (pRMHA3) containing Ttk88-HA (0.25 μg) and with combinations of Phyl (0.375 μg) and SinaH (various amounts). The amount of SinaH transfected corresponds to the levels used for Sina transfection in Figure 2 (1= 0.375 μg). It was necessary to reduce the levels of SinaH as this protein seemed more active than Sina. 18 h after induction with $CuSO_4$, cell extracts were analysed for levels of Ttk88-HA by Western blot probed with HA antibody. The lower part of the blot was probed with anti-HistoneH3 as a loading control. B. As A, except Ttk69-HA and Pointed P1-HA were transfected.

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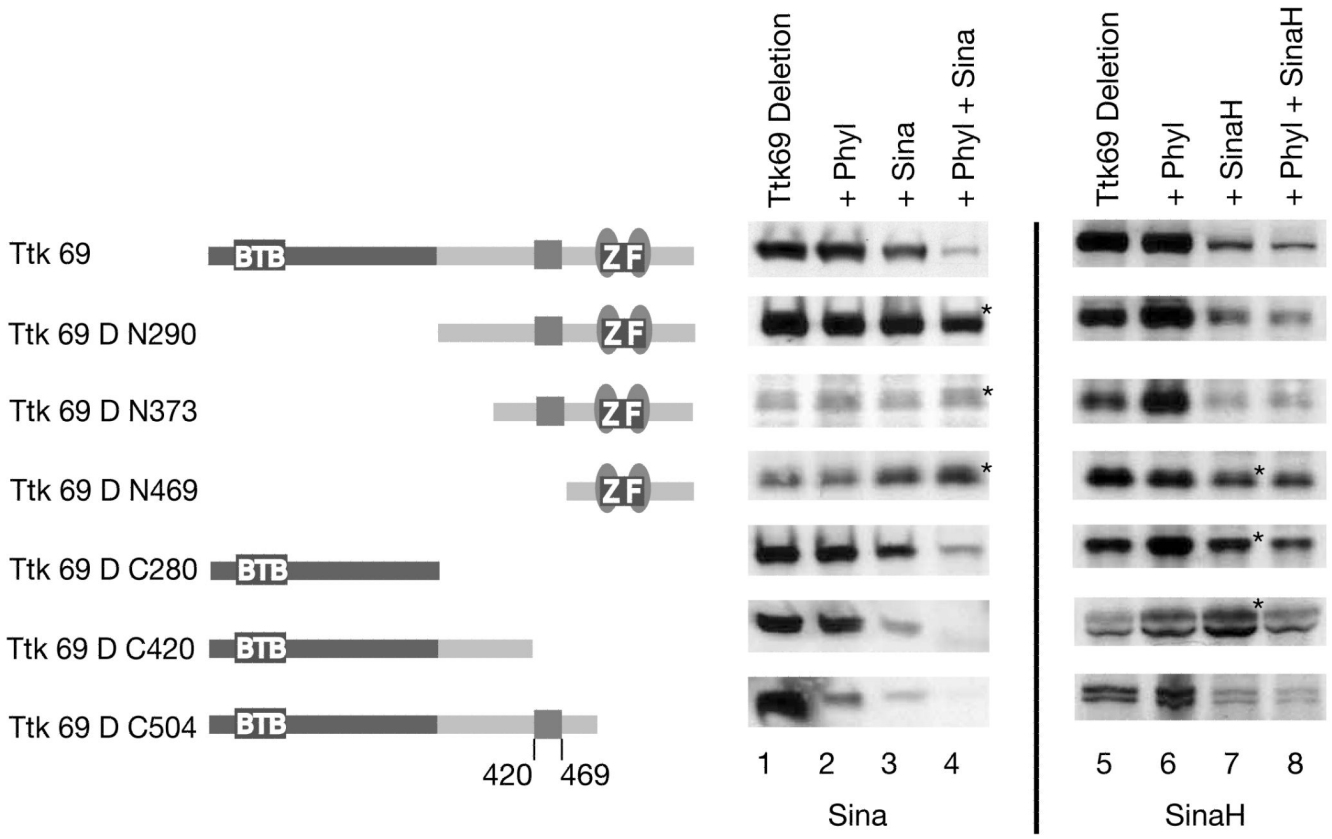

FIGURE 4. Sina and Phyl dependent degradation of Ttk69 requires the BTB domain but SinaH dependent degradation of Ttk69 requires a different region

Various HA tagged fragments of Ttk69 were transfected into S2 cells and are shown here schematically. The dark grey region corresponds to the common region between Ttk69 and Ttk88 and N terminal BTB domain is shown. The light grey region is unique to Ttk69, and the zinc fingers (ZF) are marked at the C terminal. HA 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 are Ttk69-HA deletion constructs alone (0.25 μg). Lanes 2 and 6 show levels of Ttk69-HA fragments with the addition of Phyl (0.375 μg). Lane 3: addition of Sina (the amount shown in Figure 2 as 0.2 or 0.075 μg). Lane 7: addition of SinaH (the amount shown in Figure 3 as 0.05 or 0.01875 μg). Lane 4 and 8 shows Phyl and Sina or SinaH together. Degradation by Sina and Phyl requires the common region of Ttk69 and Ttk88 as if this is deleted, degradation does not occur (marked with *). SinaH degradation is not dependent on Phyl, and requires the region marked 420-469. Deletion of this region causes the Ttk69-HA fragments to become resistant to degradation (marked with *).

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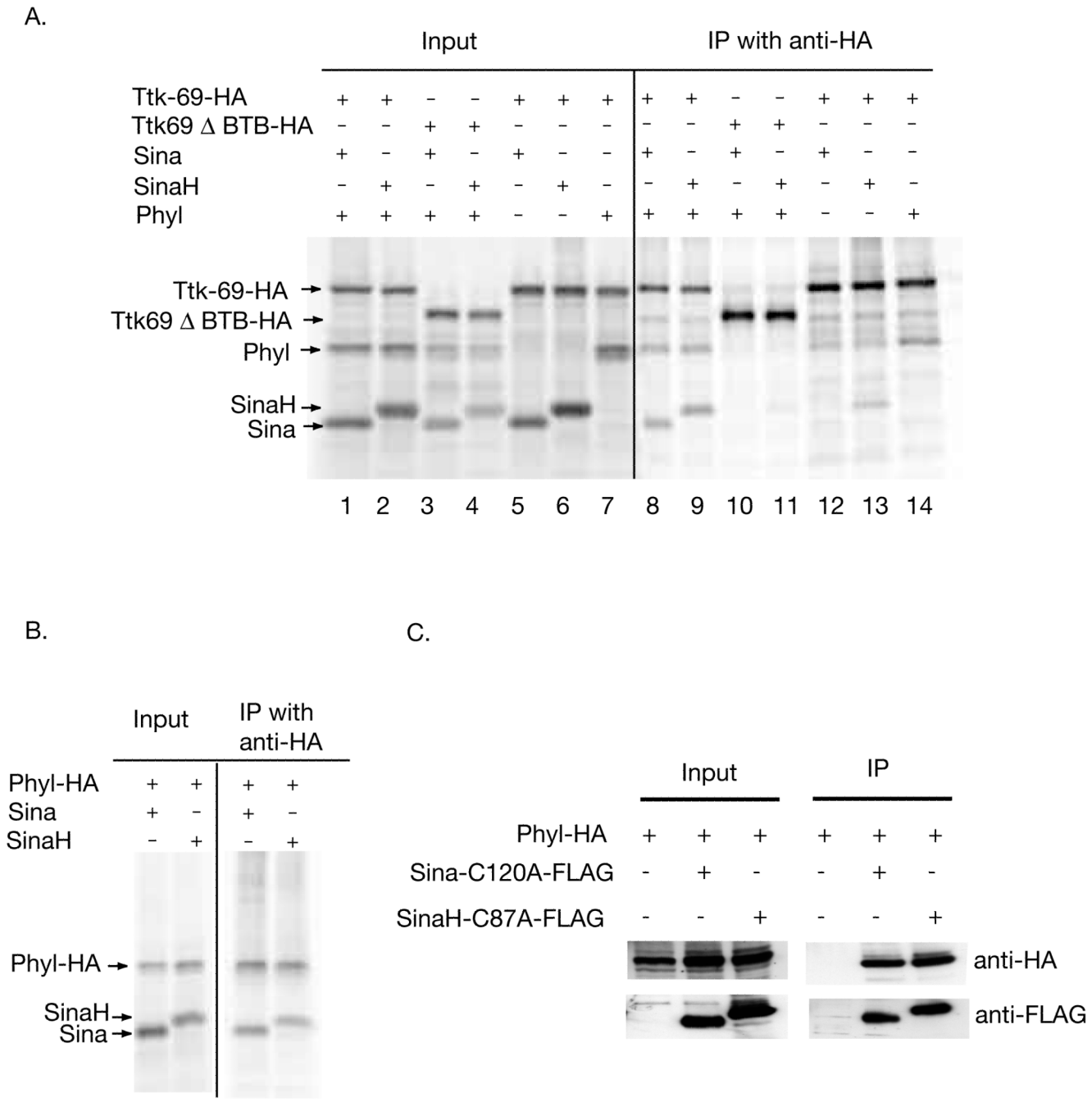

FIGURE 5. Phyl acts as an adaptor binding the BTB domain of Ttk69 and also Sina and SinaH

A. Combinations of proteins were produced by *in vitro* transcription/translation and labelled with ^{35}S methionine. Lanes 1-7 show 10% of the input. IP lanes 8-14 show the material recovered from immunoprecipitations using anti-HA agarose and therefore the proteins which can bind to the HA constructs (either full length Ttk69 or Ttk69ΔBTB). B. 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 μg pRMHA3-HA-Phyl and with 2.5 μg of either carrier pRHMA3, pRMHA3-SinaC120A-FLAG or pRMHA3-SinaHC87A-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. Anti-Flag beads alone do not interact with Phyl-HA, but both Sina and SinaH can bind Phyl-HA *in vivo*.

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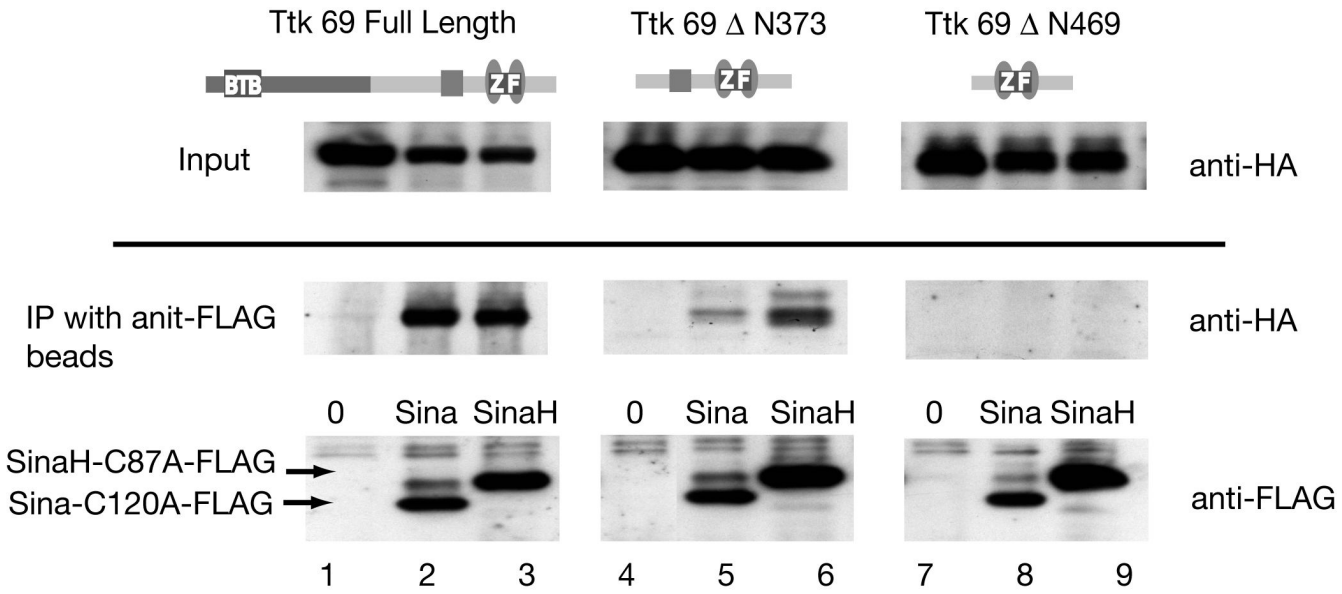

FIGURE 6. SinaH interacts with the same region of Ttk69 that is required for degradation

S2 cells were transfected with 2.5 μg of either carrier pRHMA3, pRMHA3-Sina-C120A-FLAG or pRMHA3-SinaH-C87A-FLAG and with 2.5 μg of either full length Ttk69-HA, Ttk69ΔN373-HA or Ttk69ΔN469-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. Western blots showing the material eluted from the anti-FLAG beads were probed with both anti-FLAG and anti-HA. 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 Ttk69ΔN469-HA. SinaH bound to Ttk69ΔN373-HA, the fragment containing the 50 amino acid region required for degradation (grey box), better than Sina.

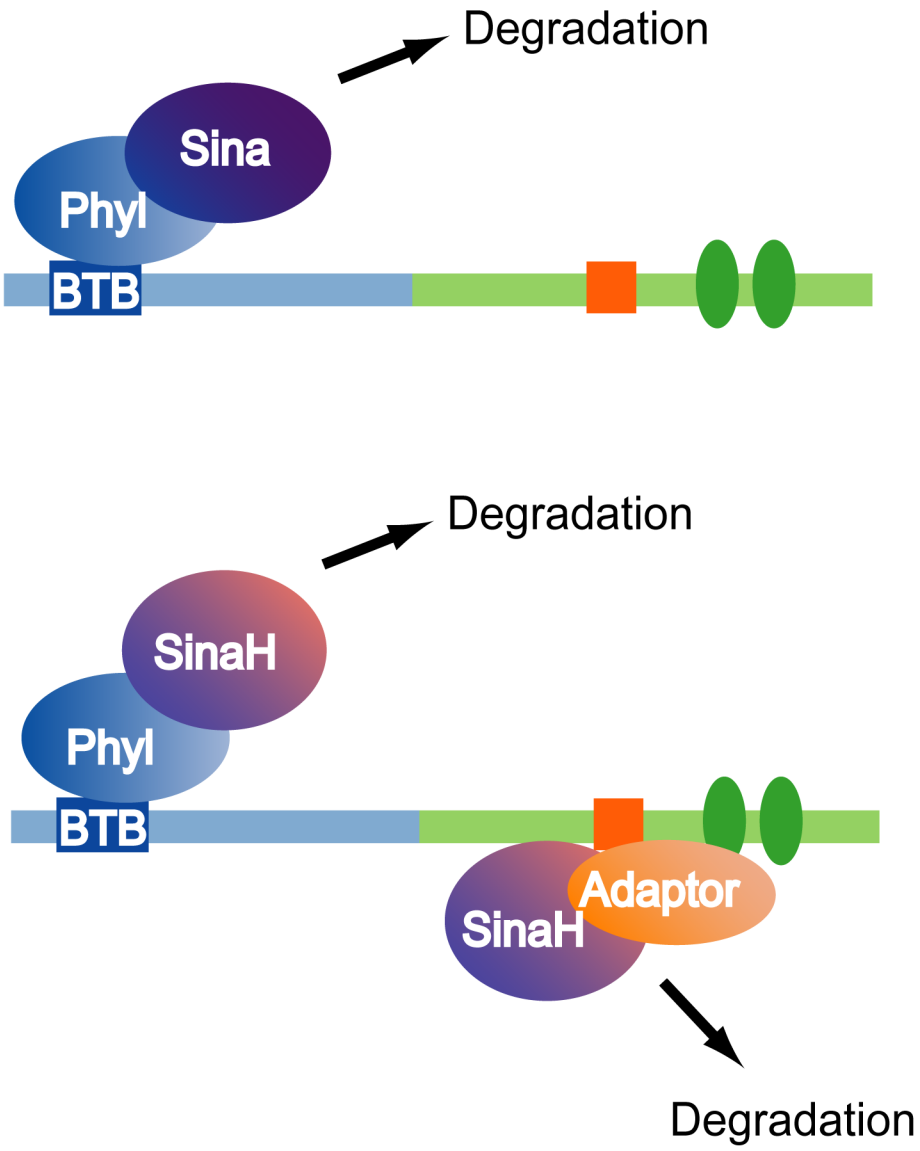

FIGURE 7. A model to show two modes of degradation for Tkk69 by SinaH

Mode one, as for Sina, requires Phyl as an adaptor to bring SinaH close to the substrate, requiring the BTB domain of the substrate. Mode two requires either direct binding of SinaH to Ttk69, or via an adaptor protein, and this mode requires a different region of the substrate molecule for its degradation.