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Report

# Processing of the *Drosophila* Hedgehog Signaling Effector Ci-155 to the Repressor Ci-75 Is Mediated by Direct Binding to the SCF Component Slimb

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## Summary

**Signaling by extracellular Hedgehog (Hh) molecules is crucial for the correct allocation of cell fates and patterns of cell proliferation in humans and other organisms [1, 2]. Responses to Hh are universally mediated by regulating the activity and the proteolysis of the Gli family of transcriptional activators such that they induce target genes only in the presence of Hh [1, 3]. In the absence of Hh, the sole *Drosophila* Gli homolog, Cubitus interruptus (Ci), undergoes partial proteolysis to Ci-75, which represses key Hh target genes [4]. This processing requires phosphorylation of full-length Ci (Ci-155) by protein kinase A (PKA), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3), as well as the activity of Slimb [5–7]. Slimb is homologous to vertebrate β-TRCP1, which binds as part of an SCF (Skp1/Cullin1/F-box) complex to a defined phosphopeptide motif to target proteins for ubiquitination and subsequent proteolysis [8–10]. Here, we show that phosphorylation of Ci at the specific PKA, GSK-3, and CK1 sites required in vivo for partial proteolysis stimulates binding to Slimb in vitro. Furthermore, a consensus Slimb/β-TRCP1 binding site from another protein can substitute for phosphorylated residues of Ci-155 to direct conversion to Ci-75 in vivo. From this, we conclude that Slimb binds directly to phosphorylated Ci-155 to initiate processing to Ci-75. We also explore the phosphorylated motifs in Ci that are recognized by Slimb and provide some evidence that silencing of Ci-155 by phosphorylation may involve more than binding to Slimb.**

## Results and Discussion

### Slimb Binds In Vitro to Ci Phosphorylated at PKA, CK1, and GSK3 Sites

The mechanism and consequences of Hh signaling have been studied extensively in the developing *Drosophila* wing imaginal disc, where Hh, secreted from posterior compartment cells, induces a strip of nearby, responsive anterior cells (AP border cells) to express a small set of target genes, including *decapentaplegic* (*dpp*), that subsequently pattern the developing wing [3, 11–13]. In anterior cells far from Hh, Ci-155 is processed slowly to Ci-75, which crucially represses potential Hh target genes, including *hh* itself and *dpp*, to ensure that they are not ectopically expressed [4, 11, 12]. Even low-level Hh signaling at the AP border blocks Ci-75 production, thereby also increasing the concentration of Ci-155. Hh further activates Ci-155 in a dose-dependent manner by facilitating its nuclear accumulation and potentially also by modifying its binding partners in the nucleus [3, 12, 14, 15]. Because formation of Ci-75 requires both Ci-155 phosphorylation and the activity of Slimb, it was proposed that Slimb might promote partial proteolysis of Ci-155 by directly binding to phosphorylated Ci-155 and catalyzing its ubiquitination [6]. However, despite some support for this hypothesis [14, 16], Ci-155 contains no obvious consensus binding site for Slimb/β-TRCP1, there are only two well-studied examples where proteasomal degradation of a ubiquitinated protein is incomplete (NF-KB precursors, p100 and p105), and ubiquitinated Ci-155 has not been detected when Ci-155 is stabilized by inhibiting the proteasome [14, 16, 17].

To determine whether Slimb can bind to Ci in a manner dependent on phosphorylation, we used a purified GST fusion protein that includes the key phosphorylation sites of Ci [7]. This GST-Ci protein undergoes a significant mobility shift in SDS polyacrylamide gels when phosphorylated by PKA and CK1 together and an even greater shift if GSK3 is also included (Figure 1B). GST-Ci bound more avidly than GST alone to $^{35}$S-labeled full-length Slimb produced by in vitro translation in a reticulocyte lysate and to HA-tagged full-length Slimb from crude extracts of transiently transfected *Drosophila* Kc cells (Figure 1B). This binding was reproducibly increased by using PKA together with CK1, and to a much greater extent by using all three protein kinases to phosphorylate GST-Ci prior to the binding assay (Figure 1B). The synergistic contribution of GSK3 was clearest in the HA-Slimb binding assay, and so we used this assay to investigate the characteristics of Ci binding to Slimb further.

Three PKA sites ("P1-3"), the neighboring three PKA-primed CK1 sites ("C1-3") and the two adjacent PKA-primed GSK3 sites ("G2,3") are required for Ci-155 to be converted to Ci-75 in *Drosophila* embryos and wing discs [5, 7, 18–21]. When the serine residues at these PKA, CK1, or GSK3 sites were replaced with alanines to produce P1-3A, C1-3A, and G2,3A (Figure 1A), we no longer saw any significant stimulation of binding of GST-Ci to HA-Slimb by any combination of PKA, CK1, and GSK3 (Figure 1C). Thus, strong binding of HA-Slimb to a Ci fragment in vitro requires the same protein kinases and the same phosphorylation sites that are required in vivo to convert Ci-155 to Ci-75.

### A Consensus Slimb Binding Site Restores Conversion of Ci-155 to Ci-75 In Vivo

We then tested whether a defined, minimal Slimb binding site from another protein could direct processing of Ci-155 to Ci-75. β-catenin is a prototypical substrate for the β-TRCP1 SCF complex, in which a dually phosphorylated motif (DpSGIHpS, where pS stands for phosphoserine) is the critical recognition element for binding [9, 10]. This motif is conserved in *Drosophila* β-catenin

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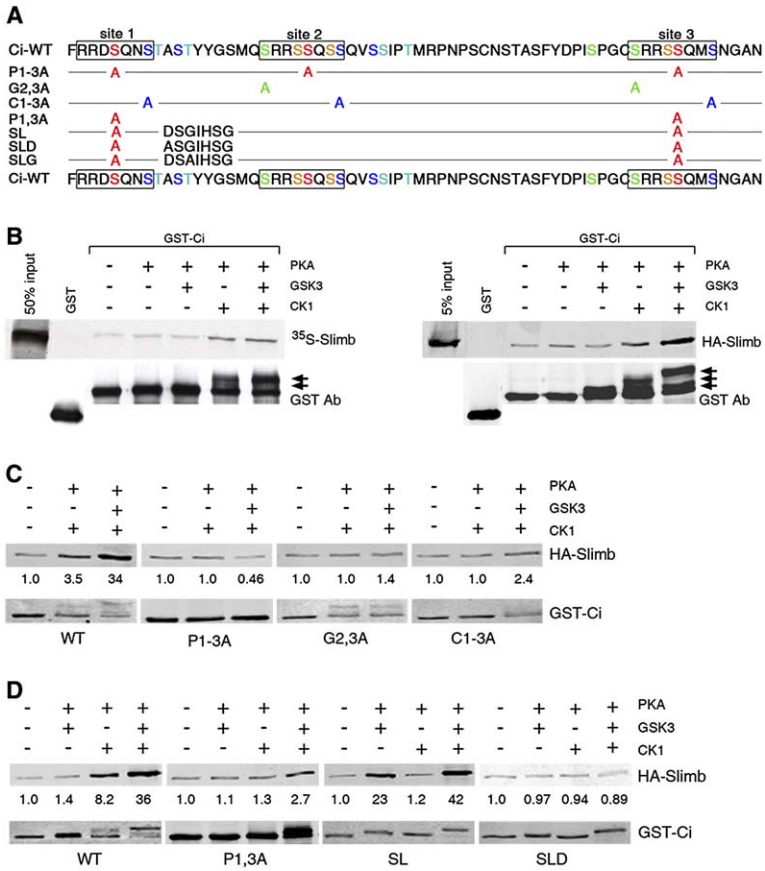


Figure 1. Phosphorylation Stimulates Binding of GST-Ci to Slimb In Vitro

(A) Amino acids 834–899 of Ci highlighting the three PKA sites and adjacent PKA-primed GSK3 and CK1 sites (boxed residues), together with changes engineered into GST-Ci proteins. Phosphorylation sites are color coded as PKA (red), PKA-primed and CK1-primed CK1 (dark blue), PKA-primed and GSK3-primed GSK3 (green), CK1-primed GSK3 or GSK3-primed CK1 (orange), and potential CK1 sites deviating from an optimal consensus (light blue).

(B) A pull-down assay was performed between GST or GST-Ci phosphorylated with the indicated protein kinases and in vitro-translated $^{35}$S-Slimb (left; autoradiograph) or HA-Slimb from a Kc tissue-culture cell extract (right; HA antibody western blot). GST proteins were visualized by western blot, with arrows indicating phosphorylated forms of altered mobility (lower panels).

(C and D) Binding of HA-Slimb to GST-Ci (WT) and GST-Ci variants listed in panel (A), showing GST-Ci proteins (lower panels) and bound HA-Slimb (top panels) visualized by western blot. The relative intensities of HA-Slimb bands are indicated, with a value of 1.0 assigned to the amount of HA-Slimb bound to each of the unphosphorylated GST-Ci proteins.

(Armadillo), and Armadillo proteolysis depends on both this sequence and Slimb activity [6, 22]. Hence, we expect the motif also to serve as a direct binding site for Slimb, the *Drosophila* homolog of β-TRCP1. To test whether this consensus Slimb/β-TRCP1 binding motif could influence the fate of Ci, we altered Ci sequence so that phosphorylation of PKA site 2 would theoretically prime three consecutive phosphorylations at GSK3 sites to produce a DpSGIHpS motif ("SL" in Figure 1A). We additionally altered PKA sites 1 and 3 to alanine residues in order to prevent the normal phosphorylation of Ci from directing conversion to Ci-75. These PKA site changes alone ("P1,3A") and a DpSGIHpS-motif variant, in which the first or third residue is changed to alanine ("SLD" and "SLG"), were also introduced into GST-Ci molecules as controls to assess the function of the Slimb consensus binding motif (Figure 1A).

GST-Ci-SL was extensively phosphorylated by the combined action of PKA and GSK3 in vitro, producing a greater mobility shift than observed for GST-Ci-WT (Figure 1D). Furthermore, unlike GST-Ci-WT and GST-Ci-P1,3A, GST-Ci-SL bound strongly to HA-Slimb when phosphorylated by only PKA and GSK3. PKA and GSK3 altered the mobility of GST-Ci-SLD to a similar extent but did not enhance binding to HA-Slimb (Figure 1D). Thus, the Slimb consensus site engineered into Ci is functional and directs Slimb binding in vitro with an apparent avidity similar to that seen for fully phosphorylated wild-type Ci.

The same alterations were engineered into full-length *UAS-Ci* transgenes that encoded HA and FLAG tags at the amino terminus and a Myc tag at the carboxy terminus of Ci to produce Ci-WT, Ci-SL, Ci-P1,3A, Ci-SLD, and Ci-SLG. Conversion of these Ci variants to Ci-75 was assayed in two ways. First, expressed proteins were assayed by western blot with HA or FLAG antibody to visualize tagged Ci-155 and Ci-75 proteins. This was done in extracts of 5–8-hr embryos expressing the *UAS-Ci* transgenes under control of a *ptc-GAL4* driver that leads to expression only in cells that normally express Ci (Figure 2A), in extracts of wing discs expressing *UAS-Ci* transgenes under control of *C765-GAL4*, which drives expression evenly throughout the wing disc (Figure 2A), and in extracts of *Drosophila* Kc cells transiently transfected with the transgenes under control of an *actin-5C* promoter (data not shown). The results were the same in each circumstance. Ci-WT and Ci-SL produced both tagged Ci-155 and Ci-75 proteins, whereas Ci-P1,3A, Ci-SLD, and Ci-SLG produced only Ci-155 (Figure 2A). Furthermore, coexpression of a constitutively active mouse PKA catalytic subunit (mC*) in wing discs increased the proportion of Ci-75 produced by Ci-WT and Ci-SL (Figure 2A), consistent with a role for PKA in promoting phosphorylation of Slimb binding sites in both proteins, but did not result in any detectable Ci-75 from Ci-SLD.

The second assay for Ci-75 production measures Ci-75 by its ability to repress transcription of a *hh-lacZ* reporter gene [23]. Thus, Ci-155 from a wild-type Ci transgene is converted to Ci-75 and represses *hh-lacZ* in posterior wing-disc clones that are unable to transduce a Hh signal because they lack activity of Smoothened (Smo)

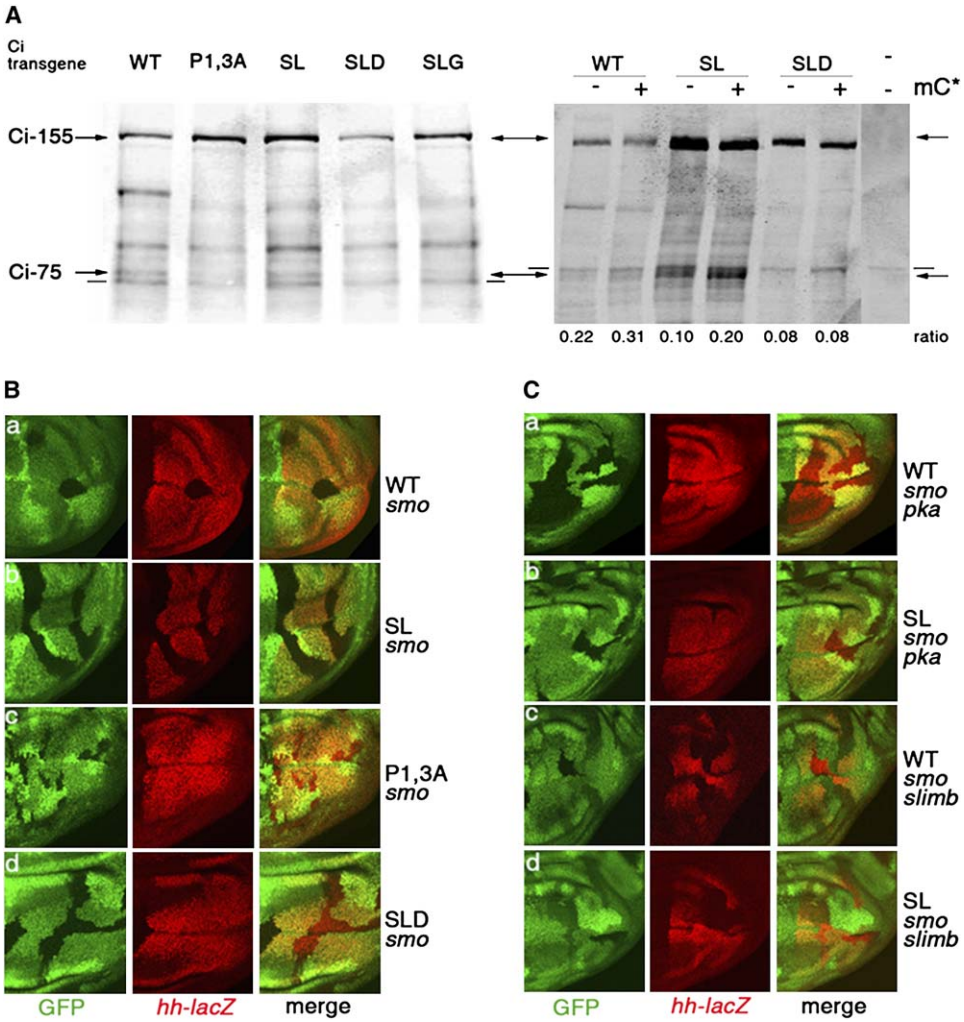


Figure 2. A Consensus Slimb Binding Site Stimulates Processing of Ci-155 to Ci-75 In Vivo

(A) Western blots with HA antibody to probe HA immune precipitates of extracts from embryos (left) and crude extracts from wing discs (right) expressing the indicated HA-tagged Ci transgenes, with or without the constitutively active mouse PKA catalytic subunit gene, *mC**. Ci-75 (arrows) migrates on top of a weak background band in each case and just above (left) or below (right) a strong background band (horizontal lines). The ratio of Ci-75 to Ci-155 band intensities after subtraction of background is shown for each lane (right; average of two such blots).

(B) *smo* mutant clones (lacking GFP, green) were generated in wing discs expressing the indicated transgenes under the control of *C765-GAL4*. *hh-lacZ* (red) expression in the posterior (right) is repressed only by Ci-WT and Ci-SL and selectively in *smo* mutant clones.

(C) *hh-lacZ* (red) is not repressed by Ci-WT or Ci-SL in *smo pka* double-mutant clones ($C_a$ and $C_b$) or *smo slimb* double-mutant clones ($C_c$ and $C_d$), marked in each case by lack of GFP (green). In ($C_c$) and ($C_d$), *hh-lacZ* is absent from two types of clones that express GFP (*smo FRT40A/smo FRT40A; FRT82B slimb C765 hh-lacZ/FRT82B UbiGFP* and *smo FRT40A/UbiGFP FRT40A; FRT82B UbiGFP/FRT82B Ubi-GFP*).

(Figure 2B). Ci-SL also repressed *hh-lacZ* specifically in posterior *smo* mutant clones, showing that Ci-SL can be converted to a transcriptional repressor and that this conversion occurs only in the absence of Hh signal transduction (Figure 2B). Neither Ci-P1,3A nor Ci-SLD repressed *hh-lacZ* in posterior *smo* mutant clones, confirming that conversion of Ci-SL to the Ci-75 repressor depends on its Slimb consensus binding site (Figure 2B). Generation of this binding site should require PKA and GSK3 activity, and its function is expected to require direct recognition by Slimb. We found that Ci-SL, like Ci-WT, does not repress *hh-lacZ* in posterior *smo* mutant clones if PKA activity is also eliminated or if these cells also lack normal Slimb activity (Figure 2C). Thus, insertion of a consensus Slimb binding site into a Ci derivative that cannot otherwise be converted into Ci-75 restores processing; processing to Ci-75 depends on both Slimb activity and phosphorylation of the Slimb binding site, and it is blocked by Hh signaling.

### Slimb Binding Sites in Ci

We then used binding assays to search for the direct Slimb recognition elements in Ci because no established Slimb/β-TRCP consensus binding sites are apparent in the sequence of Ci or Gli proteins. Each of the three PKA sites in Ci is required for detectable processing to Ci-75 in Kc tissue-culture cells [18], and so we first tested whether each site contributes to Slimb binding in vitro. Phosphorylation-dependent binding of GST-Ci to HA-Slimb was reduced substantially by loss of PKA site 1 (P1A) or site 3 (P3A) and eliminated by loss of PKA site 2 (P2A) (Figure 3B). PKA sites 2 and 3

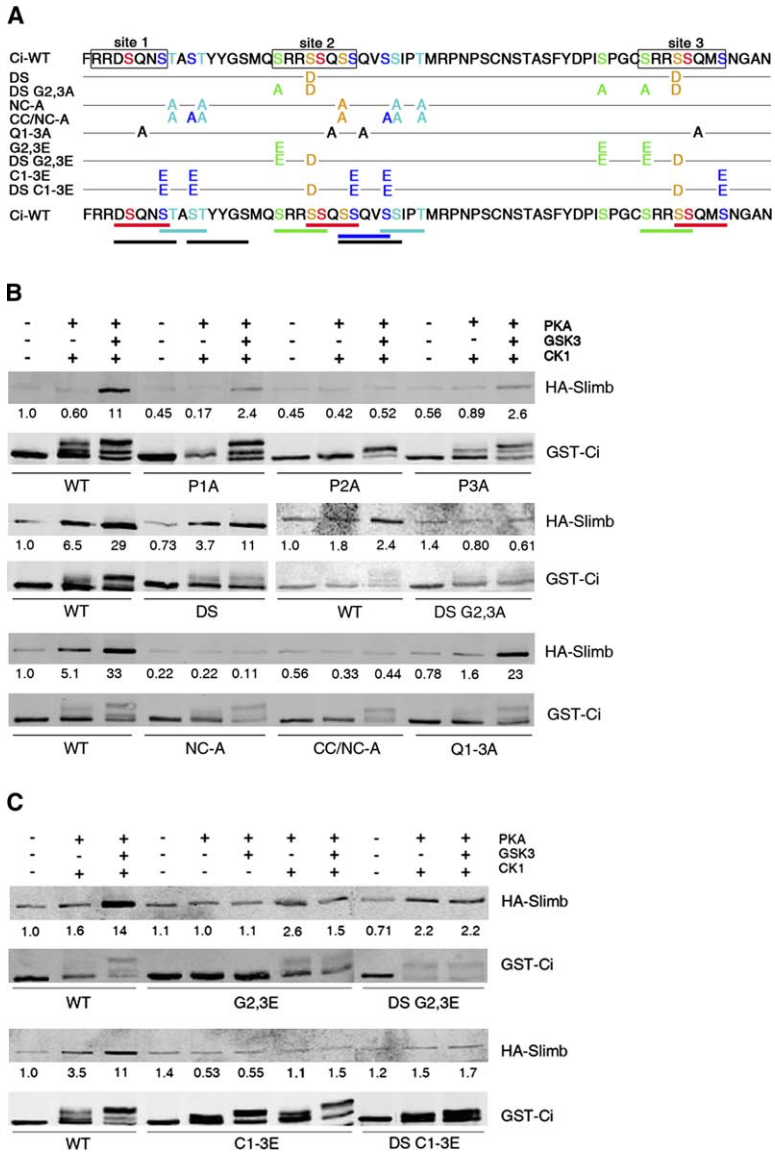


Figure 3. Phosphorylated Ci Motifs Required for Slimb Binding

(A) Amino acids 834–899 of Ci color coded as in Figure 1A, together with variants used in Slimb binding assays. Potential Slimb binding sites are indicated by bars (bottom) that are colored red [(D/pS)XXpS, centered on PKA sites], green (pSXXpSpS, including GSK3 sites), dark blue (pSpSXXpS, comprising only strict consensus CK1 sites), light blue [pS(pS/pT)XXpS, including nonconsensus CK1 sites], and black [(D/pS)(pS/pT)XXX(pS/pT)].

(B and C) GST-Ci variants shown in (A) were phosphorylated by the indicated protein kinases and incubated with HA-Slimb from Kc cell extracts. Proteins brought down with glutathione beads were blotted with HA antibody (upper panels) and GST antibody (lower panels). In each case, results for GST-Ci variants are presented alongside the internal Ci-WT control tested strictly in parallel. Band intensities corresponding to bound HA-Slimb are indicated (after subtraction of background) for each lane relative to wild-type GST-Ci with no phosphorylation (fixed at 1.0) for that experiment.

prime GSK3 sites. Those GSK3 sites, which are required for Slimb binding (Figures 1A and 1C), can in turn prime further phosphorylation by CK1 of the second serine in the motif $pS_{GSK3}RRSpS_{PKA}$. If that second serine is replaced with an acidic residue (D, to form SRRDS, "DS" in Figures 3A and 3B), binding to Slimb is still markedly stimulated by phosphorylation, but this stimulation is lost if the GSK3 sites are also altered (ARRDS, "DS G2,3A" in Figures 3A and 3B). Thus, the GSK3 sites are not required simply to prime further phosphorylation by CK1. When the PKA-primed GSK3 sites (and the single GSK3-primed GSK3 site) were changed to glutamate residues ("G2,3E" in Figure 3A), binding of GST-Ci to HA-Slimb was stimulated by combined PKA and CK1 phosphorylation and was not further enhanced by GSK3 (Figure 3C). Similar properties were observed if, in addition, the serine residues preceding PKA sites 2 and 3 were changed to aspartate ("DS G2,3E" in Figures 3A and 3C). Thus, acidic-residue replacements can at least partially mimic the contribution of phosphorylated PKA-primed GSK3 sites to Slimb binding, and those GSK3 sites are likely recognized directly by Slimb, rather than being required simply to prime further phosphorylation of Ci. Although replacement of GSK3 sites with acidic residues did reduce maximal binding to Slimb in vitro, this Ci variant (G2,3E) was processed to Ci-75 efficiently enough to repress *hh-lacZ* substantially in posterior *smo* mutant wing-disc clones (Figure S1 in the Supplemental Data available online).

In contrast to the results with GSK3 sites, replacement of all three PKA-primed CK1 sites (and the two predicted CK1-primed CK1 sites) with acidic residues ("C1-3E" in Figure 3A) abolished any stimulation of HA-Slimb binding by phosphorylation of GST-Ci, even in the sequence context RRDSXXE ("DS C1-3E" in Figures 3A and 3C), where a potential role for CK1 sites in priming phosphorylation by GSK3 is eliminated. The C1-3E variant also was not converted to Ci-75 repressor in wing discs (Figure S1). These properties may reflect a defect in direct recognition by Slimb of glutamates in place of CK1-site phosphoserines or a defect in priming further phosphorylation. Several serine and threonine residues

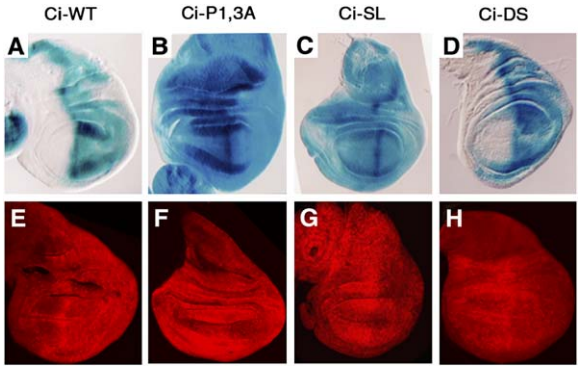


Figure 4. A Consensus Slimb Binding Site Does Not Suffice to Silence Ci-155

(A–D) The indicated Ci transgenes were expressed in wing discs, and activation of the Hh target gene reporter *ptc-lacZ* was assessed by staining for β-galactosidase activity (blue).

(E–H) Full-length Ci-155 transgene products were stained with an antibody to the C-terminal Myc epitope tag (red). Ci-SLD behaved exactly like Ci-P1,3A in these assays. Anterior is to the left, and dorsal is up.

beyond PKA sites 1 and 2 do not conform to the optimal [pS/pT]XX[S/T] consensus but may nevertheless be phosphorylated by CK1 after PKA and CK1 priming [24]. Altering five such residues to alanine, alone ("NC-A") or together with consensus CK1-primed CK1 sites ("CC/NC-A"), eliminated all phosphorylation-dependent binding to HA-Slimb (Figures 3A and 3B). The most C-terminal of these potential CK1 sites would not be expected to prime further phosphorylation, and their requirement therefore suggests they are recognized directly by Slimb.

We cannot readily determine whether the PKA sites and PKA-primed CK1 sites are, as argued above for GSK3 sites and distal CK1-primed CK1 sites, directly recognized by Slimb. However, the evident contribution of each PKA site to Slimb binding (Figure 3B) implies that each must nucleate at least one direct Slimb binding site. Surrounding the three PKA sites there are two types of motifs that are related to previously recognized or postulated Slimb/β-TRCP binding motifs (DSGXXS, DSGXXXS, TSGXXS, EEGXXS, DDGXXD, and DSGXXL) [17, 25–29]. First, the six-amino-acid motif (D/pS)(pS/pT)(Q/Y)XX(pS/pT) might be created in three places if phosphorylation occurs, as we suspect, at some non-consensus sites (black bars, Figure 3A). These motifs most closely resemble the β-TRCP1 binding site postulated for p100 (DpSAYGpS) [17], but the presence of glutamine or tyrosine at the third position in place of glycine in the ideal consensus would be expected to reduce binding by more than an alanine substitution [10]. Second, many five-amino-acid motifs are created in which two acidic residues (DpS, pSpS, or pSpT) are separated from a phosphorylated residue (pS or pT) by only two amino acid residues (colored bars, Figure 3A). Four of these eight motifs include a glutamine at the third position. However, this residue does not appear to be instrumental in Slimb binding because substitution with alanine ("Q1-3A") has little effect (Figures 3A and 3B). On the basis of the crystal structure of β-TRCP1, it is hard to predict the affinity of the designated five-amino-acid motifs for Slimb, but it is likely to be lower than for any of the motifs cited above [10]. Regardless of which precise motifs contribute most significantly to Slimb binding, it is clear from our mutational analysis that Ci must be highly phosphorylated over a region spanning almost sixty amino acid residues to generate several suboptimal binding sites that must collaborate to provide physiologically significant affinity for Slimb. This follows a precedent established for degradation of the yeast cell-cycle regulator Sic1 by binding of the $SCF_{CDC4}$ complex to multiple low-affinity phosphodegrons [30]. The requirement for extensive Ci phosphorylation could account for the essential role of the scaffolding protein Cos2 in facilitating phosphorylation and for the relatively slow conversion of Ci-155 to Ci-75 seen in vivo [4, 31].

### Regulation of Ci-155 Activity by Slimb

We have shown that Slimb binding can promote conversion of Ci-155 to Ci-75 repressor. This is a crucial role for Slimb and Ci phosphorylation in vivo because it keeps the key target gene *dpp* repressed in anterior wing-disc cells away from the AP border [12]. However, Ci-155 is only slowly converted to Ci-75 in anterior cells, so it is also imperative that Ci-155 activity is held in check prior to its partial proteolysis [3, 4]. We therefore tested whether the consensus Slimb binding site inserted into Ci-SL was effective at silencing Ci-155 activity. The *ptc-lacZ* reporter gene requires Ci-155 to be activated [12]. It is normally expressed only at the AP border, but it is ectopically activated in posterior cells if a wild-type Ci transgene is expressed evenly throughout the wing disc (Figure 4A). This selective activation in Hh signaling territory reflects both stabilization and activation of Ci-155 by Hh [12]. By contrast, Ci-P1,3A and Ci-SLD activate *ptc-lacZ* strongly in both anterior and posterior cells (Figure 4B), indicating high activity of Ci-155 even in the absence of Hh, as has been seen previously for Ci variants lacking PKA, CK1, or GSK3 sites [5, 7, 18]. Surprisingly, Ci-SL also induced *ptc-lacZ* in both anterior and posterior cells, albeit more weakly than Ci-P1,3A and Ci-SLD (Figure 4C).

We also examined the levels of Ci-155 corresponding to Ci-SL and other variants by staining wing discs for the Myc epitope attached to the C terminus of each transgene product. Myc staining was highest in posterior and AP border cells for Ci-WT (Figure 4E), as expected, reflecting stabilization of Ci-155 by Hh, whereas Myc staining was extremely high and uniform for Ci-P1,3A and Ci-SLD (Figure 4F). Surprisingly, Myc staining of Ci-SL was higher in anterior cells than posterior cells (Figure 4G) even though we have established that Ci-SL is converted to Ci-75 only in the absence of Hh signaling. This suggests that Ci-SL is not proteolyzed in anterior cells as efficiently as Ci-WT, whether this is by conversion to Ci-75 or by other means [15, 16]. The aberrant accumulation of full-length Ci-SL in anterior cells might in turn be responsible for the induction of *ptc-lacZ* in these cells, especially if Ci-155 molecules accumulate in excess over their stoichiometric binding partners Cos2 and Su(fu), which normally limit Ci-155 activity [3]. However, the consequences of increased Ci-155 concentration cannot be safely predicted [5, 7, 15, 21]. Indeed, when we examined another Ci variant, Ci-DS,

which like Ci-SL is converted to Ci-75 in the absence of a Hh signal (Figure S1), we also observed higher Myc levels in anterior cells than in posterior cells, but Ci-DS activated *ptc-lacZ* only in posterior cells (Figures 4D and 4H). Thus, induction of *ptc-lacZ* in the absence of Hh by Ci-SL may indicate a distinct role of phosphorylation in limiting the specific activity rather than the proteolysis of Ci-155, a role for which a single consensus Slimb binding site is not an adequate substitute.

In summary, we have found that processing of Ci-155 to Ci-75 is initiated by direct binding of Slimb to Ci-155 molecules that have been extensively phosphorylated by PKA, GSK3, and CK1. This presumably leads to ubiquitination of Ci-155 and its partial proteolysis by the proteasome, generating a transcriptional repressor that plays a key developmental role in cells that are not exposed to Hh. Whether phosphorylation also prevents Ci-155 from activating transcription through an additional mechanism remains to be explored, as does the mechanism by which proteolysis of Ci-155 is limited to preserve its N-terminal domains as Ci-75.

## Experimental Procedures

### Mutagenesis and Cloning

Constructs for *Drosophila* germline transformation and tissue-culture cell transfection were made with Gateway Technology (Invitrogen). The cDNAs of *ci* and *slimb* were inserted into pENTR-D/TOPO via "topo cloning." The destination vector used for germline transformation is pTFHWM, which contains pUASt regulatory elements and encodes both a composite N-terminal Flag/HA tag and a C-terminal Myc tag (www.ciwemb.edu/labs/murphy/Gateway%20vectors.html). The destination vector used for tissue-culture transfection, pAFHWM, differs only in that it contains an *actin5C* promoter. pGEX2T-$Ci_{685-920}$, described previously [7], was used to make the GST-Ci fusion proteins. Mutations were made in pENTR-D/TOPO-Ci and pGEX2T-Ci with the QuikChange Site-Directed Mutagenesis Kit (Stratagene), and products were sequenced in their entirety.

### Kc Cell Extracts

Cells were cultured at 25ºC in Schneiders *Drosophila* media + 5% FBS + 1% Penicillin-Streptomycin (GibcoBRL). Five 100 mm plates were seeded with $1 \times 10^7$ cells in a volume of 10 ml of media and were allowed to adhere overnight. Three to four hours prior to transfection, cells were given fresh media. Ten micrograms of pAFHWM-Slimb (or pAFHWM-Ci) was transfected by using the standard calcium phosphate protocol, and after 24 hr, cells were given fresh media. Cells were allowed to express for approximately another 36 hr and were then lysed in 3 ml lysis buffer (50 mM Hepes, pH 7.5, 0.2mM EDTA, 250 mM NaCl, 50 mM NaF, 0.5 mM $NaVO_3$, 0.5% NP-40, 1 mM DTT, protease inhibitors [Mini Complete, Roche]).

### In Vitro Phosphorylation and GST Pull-Down Assay

Approximately 200 ng of GST-Ci protein was bound to glutathione sepharose beads in GSK3 buffer from NEB (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 5 mM DTT) for 1 hr at 4ºC in a volume of 100 μl. Beads were then washed twice with GSK3 buffer and subjected to in vitro phosphorylation by incubating at 30º for 30 min with GSK3 buffer, 100 μM ATP, 12.5 mM $MgCl_2$, 250 U PKA, 50 U GSK3, and/or 100 U CK1 (NEB) where indicated. Beads were then washed twice with the Kc cell-lysis buffer for binding reactions with HA-Slimb cell lysate or CX buffer (50 mM Tris [pH 8], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 50 mM NaF, 1 mM DTT) for binding reactions with $^{35}$S-Slimb. Two hundred microliters of Kc cell extract or 8 μl in vitro-translated product was bound to the beads along with 1 mg/ml BSA + 0.5 μM okadaic acid as a phosphatase inhibitor for 2 hr at 4ºC. Beads were then washed three times and eluted with SDS-PAGE loading buffer and boiled for 5 min prior to loading. Proteins were visualized by western blotting with the 3F10 rat anti-HA (Roche) and rabbit anti-GST primary antibodies. Alexaflour-680 and -800 were used as secondary antibodies and visualized with the LI-COR Odyssey Infared Imager, which allowed accurate quantitation of band intensities. $^{35}$S-Slimb was visualized by autoradiography.

### Immunohistochemistry

Generally, larvae were heat shocked at the second instar for 1 hr at 37ºC to induce clones and then kept at 29º to drive high expression of proteins expressed from UAS transgenes. At late third instar, larvae were dissected into cold Brower fixation buffer and rocked at 4ºC for 1 hr. Discs were blocked in PBST + 5% BSA for 1 hr and then stained by standard methods. *hh-lacZ* gene product was stained by using rabbit anti-β-galactosidase antibody (Promega) and Alexaflour-594 secondary. Full-length Ci transgene products were stained with Cy3-conjugated mouse monoclonal antibody to Myc (Amersham).

### Wing-Disc Extracts

*C765-GAL4* females were crossed to *UAS-Ci* or *UAS-mC*; UAS-Ci* males, and progeny were incubated at 25ºC. Approximately 40 discs were dissected from late third-instar larvae into cold PBS and centrifuged at maximum speed for 30 s. The PBS was aspirated off, and the discs were lysed in 25 μl SDS-PAGE loading buffer and boiled for 5 min prior to loading.

### Immunoprecipitation from Embryo Extracts

*ptc-GAL4* females were crossed to *UAS-Ci* males to generate embryos expressing the transgenes. Embryos were collected for 3 hr on apple juice plates and then incubated for 5 hr at 29ºC. Embryos were dechorionated for 3 min in bleach with shaking, rinsed for 2 min with PBST, and then decanted into 1.5 ml tubes. Fifty microliters of embryos were homogenized in 200 μl RIPA/glycerol buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1× SDS, 50 mM Tris [pH 8], 1 mM DTT, 10% glycerol, 1 mM EDTA, protease inhibitors [Mini Complete, Roche], and 0.5 μM okadaic acid) for 1 min at 4ºC. After a 15 min centrifugation at 4ºC, the extracts were precleared with protein G beads for 1 hr at 4ºC. Immunoprecipitation was carried out by incubating with 1 μl 12CA5 HA monoclonal antibody (in ascites fluid) overnight and then adding protein G beads for 1 hr at 4ºC. Beads were washed three times with RIPA/glycerol buffer and eluted with SDS-PAGE loading buffer and boiled for 5 min prior to loading.

### Fly Crosses

Females with the genotype *ywflp; ubi-GFP FRT40A / Cyo; C765 hh-lacZ / TM6B* were crossed to *yw; $smo^2$ UAS-Ci FRT40A / CyO* males to generate *smo* mutant clones or *yw; $smo^2$ pka-$c1^{B3}$ FRT40A / CyO; UAS-Ci* to generate *smo pka* mutant clones. Females with the genotype *ywflp; ubi-GFP FRT40A / CyO; FRT82B $slimb^1$ C765 hh-lacZ / TM6B* were crossed to *yw; $smo^2$ UAS-Ci FRT40A / CyO; FRT82B ubi-GFP / TM6B* males to generate *smo slimb* clones.

### Supplemental Data

Supplemental Data include one figures and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/1/110/DC1/.

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