Most mutations that cause spinocerebellar ataxia autosomal recessive type 16 (SCAR16) destabilize the protein quality-control E3 ligase CHIP

The accumulation of misfolded proteins promotes protein aggregation and neuronal death in many neurodegenerative diseases. To counteract misfolded protein accumulation, neurons have pathways that recognize and refold or degrade aggregation-prone proteins. One U-box–containing E3 ligase, C terminus of Hsc70-interacting protein (CHIP), plays a key role in this process, targeting misfolded proteins for proteasomal degradation. CHIP plays a protective role in mouse models of neurodegenerative disease, and in humans, mutations in CHIP cause spinocerebellar ataxia autosomal recessive type 16 (SCAR16), a fatal neurodegenerative disease characterized by truncal and limb ataxia that results in gait instability. Here, we systematically analyzed CHIP mutations that cause SCAR16 and found that most SCAR16 mutations destabilize CHIP. This destabilization caused mutation-specific defects in CHIP activity, including increased formation of soluble oligomers, decreased interactions with chaperones, diminished substrate ubiquitination, and reduced steady-state levels in cells. Consistent with decreased CHIP stability promoting its dysfunction in SCAR16, most mutant proteins recovered activity when the assays were performed below the mutants’ melting temperature. Together, our results have uncovered the molecular basis of genetic defects in CHIP function that cause SCAR16. Our insights suggest that compounds that improve the thermostability of genetic CHIP variants may be beneficial for treating patients with SCAR16.

Protein aggregation is a hallmark of over 70 different diseases, including many neurodegenerative diseases (1–3). To counteract the accumulation of misfolded proteins, cells use protein quality-control pathways to refold or degrade misfolded proteins and promote survival (4, 5). Two major protein quality-control components, the chaperone and ubiquitin-proteasome systems, play critical roles either refolding or degrading misfolded proteins that accumulate in neurodegenerative diseases (1). One particular E3 ligase, C terminus of Hsc70-interacting protein (CHIP),2 sits at the interface of these pathways to facilitate the clearance of aggregation-prone proteins. CHIP binds chaperones via an N-terminal tetratricopeptide repeat (TPR) domain and recruits E2 ubiquitin-conjugating enzymes via a C-terminal U-box domain (6). Recruitment of these partners allows CHIP to facilitate the transfer of ubiquitin from the E2 to chaperone-bound misfolded proteins (6, 7).

In vivo, CHIP plays a critical role in protecting the brain against aggregation-prone proteins commonly found in age-related neurodegenerative diseases. Genetic deletion of CHIP imparts an early aging phenotype in mice, and removing CHIP in the context of models of neurodegeneration exacerbates protein aggregation and neuronal loss (8–12). Conversely, overexpression of CHIP reduces levels of mutant androgen receptor and ameliorates phenotypes in a spinal and bulbar muscular atrophy transgenic mouse model (13). Together, these data strongly argue that CHIP plays a crucial neuroprotective role.

More recently, mutations in CHIP have been identified as the cause of spinocerebellar ataxia autosomal recessive type 16 (SCAR16) (14–18). SCAR16 is a rare form of ataxia characterized by adolescent-onset degeneration of the cerebellum and cognitive impairment (14–18). To date, over 13 mutations have been identified as the cause of SCAR16 (19), but the mechanism by which these mutations disrupt CHIP function has not been defined. Here, we systematically analyzed mutations in CHIP that cause SCAR16. We found that the majority of SCAR16 mutations destabilize CHIP, resulting in a decrease in CHIP activity and decreased steady-state levels in cells. Consistent with this, the activity of most SCAR16 mutants was recovered at temperatures below their thermal melting temperature (Tm).

Together, our data demonstrate an array of defects in CHIP

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References

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This work was supported by National Institutes of Health Grants R00NS073936 and R35GM119544 (to K. M. S.); a grant from the Research and Education Program, a component of the Advancing a Healthier Wisconsin endowment at the Medical College of Wisconsin (to K. M. S.); and the Peter G. Pentchev Research Fellowship from the National Niemann-Pick Disease Foundation (to A. J. K.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

3 The abbreviations used are: CHIP, C terminus of Hsc70-interacting protein; TPR, tetratricopeptide repeat; SCAR16, spinocerebellar ataxia autosomal recessive type 16; FP, fluorescence polarization; TR-FRET, time-resolved fluorescence energy transfer; CFTR, cystic fibrosis transmembrane-conductance regulator; NPC1, Niemann-Pick C1 protein; FMRP, fragile X mental retardation protein.
function and stability that cause SCAR16 and suggest that identification of compounds that stabilize CHIP’s $T_m$ may be beneficial for treating patients with SCAR16.

Results

Mutations in SCAR16 inhibit CHIP activity

In addition to mutations that lead to aborted translation of full-length CHIP, 13 point mutations resulting in single amino acid changes in CHIP cause SCAR16 (14–18) (Fig. 1, A and B). To determine whether SCAR16 mutations decreased CHIP’s E3 ligase activity, we performed in vitro ubiquitination assays with the physiologically relevant substrate Hsp70 (20). Consistent with previous reports, wildtype CHIP facilitates the formation of long polyubiquitin chains on Hsp70, whereas SCAR16 mutants had severely decreased activity, leaving large amounts of unmodified, mono-, and diubiquitinated substrate (Fig. 1, C and D).

To facilitate ubiquitination, CHIP binds chaperones that are bound to misfolded proteins. Therefore, we reasoned that a decrease in CHIP’s affinity for chaperones may be one potential mechanism that inactivates SCAR16 mutants. To begin assessing this, we analyzed the crystal structure of CHIP bound to a C-terminal peptide of Hsp90 (21). One SCAR16 mutation (N65S) is predicted to disrupt CHIP’s interaction with chaperones; however, the vast majority of mutations reside outside of the CHIP/chaperone interface (21) (Fig. 2A). Although most mutations are not predicted to directly disrupt chaperone binding, other mutations may disrupt CHIP’s ability to bind chaperones by altering CHIP’s dynamics (22). We therefore wanted to systematically determine whether SCAR16 mutations reduced CHIP’s affinity for chaperones. To accomplish this, we utilized an established fluorescence polarization (FP) assay to measure binding between CHIP and a rhodamine (r)-labeled peptide derived from the C terminus of Hsc70 (r-SSGP-TIEEVD) (23). This peptide contains a C-terminal EEVD motif, similar to the Hsp90-derived peptide (RDDTSRMEEVD) used for cocryrstallization with CHIP. Importantly, this peptide contains the EEVD motif that is responsible for the vast majority of the affinity between Hsp70 and CHIP (23, 24). Similar to previous reports, we obtained a $K_D$ of 2.9 ± 0.2 μM for binding between CHIP and chaperone (Fig. 2B and Table 1, right panel) (23). Two mutations in CHIP’s TPR domain, N65S and L123V, robustly inhibited chaperone binding by CHIP, whereas other mutations, including E28K, A79D, N130I, M240T, and T246M, caused more modest deficits (Fig. 2B and Table 1, right panel).

Together, these data demonstrate that some SCAR16 mutations disrupt CHIP’s ability to bind chaperones.

In addition to recruitment of substrate, CHIP also recruits E2 ubiquitin-conjugating enzymes. Failure in substrate ubiquitination by SCAR16 mutants (Fig. 1C) may arise from defects in E2 recruitment or from structural defects that prevent proper orientation of substrate and E2. If substrate and E2 are not properly aligned, substrate ubiquitination would be inhibited, whereas the formation of unanchored ubiquitin chains would still occur (22). To distinguish from these two possibilities, we performed time-resolved fluorescence energy transfer (TR-FRET) ubiquitination experiments to quantitatively assess the formation of ubiquitin chains. If SCAR16 mutants are defective in E2 recruitment, we expected to see a deficiency in ubiquitin chain formation and TR-FRET signal. However, if E2s are still recruited to SCAR16 mutants but are not properly aligned next to the substrate, we expected to observe TR-FRET signal as free...
The majority of SCAR16 mutants form soluble oligomers

To assess structural changes that may alter substrate ubiquitination, we performed gel filtration analysis on SCAR16 mutants. In solution, the majority of wildtype CHIP forms a homodimer with a small population of tetramer present. However, under conditions of cellular stress, CHIP has an increased propensity to form soluble oligomers (Fig. 4A). We next performed gel filtration experiments to determine whether SCAR16 mutations altered the oligomeric state of CHIP. As expected, the majority of wildtype CHIP was present as a dimer with some tetrameric species (Fig. 4A). Like wildtype CHIP, the N65S mutation was predominately a dimer with some tetramer; however, the majority of SCAR16 mutations led to an increased population of soluble oligomers (Fig. 4, A–C). Together, these data demonstrate that the vast majority of SCAR16 mutants have an increased propensity to form larger oligomeric species.

The majority of SCAR16 mutants have decreased thermal stability

CHIP oligomerization is induced upon heat stress both in vitro and in cells (26). This led us to hypothesize that SCAR16 mutants may have a decreased thermal stability, promoting SCAR16 mutant oligomerization. To test this hypothesis, we determined the $T_m$ of wildtype and SCAR16 mutants using thermal shift analysis. Although wildtype CHIP’s $T_m$ was $39 \pm 0.5 ^\circ C$, most SCAR16 mutants’ $T_m$ values were at or below physiological temperature (Table 2). Because the majority of SCAR16 mutants have a $T_m$ at or below physiological temperature, we next tested the stability of SCAR16 mutants in cells. To assess this, wildtype and SCAR16 mutants were expressed in HEK293 cells, and levels of CHIP normalized to tubulin were assessed 48 h post-transfection. Consistent with a decrease in CHIP stability, the majority of SCAR16 mutants had decreased steady-state levels in cells (Fig. 5, A and B). Three mutations (W147C, M211I, and S236T) were expressed at similar levels as wildtype and SCAR16 mutants using $\mu M$ and $\mu mol min^{-1}$.

<table>
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<tr>
<th>CHIP</th>
<th>$K_D$ $\mu M$</th>
<th>$B_{max}$ $\mu mol min^{-1}$</th>
<th>$K_D$ $\mu M$</th>
<th>$B_{max}$ $\mu mol min^{-1}$</th>
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<tr>
<td>WT</td>
<td>3.4 ± 0.2</td>
<td>222.8 ± 4.5</td>
<td>2.9 ± 0.2</td>
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<td>E28K</td>
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<td>N65S</td>
<td>30.8 ± 13.6</td>
<td>135.7 ± 37.1</td>
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<td>A79D</td>
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<td>M240T</td>
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<td>T246M</td>
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CHIP destabilization causes SCAR16

Figure 2. Some SCAR16 mutants have defects in chaperone binding. A. Crystal structure of the TPR domain of CHIP bound to a C-terminal peptide that corresponds to the C terminus of Hsp90. Asn-65 interacts with the Hsp90 peptide and is indicated in red. Binding curves for FP assays of wildtype and SCAR16 mutants at 37 °C. Increasing amounts of wildtype or SCAR16 mutants were incubated with 20 nm rhodamine-labeled peptide corresponding to the C terminus of Hsc70. Samples were incubated for 30 min at 37 °C prior to data collection. Error bars represent S.D. mP, millipolarization units.

Table 1

Fluorescence polarization measurements of C-terminal Hsc70 peptide binding to CHIP variants

ND, not determined.
CHIP destabilization causes SCAR16

Our in vitro ubiquitination and chaperone binding assays are typically performed at 37 °C. Because we observed that many SCAR16 mutants have a decreased stability at 37 °C, we wanted to determine whether lowering the assay temperature below the $T_m$ of the SCAR16 mutants would restore activity. To test this, we repeated our binding and ubiquitination assays at 25 °C, a temperature below the $T_m$ of all SCAR16 mutants. Reducing the temperature led to a near complete recovery of chaperone binding for the N130I mutant, whereas the L123V, M240T, and T246M mutants had partially restored binding (Fig. 6, A–E, and Table 1). Similar to the FP assay, the majority of SCAR16 mutants regained ubiquitination activity at reduced temperature with the exception of the N65S mutation that disrupted Hsp70 binding and the M240T and T246M mutations that are expected to disrupt E2 binding (Fig. 7). Together, these data demonstrate that lowering the temperature below SCAR16 mutant $T_m$ restores chaperone binding and E3 ligase activity for the majority of SCAR16 mutants.

**Discussion**

Here, we have identified molecular defects in CHIP function that cause SCAR16. We show that mutations that cause SCAR16 decrease CHIP’s ability to bind chaperones and ubiquitinate substrate (Figs. 1–3 and Table 1). We further demonstrate that most SCAR16 mutations cause an increased propensity to form soluble oligomers (Fig. 4), a decrease in thermal stability (Table 2), and a reduction of steady-state levels in cells (Fig. 5). Finally, we show that, for the majority of SCAR16 mutants, defects in chaperone binding and substrate ubiquitination can be improved by lowering the reaction temperature to below CHIP’s $T_m$ (Figs. 6 and 7 and Table 1). Together, our data suggest that the majority of CHIP mutations cause SCAR16 by reducing CHIP’s stability, resulting in decreased CHIP levels and decreased activity of the remaining CHIP protein.

CHIP comprises an N-terminal TPR domain required for binding to the C-terminal EEVD motif of Hsp70 and Hsp90 chaperones, a linker region, and a C-terminal U-box domain important for recruitment of E2. Mutations that cause SCAR16 occur in each of these three domains, suggesting that the defects in CHIP function that cause SCAR16 are likely mutation-specific. Indeed, some mutations, like the N65S and L123V mutations in CHIP’s TPR domain, drastically reduce CHIP’s ability to bind chaperones as measured by fluorescence polarization (Fig. 6A and Table 1). Although the N65S and L123V mutations have major defects in chaperone binding, other mutations in CHIP’s TPR domain such as the E28K and A79D/T mutations only have minor defects in chaperone binding (Fig. 6 and Table 1) but have substantial defects in substrate ubiquitination (Fig. 1, C and D). These mutations, like most SCAR16 mutations, result in decreased thermal stability that partially inactivates and destabilizes CHIP at physiological temperature (Figs. 1C, 2, and 5). Similar to the E28K and A79D/T mutations, mutations in CHIP’s linker domain had little effect on chaperone binding but caused a decrease in CHIP’s E3 ligase activity and decreased thermal stability (Fig. 1C and Table 1). Other SCAR16 mutations in CHIP’s U-box resulted in loss of CHIP’s ability to stimulate ubiquitin chain formation (Figs. 1, C and D, and 3).

Although mutations in CHIP that cause SCAR16 resulted in decreased substrate ubiquitination (Fig. 1C), most SCAR16 mutations remained capable of generating free ubiquitin chains (Fig. 3). In our in vitro TR-FRET ubiquitination assay, we observed a laddering of ubiquitin species that originates from
monoubiquitin (Fig. 3A). Additionally, in previous work, we have found that CHIP is predominantly monoubiquitinated, not polyubiquitinated, under our assay conditions (27). Together, these data are consistent with previous reports that CHIP forms free ubiquitin chains (22). Although formation of free chains occurs in vitro, it is unclear whether CHIP generates free chains in vivo. Free ubiquitin chains are up-regulated under conditions of cell stress (28); however, it is unclear whether CHIP participates in generating these free chains. Surprisingly, in addition to U-box mutations, the two TPR domain mutants that had significant defects in chaperone binding (N65S and L123V) also had defects in free ubiquitin chain formation (Figs. 2B and 3). This suggests that binding of chaperones to CHIP’s TPR domain may be important for activating CHIP’s ability to form polyubiquitin chains. If this is the case, CHIP activity may be limited in the absence of chaperone, preventing CHIP from generating free ubiquitin chains. In the future, it will be important to determine whether binding to chaperones stimulates CHIP’s E3 ligase activity and subsequent formation of polyubiquitin chains.

In addition to examining the mechanism by which the SCAR16 mutations contribute to attenuation of substrate ubiquitination, we also examined the effects that the SCAR16 mutations might have on the thermodynamic stability of CHIP. Wildtype CHIP’s $T_m$ was surprisingly close to physiological temperature (Table 2), and most SCAR16 mutants’ $T_m$ values...
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Table 2

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<tr>
<th>CHIP</th>
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<tr>
<td>WT</td>
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<td>E28K</td>
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<td>N65S</td>
<td>39.0 ± 0.5</td>
</tr>
<tr>
<td>A79P</td>
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<td>A79T</td>
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<td>L123V</td>
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<td>T246M</td>
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Figure 5. The levels of most SCAR16 mutants are significantly decreased in HEK293 cells. A, Western blot of HEK293 cells expressing wildtype or SCAR16 mutants. Immunoblotting was performed with anti-myc or anti-tubulin antibodies as indicated. B, quantification of CHIP levels normalized to tubulin from experiments in A. Error bars indicate S.D. ** indicates p < 0.005, *** indicates p < 0.001, and **** indicates p < 0.0001 (n ≥ 3).

Our data also suggest that destabilization of CHIP results in decreased steady-state levels of some CHIP mutants and decreased CHIP activity in all mutants (Figs. 1–3 and Tables 1 and 2). In some diseases, decreasing CHIP activity and mimicking SCAR16 mutations would be beneficial. In Niemann-Pick disease type C, cystic fibrosis, and fragile X syndrome, CHIP promotes the degradation of proteins whose levels are decreased in the disease state. For example, in cystic fibrosis the most prevalent mutation in the cystic fibrosis transmembrane-conductance regulator (CFTR) is a single amino acid deletion (CFTRΔF508). CFTRΔF508 is functional; however, it has folding defects that result in its ubiquitination by CHIP and subsequent proteasomal degradation (7). Similarly, in Niemann-Pick disease type C, a single amino acid substitution in the Niemann-Pick C1 protein (NPC1), NPC111061T causes CHIP to ubiquitinate NPC111061T. This point mutation causes NPC111061T to be recognized as misfolded and subsequently ubiquitinated by CHIP and targeted to the proteasome for degradation (30). Although in fragile X syndrome an expansion of a repeat sequence in the 5′-untranslated region of the fragile X mental retardation 1 gene leads to decreased production of the fragile X mental retardation protein (FMRP) (31). FMRP is also targeted for degradation by CHIP, and reduction of CHIP levels stabilizes FMRP (32). In these diseases, small molecules that decrease CHIP stability could potentially lead to stabilization of CHIP substrates and be beneficial for patients.

To date, a large body of literature identifies the importance of CHIP in protecting against protein aggregation, especially in the context of neurodegenerative diseases (8–13). Despite the clear importance of CHIP activity, no small molecules that alter CHIP activity have been reported in the literature. Our results suggest that identification of small molecules that alter CHIP’s thermal stability may serve as an avenue to begin designing small molecules that regulate CHIP activity.

Experimental procedures

Constructs

CHIP was cloned into pGEX6p-1 as described previously (33). UbcH5c in pET28a was obtained from Addgene (plasmid 12643). Hsp70 was cloned into pMCSG7 as described previously (27). Mutations were introduced into wildtype CHIP in a pcDNA3.1 vector via a QuikChange Lightning Mutagenesis kit (Stratagene).

Ubiquitination assays

Ubiquitination reactions were performed as previously optimized for maximal CHIP activity (27, 34). Briefly, ubiquitination was performed in 10-μl reactions for 1 h at 37 °C. Reactions contained buffer A (50 mM Tris (pH 7.5), 50 mM KCl, and 0.2 mM DTT), E1mixture (0.1 μM Ube1, 2.5 mM ATP, 2.5 mM MgCl2, and 100 μM ubiquitin), 1 μM UbcH5c, 1 μM CHIP variant, and 1 μM Hsp70. Reactions were stopped by addition of Laemmli buffer and boiling followed by separation of proteins by SDS-PAGE and visualization by Western blotting with appropriate antibodies.
Western blotting

Protein lysates from HEK293 cells were generated by direct lysis in Laemmli buffer followed by sonication and boiling for 5 min at 100 °C. For in vitro ubiquitination assays, reactions were stopped by addition of 4× Laemmli buffer and boiled for 5 min at 100 °C. Both HEK293 lysates and in vitro ubiquitination assays were then resolved by SDS-PAGE and visualized by Western blotting with the indicated antibodies: anti-Hsp/Hsc70 (SC33575, Santa Cruz Biotechnology), anti-CHIP (AB10000, Millipore), and anti-tubulin (PA5-27552, Invitrogen).

Thermal shift assay

Proteins were diluted to 5 μM in PBS and placed at 4 °C. SYPRO Orange protein stain was diluted to 200× from a 5000× stock in PBS. In a 96-well plate, 45 μl of protein was combined with 5 μl of SYPRO Orange solution in triplicate. The mixture was briefly spun down to avoid bubbles before reading in a plate reader. Beginning at 25 °C, each successive 1-min cycle increased the temperature by 1 °C, ending at 95 °C. The melting curve for each protein was obtained by Gaussian curve regression set to fit points in between the minimum and maximum intensity read for each sample. The $T_m$ is the temperature that is halfway between the minimum and maximum value (35).

Fluorescence polarization

For fluorescence polarization experiments, polarization was determined by plotting FP readings as a function of CHIP protein concentration after incubation with 20 nM rhodamine B-labeled Hsc70 peptide diluted in assay buffer (50 mM HEPES, 75 mM NaCl, and 0.001% Triton X-100 (pH 7.4)). Following addition of peptide to CHIP dilutions, the plates were covered and incubated at either room temperature or 37 °C for 30 min to attain binding equilibrium. Polarization values in millipolarization units were measured using a Molecular Devices Flexstation 3 plate reader at excitation/emission of 544/612 nm. Fluorescence polarization experiments were performed using 384-well, black, low-volume, flat-bottom plates. All experiments were performed at least two independent times in triplicate. Results are shown as the average with S.D. displayed for all measurements. Experimental data were analyzed using GraphPad Prism.

TR-FRET ubiquitination assays

In vitro ubiquitination assays were performed using a ubiquitination mixture containing 2 mM ATP, 4 mM MgCl$_2$, 50 nM E1, 200 nM UbcH5c, 500 nM fluorescein-labeled ubiquitin, 50 nM terbium-labeled ubiquitin, 50 nM Hsp70, and 1 μM CHIP variant. Reactions were performed for 1 h at 37 °C and quenched with 20 mM EDTA. TR-FRET readings were taken on a Molecular Devices Flexstation 3 plate reader at excitation of 340 nm and emission at 495/520 nm following a 100-μs delay.
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after excitation. TR-FRET results are presented as a ratio of 520/495 nm fluorescence readings.

**Fluorometry**

Following TR-FRET, *in vitro* ubiquitination assays were resuspended in Laemmli buffer and run on an 8–20% gradient by SDS-PAGE. Imaging was performed on a GE Typhoon Trio Variable Mode Imager using excitation/emission at 488/532 nm.

**HEK293 transfections**

Transfections were performed in 12-well plates by combining 1 μg of DNA in 50 μl of Opti-MEM with 2 μl of Lipo-fectamine 2000 diluted in 50 μl of Opti-MEM followed by addition to HEK293 cells. Protein lysates from HEK293 cells were generated by direct lysis in Laemmli buffer followed by sonication and boiling for 5 min at 100 °C.

**Author contributions**


**Acknowledgments**

We acknowledge members of the Scaglione laboratory for helpful discussions and critiques of the manuscript.

**Addendum**

During the preparation of this manuscript, Hayer et al. (19) identified additional mutations in CHIP that cause SCAR16.

**References**


