A Three-part Signal Governs Differential Processing of Gli1 and Gli3 Proteins by the Proteasome* §

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The Gli proteins are the transcriptional effectors of the mammalian Hedgehog signaling pathway. In an unusual mechanism, the proteasome partially degrades or processes Gli3 in the absence of Hedgehog pathway stimulation to create a Gli3 fragment that opposes the activity of the full-length protein. In contrast, Gli1 is not processed but degraded completely, despite considerable homology with Gli3. We found that these differences in processing can be described by defining a processing signal that is composed of three parts: the zinc finger domain, an adjacent linker sequence, and a degron. Gli3 processing is inhibited when any one component of the processing signal is disrupted. We show that the zinc fingers are required for processing only as a folded structure and that the location but not the identity of the processing degron is critical. Within the linker sequence, regions of low sequence complexity play a crucial role, but other sequence features are also important. Gli1 is not processed because two components of the processing signal, the linker sequence and the degron, are ineffective. These findings provide new insights into the molecular elements that regulate Gli protein processing by the proteasome.

The Hedgehog (Hh) signal transduction pathway plays a central role in development, and patterns structures such as the ventral neural tube and the anterior-posterior axis of appendages (1, 2). In Drosophila the transcription factor Cubitus interruptus (Ci) mediates the effects of Hh signaling, whereas in mammals three effectors, Gli1, Gli2, and Gli3, fulfill this function. The ubiquitin-proteasome system modulates Ci activity in at least two ways. First, following activation of Ci by high levels of Hh ligand, the ubiquitin-proteasome system completely degrades Ci to limit the Hh signaling response (3, 4). Second, in the absence of Hh stimulation, the ubiquitin-proteasome system partially degrades Ci to convert it from a transcriptional activator into a repressor (5). This partial degradation or processing occurs by an unusual mechanism in which the proteasome degrades the transactivation domain of Ci but leaves intact the DNA-binding domain. Processing of Ci is a powerful way to modulate signaling because, instead of simply removing a pathway activator like complete degradation would, processing creates a potent repressor.

The mechanism of processing has been studied in vitro using model proteins, and this has led to a simple mechanical model of proteasomal processing (6–8). The proteasome normally degrades its substrates completely by running along their polypeptide chain and hydrolyzing them processively (7). Processing occurs when the proteasome runs into a folded domain that stalls its progress along the substrate's polypeptide chain, which then gives the remainder of the protein a chance to escape degradation. The amino acid sequence adjacent to the folded domain can also affect processing, and the amount of processing increases if the amino acid composition of this sequence is biased (6, 8, 9). In other words, the simpler the amino acid composition of this region (i.e., the fewer amino acids represented), the more fragment is formed. It is not known how simple sequences increase processing, but one explanation is that simpler sequences may weaken proteasome binding and allow more fragment to escape (6, 8–10).

This mechanical model is relevant to physiological proteins. For example, the NF-κB p50 subunit is generated by processing of the larger precursor p105 (11–13). This processing requires both the folded Rel-homology domain (6, 7, 14) and an adjacent simple glycine-rich region (6, 15, 16). Similarly, processing of Ci requires the folded zinc finger domain and adjacent sequences (6, 17, 18). However, we know that the mechanism is more complex in vivo, and sequence features in addition to complexity affect processing (17) so that the mechanical model can no...
longer describe the processing mechanism adequately. Here we investigate the processing signal further by following degradation of the Gli proteins.

In mammals, the ancestral Ci gene has been duplicated, which allowed the divergence of Gli protein function. Gli3 is most closely related to Ci and is robustly processed in the absence of Hh pathway stimulation (19). The Gli2 sequence is similar to that of Gli3, but the Gli2 protein is not processed as efficiently as Gli3 (20). Gli1 is less similar to Gli3, and the Gli1 protein is not processed at all but is degraded completely (21–23). Thus, it seems that the ancestor of Ci and the Gli proteins was processed and that Gli1 likely lost the features responsible for processing.

An earlier study compared Gli3 and Gli2 processing (24) and defined a region of ~200 amino acids following the zinc fingers as the processing determinant domain (PDD). Transplanting the PDD between Gli2 and Gli3 transfers the ability to be processed efficiently between the two proteins. The PDD can even cause fragment formation when placed between tubulin and an exogenous degron, which suggests that the Gli3 PDD may independently mediate protein processing (24). Quite surprisingly, we found that the Gli3 PDD does not transfer the ability to be processed from Gli3 to Gli1. This finding emphasizes that significant questions about the mechanism governing Gli3 processing remain. Therefore, we first characterized the processing signal in Gli3 and then used the information gained to determine why Gli1 is not processed. We analyzed Gli1 and Gli3 processing in the conceptual framework of the mechanical model of processing and defined a region responsible for processing consisting of three components: the zinc finger domain, an adjacent linker sequence, and the degron. We found that disruption of any of the three components individually abolished Gli3 processing. Gli1 lacks two of the three components, the linker sequence and the degron. Thus, the three-part processing signal described in this work provides novel insight into the molecular mechanism that governs Gli protein processing.

EXPERIMENTAL PROCEDURES

DNA Constructs—N-terminally FLAG-tagged human Gli1 and Gli3 cDNA sequences were expressed in pcDNA3 (Invitrogen). Constructs were generated by a combination of InFusion (Clontech), PCR, PCR-based mutagenesis, and restriction digestions. All PCR-generated constructs were verified by sequencing analysis. See supplemental Table S1 for the details of all constructs.

Cell Culture, Transfection, and Protein Analysis—HEK293T cells were seeded 48 h prior to transfection at 500,000 cells per well of a 6-well plate into DMEM containing 10% calf serum and penicillin/streptomycin with 5 μg/ml of tetracycline (Clontech), PCR, PCR-based mutagenesis, and restriction digestions. All PCR-generated constructs were verified by sequencing analysis. See supplemental Table S1 for the details of all constructs.

RESULTS

Gli Processing Is Appropriately Regulated in HEK293T Cells—In mice, Gli3 is processed to a fragment of approximately half its original size (19), whereas Gli1 is not processed (21–23). To follow Gli processing in a cell culture model, we used expression vectors containing the coding region of N-terminally FLAG-tagged human Gli1 or Gli3 (Fig. 1A) so that both full-length (Gli3, 190 kDa; Gli1, 150 kDa) and processed forms (Gli3, 83 kDa) could be detected by immunoblotting.

When FLAG-tagged Gli3 was transfected into cells, we observed the full-length form as well as a fragment of the size that corresponds to the processed form. For Gli1, we observed only the full-length form (Fig. 1B). Gli3 processing depended on the proteasome because addition of the proteasome inhibitor lactacystin eliminated Gli3 fragment formation (Fig. 1B). Lactacystin treatment was used throughout this work to determine the proteasome dependence of processing. Gli3 processing depends on PKA activity (19, 25, 26), because PKA activates the degron responsible for Gli3 and Gli2 processing by allowing its recognition by the E3 ubiquitin ligase SCFβTrCP (20, 25, 26). Here, stimulation of PKA by either the addition of forskolin or co-transfection of constitutively active PKA induced formation of processed Gli3 (Fig. 1C). Gli3 processing depended on the same PKA sites as in mice (27) because mutating the relevant four serines to alanines abolished Gli3 fragment formation (Fig. 1C). Thus, Gli protein processing and the regulation of processing appear to be intact in this cell culture model, and therefore, HEK293T cells are a suitable system in which to study Gli protein processing by the proteasome.

Insertion of the Gli3 PDD into Gli1 Does Not Induce Processing—To determine why Gli1 is not processed, we attempted to alter Gli1 to induce its processing. The Gli3 PDD and the equivalent region in Gli1 are quite divergent (supplemental Fig. S1). This suggested that this sequence may account for the differences in processing between Gli1 and Gli3 and that introducing the Gli3 PDD into Gli1 would induce its processing. Surprisingly, we found that Gli1 containing the Gli3 PDD was not processed (Fig. 1D). We then attempted to induce Gli1 processing by transplanting a much larger region of Gli3, namely the entire C-terminal part of the protein from the zinc fingers to the C terminus. This region of Gli3 did induce Gli1...
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A. Three-part Signal Governs Gli1 and Gli3 Processing

Gli1 and Gli3 expression and processing were assessed by immunoblotting with a FLAG antibody after cell lysis and SDS-PAGE. Results in this and all subsequent figures are representative of at least three independent experiments. A, schematic diagrams of Gli1 (top) and Gli3 (bottom). The zinc fingers are depicted as small rectangles, the PKA phosphorylation sites are shown as asterisks, and the FLAG tag is depicted as a green rectangle. The FLAG tag is omitted from all other figures for clarity. B, Gli3 was processed and Gli1 was not processed in HEK293T cells. The processed fragment sometimes appears as a doublet in other investigations of Ci/Gli3 processing as well (5, 6, 17, 24). Gli3 processing was dependent on the proteasome; addition of the proteasome inhibitor lactacystin (Lac) abolished processing. C, Gli3 processing was dependent on PKA activity. Activation of PKA, either through the addition of forskolin (FSK) or co-transfection of constitutively active PKA, induced Gli3 processing. Mutation of the first four PKA sites in Gli3 (Gli3(PKD Gli3)) abolished processing. Mock, cells transfected with empty pcDNA3. D, in this and all subsequent figures, schematic diagrams of the constructs are shown in the order top to bottom that they are depicted left to right in the blot, and the first construct in each set of diagrams is wild-type Gli1 or Gli3. Gli1 containing the Gli3 processing determinant domain (Gli1(PDD Gli3)) was not processed. Gli1 containing the Gli3 zinc fingers and C terminus was processed (Gli1(C term Gli3)). E. The processing signal in Gli3. The features between the zinc finger domain (ZFs, small rectangles) and the PKA site region (asterisks) are enlarged. The two simple sequence regions are labeled as 1 and 2. The four lysines of the Gli3 processing degron, the previously defined processing determinant domain (PDD) (24), and the predicted end of the processed form of Gli3 (19) are indicated. The components of the processing signal, as we define them, are indicated below the diagram.

Gli3 Processing Is Required for Gli3 Processing—Next, we investigated the 140 amino acids between the zinc fingers and the degron in Gli3, which we call the linker sequence of the processing signal. Gli3 processing was disrupted when the 100 amino acids immediately after the zinc fingers were replaced with a complex, unstructured region from cytochrome b$_{2}$ (Fig. 3A). Again, the replacement of this region inhibited processing but not degradation of full-length Gli3 (supplemental Fig. S2). This region of Gli3 contains two stretches of simple sequences, and when these two stretches were deleted or replaced with a complex sequence, processing was severely impaired (Fig. 3A). Replacement of the two simple regions with the glycine-rich region from p105 rescued proteasome- and PKA-dependent processing (Fig. 3A). This suggests that these simple stretches are required for Gli3 processing.

Although simple regions were necessary, they were not sufficient to drive Gli3 processing. Replacing the entire 140-amino acid linker sequence with a complex, unstructured sequence containing a simple sequence of 35 glycines at the appropriate place prevented Gli3 processing (Fig. 3B). Additionally, replacing the 40 most C-terminal amino acids of the linker sequence (residues 101–140 after the zinc fingers) with a complex sequence, which left the endogenous simple stretches intact, also inhibited Gli3 processing (Fig. 3B). Thus, there are additional parts of the linker sequence, other than simple regions, that are required for processing.

FIGURE 1. Gli3 processing is appropriately regulated in HEK293T cells, and the Gli3 PDD does not induce Gli1 processing. In this and all subsequent figures, HEK293T cells were transfected with N-terminally FLAG-tagged Gli1 or Gli3 and constitutively active PKA, unless otherwise indicated. Gli1 and Gli3 expression and processing were assessed by immunoblotting with a FLAG antibody after cell lysis and SDS-PAGE. Results in this and all subsequent figures are representative of at least three independent experiments. A, schematic diagrams of Gli1 (top) and Gli3 (bottom). The zinc fingers are depicted as small rectangles, the PKA phosphorylation sites are shown as asterisks, and the FLAG tag is depicted as a green rectangle. The FLAG tag is omitted from all other figures for clarity. B, Gli3 was processed and Gli1 was not processed in HEK293T cells. The processed fragment sometimes appears as a doublet in other investigations of Ci/Gli3 processing as well (5, 6, 17, 24). Gli3 processing was dependent on the proteasome; addition of the proteasome inhibitor lactacystin (Lac) abolished processing. C, Gli3 processing was dependent on PKA activity. Activation of PKA, either through the addition of forskolin (FSK) or co-transfection of constitutively active PKA, induced Gli3 processing. Mutation of the first four PKA sites in Gli3 (Gli3(PKD Gli3)) abolished processing. Mock, cells transfected with empty pcDNA3. D, in this and all subsequent figures, schematic diagrams of the constructs are shown in the order top to bottom that they are depicted left to right in the blot, and the first construct in each set of diagrams is wild-type Gli1 or Gli3. Gli1 containing the Gli3 processing determinant domain (Gli1(PDD Gli3)) was not processed. Gli1 containing the Gli3 zinc fingers and C terminus was processed (Gli1(C term Gli3)). E. The processing signal in Gli3. The features between the zinc finger domain (ZFs, small rectangles) and the PKA site region (asterisks) are enlarged. The two simple sequence regions are labeled as 1 and 2. The four lysines of the Gli3 processing degron, the previously defined processing determinant domain (PDD) (24), and the predicted end of the processed form of Gli3 (19) are indicated. The components of the processing signal, as we define them, are indicated below the diagram.
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The Location, but Not the Identity, of the Gli3 Degron Is Essential for Processing—Lastly, we investigated the role of the degron in Gli3 processing. PKA phosphorylates six sites in the degron, which primes the region for phosphorylation by casein kinase 1 and glycogen synthase kinase 3 (19, 25, 26). This phosphorylation creates binding sites for the E3 SCF<sup>HP14CP</sup>, which mediates ubiquitination of four nearby lysines (25). Both the phosphorylation sites and the lysines are required for Gli3 processing (19, 25--27), but the role of the degron has not been characterized further.

First, we investigated if the location of the degron matters by moving the entire degron to the C terminus of Gli3. This abolished processing, even when a copy of the region containing the lysines was also left at the endogenous location (Fig. 4A), but did not abolish degradation of full-length Gli3 (supplemental Fig. S2). Second, we examined if the nature of the degron is important for processing by inserting the degron from IκBα, a protein whose degradation is well characterized (29, 30). When the Gli3 PKA site region was replaced with the IκBα degron, Gli3 was robustly processed (Fig. 4B). Stimulation of the IκBα degron with activated IκBα kinase (IKKβ) appeared to increase processing (Fig. 4B). Wild-type Gli3 processing was unaffected by IKKβ overexpression (supplemental Fig. S3), as expected. Therefore, the location of the degron but not its identity appears to be critical for efficient processing.

The Gli1 Zinc Finger Domain, but Not the Linker Sequence, Can Mediate Processing—The results described so far suggest that Gli3 processing is controlled by a three-part signal. Gli1 is degraded by the proteasome (supplemental Fig. S2 and Refs. 31--33), and perhaps Gli1 is not processed because one or more of these signal parts are missing. Therefore, we examined the role of each part of the signal in Gli1 individually. First, we tested if the Gli1 zinc finger domain can mediate processing by replacing the Gli1 zinc fingers with those of Gli3 (Fig. 4B), but did not induce Gli1 processing (Fig. 5A). Therefore, the zinc fingers are unlikely to be responsible for the absence of Gli1 processing, and another component of the signal is lacking.

We next examined if the Gli1 linker sequence is responsible for the lack of Gli1 processing. Replacement of the 100 amino acids immediately after the zinc fingers in Gli3 with the equivalent sequence from Gli1 disrupted Gli3 processing (Fig. 5B). Thus, the Gli1 linker sequence cannot mediate Gli3 processing, which suggests that it may be the deficient component of the processing signal in Gli1. As mentioned previously, the Gli3 PDD (24), which contains the Gli3 linker sequence and the lysines of the processing degon, did not induce Gli1 processing (Fig. 5B). Thus, the Gli1 linker sequence is incapable of mediating Gli3 processing, and the Gli3 PDD does not induce Gli1 processing.

Modification of the Gli1 Degron Can Stimulate a Small Amount of Processing—Finally, we investigated the role of the Gli1 degron, the last component of the processing signal. To
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Changing Two Processing Signal Components in Combination Induces Gli1 Processing—Because modification of no single component of the processing signal could cause robust Gli1 fragment formation, we tested whether altering two components might do so. Indeed, introducing the Gli3 PDD together with the IκBα degron led to robust processing (Fig. 6A). Similarly, introducing the Gli3 linker sequence and the Gli3 degron also induced Gli1 processing (Fig. 6B), but neither component alone could do so (Fig. 5, B and C).

Next, we altered the folded domain and the linker sequence in combination. We inserted what should be an effective linker sequence, the glycine-rich region from p105, and replaced the zinc finger domain with E. coli DHFR, which is likely more resistant to unfolding by the proteasome than the zinc fingers (7). Introducing both components together induced Gli1 processing (Fig. 6C), but neither component alone could do so (Figs. 5A and 6C). Therefore, it appears that modification of two parts of the processing signal in combination is sufficient to induce Gli1 processing. Components of the processing signal that do not induce processing on their own, like the Gli3 PDD, can have a large effect on processing when combined with another processing signal component. Overall, these results suggest that, although all three components of the processing signal can contribute to Gli1 processing, wild-type Gli1 is not processed because two components of the processing signal, the linker sequence and the degron, are ineffective.

DISCUSSION

Gli1 and Gli3 are closely related proteins, but Gli3 is processed by the proteasome (19) and Gli1 is not (21–23). To explain the contrasting behavior of these two related proteins, we defined a processing signal consisting of three components: the zinc finger domain, an adjacent linker sequence, and the degron. Disruption of any one of these three components abolished Gli3 processing. Gli1 has lost two of these components and is processed robustly only when two of them are strengthened.

Processing of model substrates in vitro as well as Ci and p105 processing depend on the presence of a domain resistant to unfolding (6, 7, 14, 17). Here, we established the requirement for a folded domain as the first component of the processing signal in Gli3. Previous work on Gli3 is consistent with this conclusion, because the experiments that characterized the Gli3 PDD as a transferable processing signal placed the PDD next to a folded domain in the exogenous protein (24). In the context of the processing reaction, the zinc fingers in Gli3 function only as a physical barrier to the proteasome because they can be replaced by an unrelated structure, E. coli DHFR. Similar observations have been made for Ci; the protein is still processed when the zinc fingers are replaced with the small enzyme barnase (17). The Gli3 zinc finger domain is not optimized for a folded domain as the first component of the processing signal, but the degron is likely the only component of the processing signal that is missing in Gli1.

determine if the Gli1 degron is sufficient to mediate processing, we swapped the Gli1 PKA site region into Gli3. This abolished Gli3 processing (Fig. 5C) and implied that the degron in Gli1 was deficient. In an attempt to induce Gli1 processing, we first swapped the Gli3 PKA site region into Gli1, but this did not strengthen the degron sufficiently to cause Gli1 processing (Fig. 5C). Next, we replaced the Gli3 PKA site region with the degron derived from IκBα. This degron induced a very small amount of Gli1 processing only when the IκBα degron was stimulated with activated IKKβ (Fig. 5D). In conclusion, it appears that the Gli1 degron is ineffective because it cannot mediate Gli3 processing, but the degron is likely not the only component of the processing signal that is missing in Gli1.
The second component of the Gli3 processing signal is the linker sequence, which stretches from the end of the zinc fingers to the lysines of the degron and is 140 amino acids long (Fig. 1E). In the simple mechanical model, processing is affected by the amino acid sequence to which the proteasome binds before the fragment is released (6, 8, 9). The last proteolytic cleavage in Gli3 processing occurs roughly 80 amino acids downstream of the zinc fingers (19), and the remaining “tail” presumably defines the last proteasome interaction site. Simple sequences in this region stimulate processing perhaps because they weaken the interaction of Gli3 with the proteasome, as has been proposed (6, 8–10). However, we also found that the C-terminal part of the linker sequence, past the cleavage site, affects processing, possibly because it serves as a particularly good proteasome initiation region (Ref. 24 and discussed below). These findings agree with the observation that deleting residues 43–111 after the zinc fingers at the endogenous locus reduces Gli3 processing in the developing mouse embryo by about half (34).

The situation for Ci is comparable; simple sequences adjacent to the zinc fingers are required for robust processing, but sequence features other than complexity are also important (6, 17). For instance, replacement of the region following the zinc fingers with a complex linker sequence can either inhibit or permit processing, depending on the particular sequence used (17). In fact, a single lysine just C-terminal to this region is required for Ci processing, whereas several other nearby lysines are dispensable (17). The mechanistic role of this lysine has not yet been determined, although it may be the site of polyubiquitination or some other covalent modification (17). Thus, both the simplicity of amino acid composition and other sequence features appear to play a role in Gli3 and Ci processing.

The third component of the Gli3 processing signal is the degron. In addition to the processing degron, Gli3 contains degrons recognized by SPOP, the substrate-binding adaptor of a cullin 3-based E3 (35–37). Overexpression of SPOP can enhance Gli3 processing, but only when the processing degron is intact (36). The Gli3 SPOP degrons are dispensable for processing, because a chimeric GST–Gli3 lacking both SPOP binding sites (36) is processed (24).

Processing does require the degron recognized by the E3 SCF<sup>B</sup>TrCP in the PKA site region (25, 26). Rather surprisingly, the location but not the identity of this degron determines Gli3 processing. Replacing it with the I<sub>B</sub>δ degron increases fragment formation, even though the I<sub>B</sub>δ degron leads to complete degradation in its native context (29, 30) and in exogenous contexts when used as a transferable degradation signal (38, 39). Both Gli3 processing and I<sub>B</sub>δ degradation are mediated by the same E3, SCF<sup>B</sup>TrCP (25, 26, 40, 41), but this does not necessarily mean that both degrons induce the same ubiquitin modification. Polyubiquitin chain topology depends on the E2 recruited by SCF<sup>B</sup>TrCP (42). Further, target lysine preference can be affected by the sequence context of the target lysines (43), which of course differs between the two degrons, and in this experiment the target lysines were transferred with the rest of the I<sub>B</sub>δ degron into Gli3.

Unlike degron identity, the location of the Gli3 processing degron clearly matters, because moving the degron to the C terminus of Gli3 abolished processing. The simple mechanical model of processing requires the presence of a degron but does not predict that its location would matter, as long as the proteasome eventually encounters the folded domain and adjacent simple sequences. Moving the Gli3 degron presumably changes the site of ubiquitin chain attachment within the substrate and, because the proteasome initiates degradation near the ubiquitin modification (44), changes the place where the proteasome first engages Gli3. Thus, the processing degron in Gli3 may direct the proteasome to begin degrading the substrate internally, most likely in the C-terminal half of the linker sequence of the processing signal. Moving the processing degron to the C terminus likely causes the proteasome to initiate degradation at...
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In vitro experiments suggest that the location of proteasome initiation can strongly affect its processivity, especially when initiation between domains means that the proteasome is challenged with two folded structures simultaneously (45).

In fact, the proteasome has to initiate internally when it processes the yeast transcription factors Spt23 and Mga2 (46, 47) and may also do so when it processes p105 (14, 48–50) because the ubiquitination sites that lead to p105 processing are located in the center of the protein (16). Internal initiation could explain why residues at the C terminus of the linker sequence are required for Gli3 processing: they may provide a particularly effective initiation site for the proteasome (24).

If a three-component signal programs Gli3 processing, then Gli1 would fail to be processed if it lacked any one component. In fact, Gli1 appears to have lost two components of the signal, namely the degron and the linker sequence. The Gli1 region that corresponds to the processing degron in Gli3 lacks many of the PKA and ubiquitination sites found in Gli3, which suggests that it drives ubiquitination less effectively. However, simply introducing the PKA and ubiquitination sites found in Gli3, which suggests that corresponds to the processing degron in Gli3 lacks many of the PKA and ubiquitination sites found in Gli3, which suggests that it drives ubiquitination less effectively. However, simply inserting the IkBa degron, which drove Gli3 processing powerfully, into Gli1 induced only a very small amount of fragment formation. Instead, robust Gli1 processing required introduction of the Gli3 PDD together with the IkBa degron. Thus, it seems that the Gli3 PDD is in fact a modular, transferable processing signal, but only when it is combined with a folded domain and an appropriate degron.

In principle, Gli1 could have lost the ability to be processed either because it is not efficiently targeted to the proteasome or because it is targeted to the proteasome but degraded completely. Our results suggest that Gli1 is targeted to the proteasome and degraded completely. We and others (31–33) have shown using cycloheximide chase experiments that Gli1 is degraded by the proteasome (supplemental Fig. S2). Further, Gli1 must contain an effective degron because inserting the more stable DHFR domain while simultaneously enhancing the linker sequence induced Gli1 processing. We do not know whether degradation of this construct is due to remnants of the Gli3 processing degron in Gli1, or whether a different degron (31–33) can drive processing with low efficiency. Overall, it appears that the linker sequence and degron components of the Gli1 processing signal have been weakened but not entirely abolished, and that all of the components of the processing signal can contribute to fragment formation.

It is interesting that the zinc finger component of the processing signal in Gli1 has not been weakened relative to Gli3. Perhaps the stability of the zinc fingers cannot be reduced without negatively affecting other required functions, such as DNA binding.

In summary, we have shown that the differential processing of Gli3 and Gli1 can be explained in terms of a three-part signal consisting of the zinc finger domain, an adjacent linker sequence, and the degron. Disruption of any of these three components abolishes Gli3 processing. Strengthening any two of these three components can induce Gli1 processing. These results thus provide insight into the general mechanism governing Gli protein processing by the proteasome.

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