Multiple Ser/Thr-rich degrons mediate the degradation of Ci/Gli by the Cul3-HIB/SPOP E3 ubiquitin ligase

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Communicated by Gary Struhl, Columbia University College of Physicians and Surgeons, New York, NY, October 19, 2009 (received for review September 8, 2009)

The Cul3-based E3 ubiquitin ligases regulate many cellular processes using a large family of BTB domain-containing proteins as their target recognition components, but how they recognize targets remains unknown. Here we identify and characterize degrons that mediate the degradation of the Hedgehog pathway transcription factor cubitus interruptus (Ci)/Gli by Cul3-Hedgehog-induced MATH and BTB domain-containing protein (HIB)/SPOP. Ci uses multiple Ser/Thr (S/T)-rich motifs that bind HIB cooperatively to mediate its degradation. We provide evidence that both HIB and Ci form dimers/oligomers and engage in multivalent interactions, which underlies the *in vivo* cooperativity among individual HIB-binding sites. We find that similar S/T-rich motifs are present in Gli proteins as well as in numerous HIB-interacting proteins and mediate Gli degradation by SPOP. Our results provide a mechanistic insight into how HIB/SPOP recognizes its substrates and have important implications for the genome-wide prediction of substrates for Cul3-based E3 ligases.

BTB | E3 ligase | Hedgehog | Gli2 | Gli3

Protein degradation through polyubiquitination plays fundamental and diverse roles in many cellular processes, including signal transduction, cell cycle progression, and metabolic pathways (1). Attachment of ubiquitin (Ub) molecules to a target protein involves activation by an Ub-activation enzyme (E1) and subsequent transfer by an Ub-conjugating enzyme (E2) in conjunction with an Ub ligase (E3) that recognizes the target protein. One large family of E3 ligases consists of multi-subunit complexes organized by the Cullin (Cul) family of scaffolding proteins. A paradigm for this class of E3 ligases is the SCF complex, which contains core components Skp1, Cul1, and Roc1/Rbx1, and a variable F-box protein serving as the substrate recognition subunit (2). Another class of modular E3 ligases consists of the Cul3-based ligases that use the BTB family of proteins as the substrate recognition subunits (3). The Cul3-based ligases regulate important developmental signaling pathways, including Hedgehog (Hh) and Wnt pathways (4, 5).

The Hh signaling pathway is regulated by multiple E3 ligases (5). In the absence of Hh, the transcription factor Ci/Gli is sequentially phosphorylated by PKA, GSK3, and CK1, which creates docking sites for SCF complex containing the F-box protein Slimb/ β -TRCP (6–9). SCF^{Slimb/ β -TRCP}-mediated ubiquitination targets Ci/Gli for proteolytic processing that generates a C-terminally truncated repressor form (Ci^R/Gli^R) (5). Hh signaling blocks Ci/Gli phosphorylation and SCF^{Slimb/ β -TRCP}-mediated processing, and hence the production of Ci^R/Gli^R. In addition, high-level Hh signaling activity converts the accumulated full-length Ci into an active but labile form (10). Degradation of the full-length Ci is mediated, at least in part, by the Hh-induced MATH and BTB domain-containing protein (HIB; also called Rdx or dSPOP) (11–13). In wing and leg imaginal discs, HIB is induced by Hh, which forms a negative feedback loop to fine-tune the Hh signaling output (11, 12). In eye discs, HIB is highly expressed in differentiating cells posterior to the

morphogenetic furrow (MF), where HIB acts together with Cul3 to degrade Ci, thereby limiting the duration of Hh signaling (11, 12, 14). The Cul3-HIB regulatory circuit appears to be conserved, because Gli proteins such as Gli2 and Gli3 can be degraded by HIB when expressed in *Drosophila*, and the mammalian homolog of HIB, SPOP, can functionally replace HIB in degrading Ci (11).

How Cul3-based E3 ligases recognize their substrates is unknown, and the specific degrons in their target proteins remain to be identified for individual BTB proteins that function as target-recognition components. Here we investigate the degrons that mediate Ci degradation by Cul3-HIB. We identify multiple Ser/Thr (S/T)-rich degrons in both the N- and C-terminal regions of Ci. We find that *in vivo* binding and biological function depend on cooperativity among HIB-binding sites. We provide evidence that both HIB and Ci form dimers/oligomers and engage in multivalent interactions. Similar S/T-rich motifs are present in Gli proteins, as well as in numerous HIB-interacting proteins, and mediate Gli degradation by SPOP.

Results

Identifying HIB-Binding Sites in Ci. In a previous study, we found that HIB targets Ci for degradation through both its N- and C-terminal regions. The Ci N-terminal region contains a 49-aa domain called NR (N-terminal regulatory element) that is conserved among all Gli family members (15). NR appears to contain a destruction signal (called the D_N degron) that targets Gli1 for degradation (15, 16). However, we found that deleting NR in the context of a Ci-Gal4 fusion protein, CiGA, in which the N-terminal half of Ci was fused to the Gal4 activation domain, did not affect HIB-mediated degradation of Ci (Fig. S1 in *SI Appendix*).

We then applied yeast two-hybrid screening to identify HIB-binding sequences in the Ci N-terminal region. Combining deletion and site-direct mutagenesis, we identified 2 small fragments (₃₆₈PEQPSSTSGGV₃₇₈ and ₃₇₉AQVEADSASS₃₈₈) that mediate HIB binding to Ci1–440 (Fig. S2 *A–D* in *SI Appendix*). Both fragments contain a stretch of S/T residues preceded by 1 or 2 acidic residues. Substitution of E369, S372, S373, T374, or S375 with A abolished HIB binding, whereas mutating P368 or Q370 to A did not affect HIB binding (Fig. S2 *E* and *F*), suggesting that the acidic and S/T residues are essential.

Author contributions: Q.Z., Q.S., Y.C., and J.J. designed research; Q.Z., Q.S., Y.C., T.Y., S.L., and B.W. performed research; Q.Z., Q.S., Y.C., T.Y., S.L., and J.J. analyzed data; and J.J. wrote the paper.

The authors declare no conflicts of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0912008106/DCSupplemental.

Fig. 1. HIB interacts with multiple S/T-rich motifs in Ci. (A) Diagram of full-length Ci with 6 HIB-binding sites (S1–S6) indicated by individual bars and the sequences of individual sites shown underneath. (B) GST fusion proteins carrying individual HIB-binding sites were incubated with cell extracts from S2 cells expressing HA-HIB-N. The bound HA-HIB-N proteins were analyzed by Western blot analysis using anti-HA antibody (Upper). Equal amounts of GST fusion proteins were used (Lower). (C) Different amounts of GST fusion proteins containing a wild-type (WT) or mutant S4 with A386 to S substitution (AS) were incubated with cell extracts containing HA-HIB-N, followed by Western blot analysis using anti-HA antibody. (D and E) S2 cells were cotransfected with the indicated Ci- and HIB-expressing constructs. Cell lysates were subjected to immunoprecipitation, followed by Western blot analysis with the indicated antibodies. In (D), the asterisk indicates IgG.

With the exception of S372, mutating S373, T374, or S375 to D abolished HIB binding (Fig. S2F).

A similar sequence, ¹³⁵⁹FPDVSSST₁₃₆₆, is located within a C-terminal fragment, Ci1239–1377, known to bind HIB in yeast (11). GST fusion proteins containing ³⁶⁸PEQPSSTSGGV₃₇₈, ³⁷⁹AQVEADSASS₃₈₈, or ¹³⁵⁹FPDVSSST₁₃₆₆ pulled down HIB (Fig. 1B and Fig. S3D and E in SI Appendix), suggesting that these motifs suffice to interact with HIB. GST-³⁷⁹AQVEADSASS₃₈₈ bound HIB less effectively, but A₃₈₆ to S substitution significantly enhanced HIB binding (Fig. 1C). Thus, an optimal HIB-binding site consists of 4 contiguous S/T residues preceded by 1 or more acidic residues.

Surprisingly, mutating ³⁶⁸PEQPSSTSGGV₃₇₈ and ³⁷⁹AQVEADSASS₃₈₈ in the context of Ci1–440 (Ci1–440m34) reduced, but did not abolish, HIB binding in a GST pull-down assay (Fig. S3A and B in SI Appendix). Likewise, mutating ¹³⁵⁹FPDVSSST₁₃₆₆ in the context of Ci1239–1377 (Ci1239–1377m6) also failed to abolish HIB binding (Fig. S3A and C), suggesting that both regions contain additional HIB-binding sites. Further mapping using smaller fragments or short S/T-rich sequences fused to GST allowed us to identify 3 additional HIB-binding sites, ⁴⁶PTDVSSSVTVPS₅₇, ²¹⁶ALSSSPYSDSFD₂₂₇, and ¹²⁶⁷ISQSQMSPST₁₂₇₆ (Fig. S3B–D). ⁴⁶PTDVSSSVTVPS₅₇ matches the HIB-binding consensus sequence more closely and exhibits stronger binding than the other 2 sites (Fig. 1B and Fig. S3D and E). Collectively, we term the 6 identified HIB-binding sites S1–S6 based on their locations (Fig. 1A). Using a coimmunoprecipitation assay (CoIP), we found that mutating the 4 N-terminal sites (S1–S4) in Ci1–440 (CiNm1–4), 2 C-terminal sites (S5 and S6) in Ci1160–1377 (CiCm56), or all 6 sites in full-length Ci (CiM1–6) abolished HIB binding (Fig. 1D and E), suggesting that these are the major HIB-binding sites in Ci.

Fig. 2. HIB-binding sites regulate Ci stability in vivo. (A–D) Eye imaginal discs expressing HACi (A), HACim346 (B), HACim125 (C), or HACim1–6 (D) with eq-Gal4 were immunostained with anti-HA (red) and anti-Ci (blue) antibodies. Mutating HIB-binding sites stabilized Ci posterior to the MF (arrows). (E–H') Eye imaginal discs containing *hib* mutant clones and expressing HACi (E and E'), HACim346 (F and F'), HACim125 (G and G'), or HACim1–6 (H and H') with GMR-Gal4 were immunostained with anti-HA (red) and anti-GFP (green) antibodies. *hib* mutant clones were recognized by the lack of GFP expression (arrows). (I–L) Adult eyes derived from imaginal discs expressing HACi (I), HACim346 (J), HACim125 (K), or HACim1–6 (L) with GMR-Gal4. Expression of Ci variants with HIB-binding sites mutated resulted in rough eyes. (M) S2 cells were cotransfected with the indicated Ci- and HIB-expressing constructs. Cell lysates were subjected to immunoprecipitation, followed by Western blot analysis with indicated antibodies.

In Vivo Function of HIB-Binding Sites. When expressed in eye discs using eq-Gal4, the Ci variant lacking all 6 sites (HACim1–6) was stabilized posterior to the MF (Fig. 2D), whereas Ci mutant lacking S1, S2, and S5 (HACim125) or S3, S4, and S6 (HACim346) was partially stabilized posterior to the MF (Fig. 2B and C). When expressed posteriorly to the MF using GMR-Gal4 in eye discs carrying *hib* mutant clones, both HACim125 and HACim346 were still up-regulated in *hib* mutant cells (Fig. 2F and G'). In contrast, the level of HACim1–6 was not increased in *hib* mutant clones (Fig. 2H and H').

We have shown previously that expressing stabilized Ci using GMR-Gal4 led to abnormal eye morphology (11). Consistently, expressing HACim346 (four of eight transgenic lines) or HACim125 (three of six transgenic lines) caused eye roughness (Fig. 2J and K), whereas expressing HACi (seven of seven lines) gave rise to normal eyes (Fig. 2I). Expressing HACim1–6 (11 of 11 lines) caused lethality, and a few escapers exhibited severe rough eye phenotypes (Fig. 2L). CoIP in S2 cells indicated that HACim125 and HACim346 bound Myc-tagged HIB (Myc-HIB) with reduced affinity compared with HACi, whereas HACim1–6 failed to bind Myc-HIB under the same condition (Fig. 2M). Taken together, these results suggest that multiple HIB-binding sites are used for optimal HIB recruitment and efficient Ci degradation.

Contribution of Individual HIB-Binding Sites to Ci Regulation. We applied a *ptc-luc* luciferase assay to determine the relative

Fig. 3. Contribution of individual S/T-rich motifs to HIB binding and Ci repression. (A) Diagrams of CiGA and full-length Ci constructs, with wild-type and mutated HIB-binding sites indicated by bars and crosses, respectively. (B and C) *ptc-luc* reporter assays in S2 cells expressing indicated CiGA (B) or full-length Ci (C) constructs in the presence or absence of HIB coexpression. The y-axis represents normalized *ptc-luc* activity. (D and E) S2 cells were cotransfected with wild-type or mutant Myc-CiN (D) or HACi (E) constructs and Flag- (D) or Myc-tagged HIB (E). Cell lysates were subjected to Western blot analysis directly (Lower) or immunoprecipitation followed by Western blot analysis with indicated antibodies. Arrows and asterisks indicate pulled-down proteins and IgG, respectively.

contribution of individual HIB-binding sites to Ci regulation (Fig. 3A–C). CiGA lacking S1 and S2 (CiGA m12) was repressed by HIB (Fig. 3A and B). In contrast, CiGA lacking S3 and S4 (CiGA m34) or all 4 sites (CiGA m1–4) was resistant to HIB (Fig. 3A and B). Furthermore, Myc-tagged Ci1–440 (Myc-CiN) lacking S1 and S2 (Myc-CiNm12) was efficiently pulled down by Flag-tagged HIB (Fg-HIB), whereas mutating S3 and S4 (Myc-CiNm34) or all 4 sites (Myc-CiNm1–4) abolished HIB binding (Fig. 3D). Mutating S1 and S2 did not affect HIB-mediated degradation of CiN or CiGA, whereas mutating S3 and S4 diminished CiN and CiGA degradation (Fig. S4A and B in [SI Appendix](#)). In addition, mutating S3 and S4, but not S1 and S2, affected HIB-mediated ubiquitination of CiN (Fig. S4C). Mutating either S3 or S4 in the context of CiGA (CiGA m3 or CiGA m4), or CiGA m12 (CiGA m123 or CiGA m124) affected HIB-mediated repression only slightly (Fig. 3B). Consistent with this finding, both CiNm123 and CiNm124 bound effectively to HIB, albeit with slightly reduced affinity (Fig. 3D, lanes 5 and 6). Thus, S3 and S4 appear to be the critical sites in CiN that act partially redundantly to mediate HIB binding and Ci degradation.

We also examined the relative contribution of the C-terminal sites in the context of a full-length Ci lacking the 4 N-terminal sites (Cim1–4; Fig. 3A). Mutating S6 (Cim1–4,6) did not significantly affect HIB-mediated inhibition, whereas mutating S5 (Cim1–5) abolished it (Fig. 3A and C), suggesting that S5 is more critical than S6 in the C-terminal region. Both Cim346 and Cim125 were suppressed by HIB (albeit less effectively than the wild-type Ci), whereas Cim1–6 was resistant to HIB-mediated inhibition (Fig. 3A and C), consistent with its relative stability in vivo (Fig. 2).

The finding that mutating S6 had less effect than mutating S5 was surprising, because S6 exhibits higher binding affinity for HIB in vitro (Fig. 1B). However, in corroboration with its in vivo activity, Cim1–4,6 bound HIB much better than Cim1–4,5 did (Fig. 3E), indicating that S5 is more critical than S6 in mediating HIB binding in vivo. Consistent with S3/4 and S5 being critical for HIB binding, mutating these sites in the context of full-length Ci (Cim345) abolished HIB-mediated inhibition (Fig. 3A and C).

HIB Forms a Dimer Through Multiple Interactions. BTB domains in several BTB-ZF family members form homodimers (17, 18). Indeed, the crystal structure of the BTB domain of PLZF reveals a tightly intertwined dimer with an extensive hydrophobic interface (19). Using CoIP, we found that Myc-HIB-F interacted strongly with HA-HIB-F and weakly with HA-HIB-N and HA-HIB-C, but did not interact with HA-MATH (Fig. 4A and

Fig. 4. Both HIB and Ci form dimers. (A) Diagrams of full-length and truncated forms of HIB. (B–E) S2 cells were transfected with indicated Myc- and HA-tagged HIB constructs (B and C) or Ci constructs (D and E). Cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. Asterisks indicate IgG. (F) FRET efficiency of the indicated CFP/YFP-tagged constructs expressed in S2 cells.

Fig. 5. Multivalent interactions between HIB and Ci. (A) *cis*-cooperativity among C-terminal HIB-binding sites. S2 cells were transfected with Flag-HIB and Myc-tagged Ci1160–1377 (CiC) or its variants with the indicated mutations, followed by immunoprecipitation and Western blot analysis with the indicated antibodies. (B) *trans*-cooperativity between N-terminal HIB-binding sites mediated by dimerization. S2 cells were transfected with Flag-HIB and indicated Ci constructs, followed by immunoprecipitation and Western blot analysis with the indicated antibodies. Asterisks indicate IgG. (C) *ptc-luc* reporter activity of the indicated CiGA derivatives in the presence or absence of HIB coexpression. The y-axis represents normalized *ptc-luc* activity. (D) S2 cells were transfected with HA-CiN or Flag-CiC, a fixed amount of Myc-HIB-N, and increasing amounts of Myc-HIB-F, followed by immunoprecipitation and Western blot analysis with the indicated antibodies. (E) S2 cells were transfected with Ci and MATH domain constructs, followed by immunoprecipitation and Western blot analysis with the indicated antibodies. The addition of a dimerization domain to MATH resulted in binding to CiN, but not to Ci268–440. (F) S2 cells were transfected with both HA-tagged and Flag-tagged HIB-N, as well as Myc-tagged CiN, CiC, or their mutant forms, followed by immunoprecipitation and/or Western blot analysis with the indicated antibodies. The interaction between 2 HIB-N molecules was greatly enhanced by either CiN or CiC. (G) A model for multivalent interactions between HIB and Ci. NDD, N-terminal dimerization domain; ZF, zinc fingers; M, MATH. See the text for details.

B). Consistently, both HIB-N and HIB-C self-associated weakly compared with HIB-F, whereas the MATH domain did not self-associate (Fig. 4C), suggesting that HIB exists as a dimer/oligomer through both N- and C-terminal interactions. (Herein, the term “dimer” is used for simplicity.)

Cooperative Binding of HIB Through Multiple S/T-Rich Motifs. The observation that HIB exists as a dimer raised an interesting possibility that HIB may bind Ci through multivalent interactions. In this scenario, 2 intrinsically weak binding sites could interact with 2 MATH domains in a HIB dimer to achieve high occupancy. For example, S5 may cooperate with S6 to bind HIB, and when S6 is mutated, S5 may cooperate with other S/T motifs in the vicinity. Consistent with this, mutating S6 (CiCm6) reduced HIB binding, whereas mutating S5 (CiCm5) in the context of Ci1160–1377 (CiC) abolished HIB binding (Fig. 5A). To identify other S/T-rich motifs that might cooperate with S5, we generated a series of C-terminally truncated CiC fragments and examined their interaction with HIB (Fig. S5A in *SI Appendix*). Longer fragments, including Ci1160–1306, still interacted with HIB, whereas Ci1160–1287, which contains intact S5, failed to bind HIB (Fig. 5B), suggesting that the S/T-rich motif $_{1284}\text{FSTVNMQPITTS}_{1295}$ (denoted by “w” hereinafter) may cooperate with S5. In the context of CiC, mutating w (CiCw) reduced HIB binding, whereas mutating both w and S6 (CiCm6w) abolished HIB binding (Fig. 5A). Similarly, in the context of Ci1–4, mutating both w and S6 (Ci1–4,w) abolished HIB binding as well as HIB-mediated repression (Fig. S6 in *SI Appendix*). These results suggest that S5 cooperates with both S6 and w to bind HIB.

The N-Terminal Region of Ci Mediates Dimerization. We next investigated how the N-terminal sites might cooperate to bind HIB. The observation that S3/4 alone can mediate HIB binding led us to speculate that these sites might cooperate *in trans* through Ci dimerization (Fig. 5G). A previous study implied that Gli

proteins might form homodimers or heterodimers through an interaction between the first 2 zinc fingers (20). We found that HA-Ci1–686 associated with Myc-Ci440–1160, but not with Myc-Ci1160–1377, in CoIP (Fig. 4D). Furthermore, Myc-Ci associated with HA-Ci and HACiΔ1–440, but not with HACiΔ1–620 (Fig. 4E), suggesting that the first 2 zinc fingers mediate Ci dimerization. Interestingly, we also observed that HA-Ci1–686 associated with Myc-Ci1–440 (Myc-CiN) (Fig. 4D), suggesting that the N-terminal region of Ci can self-associate as well. Further deletion analyses suggest that the N-terminal region between aa 1 and aa 212 can mediate dimerization (Fig. S7 in *SI Appendix*).

To confirm that full-length Ci forms a dimer in intact cells, we applied fluorescence resonance energy transfer (FRET) analysis (21). We generated CFP- and YFP-tagged Ci with the fluorescence protein fused either to the Ci N-terminus (Ci-CFP^N/Ci-YFP^N) or C-terminus (Ci-CFP^C/Ci-YFP^C). As a positive control, we generated CFP- and YFP-tagged forms of Costal2 (Cos2-CFP^C/Cos2-YFP^C), a kinesin-like protein thought to form a dimer (22, 23). We observed high FRET between Ci-CFP^N and Ci-YFP^N (11.0%) that was comparable to the FRET between Cos2-CFP^C and Cos2-YFP^C (10.2%) (Fig. 4F). These FRET values also are comparable to those of Smo and Fz2, which form dimers in S2 cells (24). FRET between Ci-CFP^C and Ci-YFP^C was relatively low but significant (4.2%; Fig. 4F). In contrast, we did not observe significant FRET between Ci-CFP^N and Ci-YFP^C (Fig. 4F). Similar results were obtained using a Ci variant lacking 3 PKA sites (Ci-3^P) that was no longer processed into Ci^R and remained as a full-length form (Fig. 4F). Together with the CoIP data, these observations suggest that Ci forms a paralleled dimer/oligomer mediated by N-terminal interactions.

Ci Dimerization Promotes Trans-Cooperativity Between HIB-Binding Sites. Strikingly, a CiGA variant lacking the N-terminal dimerization domain (CiGAΔ1–268) was resistant to HIB-mediated repression in a *ptc-luc* reporter assay (Fig. 5C). Furthermore,

Myc-Ci268–440 failed to bind HIB even though it contains intact S3/4 (Fig. 5B). The addition of a heterologous dimerization motif, the GCN4 leucine zipper dimerization motif (CC), but not its mutant version CCm (25), restored binding of Myc-Ci268–440 to HIB and rendered HACiGAΔ1–268 responsive to HIB-mediated repression (Fig. 5B and C), suggesting that dimerization allows S3/4 to cooperate *in trans* to bind HIB.

HIB Binds Ci Through Multivalent Interactions. A likely explanation for the observed cooperativity among HIB-binding sites is that 2 sites may interact simultaneously with 2 MATH domains within a HIB dimer. Consistent with this multivalent interaction model, we observed that the strength of HIB dimerization was correlated with its binding affinity to Ci. For example, HIB-F appeared to bind CiN much more strongly than HIB-N, and easily outcompeted HIB-N in a competition assay (Fig. 5D). MATH did not exhibit any discernible binding to CiN or CiC, but the addition of a heterologous dimerization domain to MATH (MATH-CC) restored binding to both CiN and CiC, but not to monovalent substrates, such as Ci268–440 and CiCm5 (Fig. 5E and Fig. S8 in *SI Appendix*). Providing further support for the multivalent interaction model, the association between 2 weakly bound HIB-N molecules was greatly enhanced by multivalent substrates (CiN or CiC), but not by monovalent substrates (Ci268–440 or CiCm5) (Fig. 5F).

Similar S/T-Rich Motifs Are Present in Many HIB-Interacting Proteins. We found that similar S/T-rich motifs are present in multiple copies in a large number of HIB-binding proteins identified by a genome-wide protein–protein interaction study (Table S1 in *SI Appendix*) (26). For example, the MAP kinase phosphatase Puckered (Puc) has 8 S/T-rich motifs, 2 of which, ₉₆DEVSTTTSSST₁₀₆ and ₃₇₇ELDSPSTSSSS₃₈₈, match the consensus for optimal HIB-binding sites (Table S1). Interestingly, mutating these 2 sites (Puc-2m) abolished Puc binding to HIB in S2 cells (Fig. S9A in *SI Appendix*). Recently, Liu et al. (13) showed that HIB promoted degradation of Puc in cultured cells and genetically interacted with the TNF/JNK pathway in eye development, although it remains to be seen whether Puc is up-regulated in *hib* mutant cells. We did not observe effective degradation of Puc by HIB in S2 cells, however (Fig. S9B). A likely explanation for this finding is that Puc is localized primarily in the cytoplasm, whereas HIB is localized predominantly in the nucleus of S2 cells (Fig. S9C). This is consistent with our previous observation that HIB is localized primarily in the nucleus of wing and eye imaginal disc cells (11). It is possible that a small fraction of Puc is localized in the nucleus and is regulated by HIB. It also is possible that the subcellular localization of HIB could be context-dependent and that in certain cell types, HIB also might localize outside the nucleus to promote degradation of its substrates.

Regulation of Gli Proteins by SPOP. We have previously shown that the Gli proteins are degraded posterior to the MF when expressed in eye discs (Fig. S10A and A' in *SI Appendix*) (11). However, in contrast to Gli2 and Gli3, which were stabilized in *hib* mutant clones (Fig. S10C and C') (11), Gli1 was not stabilized in *hib* mutant cells posterior to the MF (Fig. S10B and B'), suggesting that Gli1 was not degraded by HIB. In *Gli-luc* reporter assays, we found that Gli1 activity was not significantly inhibited by SPOP, whereas Gli2 activity was readily blocked by SPOP (Fig. S10D). Furthermore, both Gli2 and Gli3 interacted with SPOP and were readily degraded by SPOP (Fig. S10E and F). In contrast, Gli1 did not interact with SPOP and was resistant to SPOP-mediated degradation under similar conditions (Fig. S10E and F). Both Gli2 and Gli3 contain many S/T-rich motifs similar to those present in Ci (Table S2 in *SI Appendix*). In contrast, Gli1 has much fewer S/T-rich motifs (Table S2),

consistent with it being a poor target for SPOP. Mutating a subset of S/T-rich motifs affected SPOP binding to Gli2 and Gli3 and rendered Gli2 resistant to SPOP-mediated degradation (Figs. S10D and G–I, S11, and S12 in *SI Appendix*), suggesting that Gli2 and Gli3 are regulated by SPOP through the S/T-rich degrons.

Discussion

In this study, we identified S/T-rich motifs as degrons for the Cul3-HIB/SPOP E3 ligase and found that they are present in a numerous HIB-binding proteins, including Ci/Gli proteins. To the best of our knowledge, these are the first set of degrons identified for Cul3-based E3 Ub ligases.

Our *in vitro* binding assays indicated that an optimal HIB/SPOP-binding site contains 4 contiguous S/T residues (mostly Ser), preceded by 1 or more acidic residues. However, we found that *in vivo* binding and biological function depend critically on cooperativity among multiple S/T-rich motifs. Interestingly, we observed 2 types of cooperativity: (i) *cis*-cooperativity among HIB-binding sites, which is exemplified by the S/T-rich motifs in the C-terminal region of Ci, and (ii) *trans*-cooperativity between HIB-binding sites promoted by dimerization, which is exemplified by S3/4 in the Ci N-terminal region (Fig. 5G). We demonstrated that both HIB and Ci form dimers and provided evidence that they engage in multivalent interactions, which explains why intrinsically weak binding sites can bind cooperatively to HIB to achieve high occupancy *in vivo*. It also is possible that HIB might form high-order oligomers that interact with multiple sites in Ci through both *cis*- and *trans*-cooperativity. Oligomerization of BTB domain-containing proteins has been observed for the transcription factor GAGA, which cooperatively interacts with multiple sites on the promoters of its target genes (18).

Cooperative binding through multiple sites is likely a general mechanism for Cul3-HIB/SPOP, as well as other Cul3-based E3 Ub ligases, to recognize their substrates, because BTB domains tend to form dimers/oligomers. Indeed, multiple S/T-rich motifs are present in Gli2 and Gli3 as well as in numerous HIB-interacting proteins identified through a genome-wide yeast two-hybrid screen (26). The requirement of multiple sites that bind cooperatively to HIB may provide a mechanism for regulating substrate specificity and binding affinity. The S/T-rich motifs do not conform a strict consensus and thus are likely present in many proteins; however, a good substrate requires the presence of at least 2 binding sites situated in favorable positions that permit either *cis*- or *trans*-cooperativity.

Our finding that HIB/SPOP interacts with S/T motifs also raises the interesting possibility that substrate recognition by this class of E3 might be modulated by phosphorylation. While substitution of S372 to D in the Ci S3 site retained HIB binding, substitution of the remaining 3 S/T residues to D abolished HIB binding (Fig. S2 in *SI Appendix*). It is possible that S/T to D substitution in these positions may not mimic phosphorylation to confer HIB/SPOP binding. Alternatively, phosphorylation at these positions may attenuate or abolish binding, making HIB/SPOP binding to S/T-rich motifs negatively regulated by phosphorylation. Further investigation is needed to determine whether substrate recognition by HIB/SPOP is regulated by phosphorylation in any cellular or developmental context.

Experimental Procedures

Mutations and Transgenes. An *hib* null allele, *hib*^{Δ6}, was used as described previously (11). *eq-Gal4*, *GMR-Gal4*, *UAS-HA-Ci*, *UAS-Myc-Gli1*, *UAS-Myc-Gli2*, *UAS-Myc-Gli3*, *UAS-ZnGA*, *UAS-HIB-F*, *UAS-HIB-N*, and *UAS-HIB-C* have been described previously (11, 14, 27, 28). Other constructs used in the report are described in *SI Appendix*. Amino acid substitutions in individual HIB-binding sites are listed in Table S3 in *SI Appendix*.

Cell Culture, Transfection, Immunoprecipitation, Western Blotting, and in Vivo Ubiquitination Assays. NIH 3T3 cells were cultured in DMEM containing 10% bovine calf serum and antibiotics at 5% CO₂ in a humidified incubator. Transfection of NIH 3T3 cells was carried out using FuGENE6 (Roche). S2 cell culture, transfection, immunoprecipitation, immunoblotting, and in vivo ubiquitination assays were performed following standard protocols as described previously (11, 29). A typical transfection experiment used 4 μ g of DNA for ub-Gal4 and 2 μ g of DNA for each *pUAST* expression vector. For immunoprecipitation assays involving full-length HIB/SPOP, cells were treated with a proteasome inhibitor, MG132, at 50 μ M for 4 h before harvesting. The following antibodies were used for immunoprecipitation and immunoblotting: mouse α Myc and α HA (Santa Cruz Biotechnology), and mouse α Flag (Sigma).

Luciferase Assay. For *ptc-luc* reporter assays, S2 cells were transfected with 1 μ g of *ptc-Luc* (30) and 50 ng of *RL-PolIII renilla* constructs in 12-well plates

together with 0.5 μ g of Ci constructs with or without an HIB-expressing construct. Cells were incubated for 48 h after transfection. The reporter assays were performed using the Promega Dual-Luciferase Reporter Assay System. Measurements for each sample were performed in triplicate using FLUOstar OPTIMA (BMG LABTCH). *Gli-luc* assays were performed essentially as described previously (31).

Immunostaining. Immunostaining of imaginal discs was done following standard protocols (32). The following antibodies were used: rat anti-Ci (2A) (a gift from R. Holmgren), mouse anti-Flag (M2; Sigma), and mouse anti-HA (F7), mouse anti-Myc (9E10), and rabbit anti-GFP (Santa Cruz Biotechnology).

ACKNOWLEDGMENTS. We thank Drs. Tony Oro, Lawrence Lum, and Bob Holmgren for reagents and Dr. Xuewu Zhang for discussions. This work was supported by National Institutes of Health Grant GM067045 (to J.J.) and Welch Foundation Grant I-1603 (to J.J.). J.J. is a Eugene McDermott Endowed Scholar in Biomedical Science at University of Texas Southwestern.

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