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Catching a Gli-mpse of Hedgehog

Minireview

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According to a recent newspaper article, hedgehogs are not overblessed with intelligence. The Hedgehog (Hh) proteins, however, appear to have thought long and hard on how to regulate cell fate during animal development. Recent work is just beginning to uncover how the vast language of Hh signaling is mediated and interpreted by transcription factors of the Gli family that regulate cell type differentiation and pattern formation.

In the wing disc of fly embryos, Hh is involved in the specification of cells along the anteroposterior (A-P) boundary to express the TGFβ family member *Decapentaplegic* (*Dpp*; e.g., Nellen et al., 1996), which acts as a morphogen to specify cell fates within both the A and P compartments (Figure 1). In parasegmental patterning as well as in wing disc development, *Hh* is expressed by posterior compartment cells that also express the homeobox gene *Engrailed* (*En*). Here En represses anterior development and activates Hh transcription, albeit indirectly. In vertebrates, there are several *Hh* genes. One of these, *Sonic Hedgehog* (*Shh*), is expressed by embryonic organizing centers such as the notochord and posterior mesenchyme of the limb where it acts to organize pattern. For example, Hh from the notochord induces immediately overlying midline cells of the neural plate to become floor plate and more distant cells to become ventral neurons. In vertebrates and insects, there is evidence for the short and long range action of Hhs as well as evidence for graded effects in vertebrates (Roelink et al., 1995; Struhl et al., 1997, and references therein).

How do Hedgehogs impart spatial information to developing cell groups? The work of many laboratories suggests a preliminary pathway (Figure 1A) in which extracellular Hhs binds to the transmembrane protein Patched (Ptc) preventing its normal inhibition of Smoothened (Smo), another transmembrane protein of the receptor complex. This allows Smo to signal through the positive actions of Fused (Fu) and Cubitus interruptus (Ci), a Gli family member, negating also the inhibitory effects of Costal2 (Cos2) and Protein Kinase A (PKA). Three papers published in *Cell* (Aza-Blanc et al., 1997; Robbins et al., 1997, and Sisson et al., 1997 [both in this issue]) address the molecular mechanisms of Ci function in response to Hh signaling. This work and parallel studies on the functions of vertebrate Gli proteins begin to shed light onto the mechanisms involved in the interpretation of Hh signals. Surprisingly, the way Gli family proteins regulate cell fate in different animals may reflect variations on a theme.

Zinc Finger Transcription Factors of the Gli Family Mediate Hedgehog Signaling

Analyses of Ci function in flies have shown that this Gli family member is a DNA-binding transcription factor that is necessary and sufficient to mediate the Hh signal (Alexandre et al., 1996; Domínguez et al., 1996; Hepker et al., 1997; von Ohnen et al., 1997). Clones lacking Ci function in the A compartment of the wing disc express Hh without En and organize a new boundary zone and pattern around them, partly through the expression of Dpp. Conversely, overexpression of Ci in A or P clones directs the development of ectopic boundary cells expressing Hh target genes and pattern reorganization. Analyses of endogenous full-length *Gli* cDNAs in frog embryos has similarly suggested that Gli1, but not Gli3, mediates Shh signaling (Lee et al., 1997). Widespread Gli1 expression in the dorsal neural tube mimics widespread Shh expression as both induce the ectopic differentiation of floor plate cells and ventral neurons within the neural tube. Thus, a critical question in understanding how cells interpret the Hh signal and choose a fate is how the function of transcription factors of the Gli family is regulated.

Control of the Mediation and Response to Hedgehog Signaling or How to Regulate Gli Function

Ci has been reported to be found mostly in the cytoplasm and to be posttranscriptionally regulated, as the RNA is evenly found throughout the A compartment of wing discs but the protein is found at higher levels in cells that receive the Hh signal at the boundary region (e.g., Hepker et al., 1997). These observations suggest that there must be mechanisms that regulate the availability of Gli factors to the nucleus although Ci protein

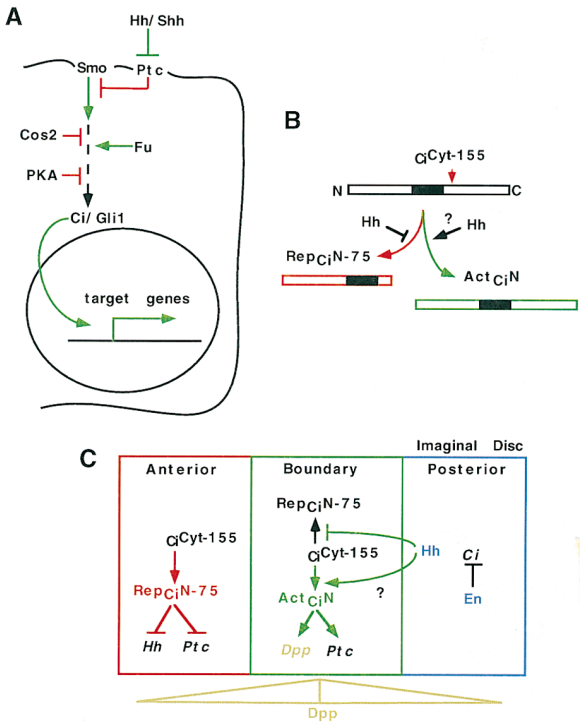

Figure 1. Hedgehog Signaling Pathway and Processing of Ci
See text for details.

does not massively translocate to the nucleus upon Hh signaling.

Aza-Blanc and colleagues (1997) report the remarkable discovery that aspects of Ci function are regulated by proteolysis (Figure 1B). Antibodies to the N and C termini and to the zinc finger domain detect an incongruent distribution of Ci protein. C-terminal antibodies showed the well-known distribution of Ci protein with higher levels in boundary cells. However, zinc finger domain and N-terminal antibodies showed a more homogenous distribution throughout the A compartment. This is due to the processing of full-length 155 kDa Ci (Ci^{155}) into a smaller form lacking most of the C termini (Ci^{75}). Because the smaller form is nuclear and this cleavage takes place mostly in A cells other than those in the boundary region, cytoplasmic Ci protein is preferentially detected near the Hh-expressing P compartment.

The significance of Ci cleavage is profound. $Ci^{N\text{-}75}$ binds DNA and represses transcription of *Hh*, *Wg*, and *Ptc*, thus ensuring the development of A cells as anterior (Figure 1C). Moreover, this cleavage is negatively regulated by Hh signaling, insuring that only A cells close to Hh-expressing P cells at the boundary will not process Ci and will not repress transcription of target genes. Expression of an artificially truncated form of Ci ($Ci^{N\text{-}76}$) in boundary cells appears to inhibit the response of A cells to Hh, and ectopic expression of $Ci^{N\text{-}76}$ in posterior cells inhibits *Hh* expression. In normal development, the transition of cytoplasmic to nuclear Ci in A cells located progressively more distal from P cells is likely to reflect the range of action of Hh.

Ci has positive effects in A cells close to the A-P boundary where Hh signaling prevents cleavage into a smaller repressive form. How does Ci positively affect Hh signaling? Ectopic expression of full-length Ci (Ci^{155}) in A cells is sufficient to induce the transcription of the Hh target gene *Dpp*, and in P cells it induces inappropriate *Ptc* transcription. Ci^{155} therefore has properties that are distinct from $^{Rep}Ci^{N\text{-}75}$. Ci^{155} is cytoplasmic and the question arises as to how a cytoplasmic protein mediates positive transactivation. Full-length cytoplasmic Ci ($Ci^{Cyt\text{-}155}$) could be normally modified by Hh signaling to produce small amounts of a nuclear form ($^{Act}Ci^{N}$) that are sufficient to transactivate (Figures 1B and 1C). In addition to possible processing, changes in the phosphorylation state of Ci could be important for nuclear transport and/or transactivation. Consistent with this idea, PKA negatively regulates Hh signaling and Ci displays several putative PKA phosphorylation sites. Alternatively, nuclear Ci may be masked by modification of the epitopes that the available antibodies recognize.

Complex Formation by Fu, Cos2, and Ci: A Retention/Modification/Cleavage Site Docked at Microtubules

The availability of Ci to nuclei either as a short repressive form or a longer activating form is subject to tight regulation. The papers of Aza-Blanc et al. (1997), Robbins et al. (1997), and Sisson et al. (1997) provide the intriguing identification of a large molecular complex involving at least three components of the Hh signaling pathway: Fu, Cos2, and Ci. These papers report the cloning and identification of Cos2 as a kinesin-related protein, the existence and biochemical determination of the binding

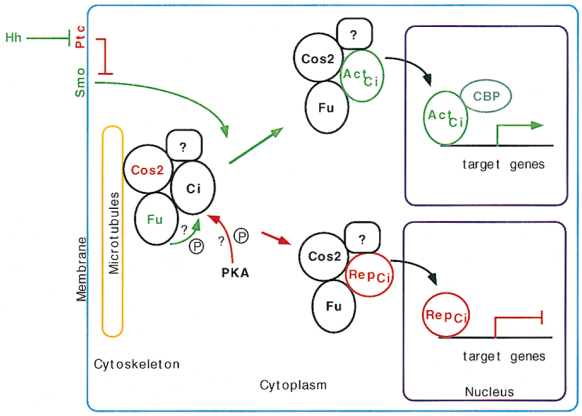

Figure 2. Complex Formation of Fu, Cos2, and Ci, Association with Microtubules, and Effects of Hh Signaling

See text for details.

of a multiprotein complex to microtubules, and its dependence on Fu function. Together, these results suggest the existence of a microtubule-associated docking/processing site regulating the availability of Ci to nuclei and its processing/modification (Figure 2).

Cos2 encodes a kinesin-related protein localized in the cytoplasm (Sisson et al., 1997). *Cos2* RNA is found throughout the imaginal discs and early embryo, but protein accumulates in anterior compartment cells showing that Cos2 is posttranscriptionally controlled. Unlike classical kinesins, however, Cos2 may lack motor activity and its binding to microtubules does not appear to be mediated by ATP hydrolysis. Like classical kinesins, Cos2 associates tightly with microtubules. Robbins et al. (1997) provide direct biochemical evidence that Ci, Fu, and Cos2 are normally found in a complex that docks at microtubules, possibly through the action of Cos2. Moreover, the tight binding of this complex to microtubules is prevented by Hh signaling. Thus, Hh signaling prevents association of the complex to the cytoskeleton and cleavage of Ci into a repressive form (Figure 2).

These results raise the question of how complex formation, association to microtubules, and processing regulate Ci function. Ci has a cytoplasmic retention domain that may interact with other elements in the complex, and the C-termini of Fu is required for association to Cos2, which is likely in turn to account for the association of the complex to microtubules. However, complex formation and microtubule association are distinct. Robbins et al. (1997) found complexes under conditions that depolymerize microtubules, and Aza-Blanc et al. (1997) found complex association with both $Ci^{Cyt\text{-}155}$ and $Ci^{N\text{-}75}$. Consistent with the latter, the presence of Cos2, and thus binding to microtubules, is not required for positive Ci function: somatic Cos2 clones in A cells cause elevated levels of cytoplasmic Ci and pattern respecification, likely due to the activation of Dpp, a Ci target. Since the positive transactivating form of Ci is unknown (even though elevated levels of full-length Ci, which may or may not remain full-length, are sufficient for this function), it remains unclear whether it is also associated with a multiprotein complex. Docking of the complex at

microtubules may provide a physical basis for cytoplasmic retention of Ci. In addition, association with microtubules may provide a way to bring the complex in close proximity to the Hh receptor proteins Ptc and Smo in the membrane, which could facilitate signaling from these to Fu and Cos2.

What is then the role of the complex? It could serve as an assembly site for all the components regulating and carrying out the cleavage and modifications of Ci. Consistent with this, mutations that inhibit Fu function prevent complex formation. It is therefore possible that there are a series of phosphorylation events mediated by Fu and other kinases that regulate the state of Ci and that these events are regulated by Hh signaling (Figure 2). While more research is needed, a preliminary scheme could flow as follows (Figure 2): in the absence of Hh signaling, Fu, Cos2, and Ci are found in a complex that binds to microtubules. In this complex Ci is processed to a smaller form that translocates to the nucleus, possibly after dissociation from the complex, and represses target gene transcription. Upon Hh signaling, extracellular Hh binds to Ptc, inactivating its repression of Smo, which then sends a signal that activates Fu and represses Cos2, and the complex dissociates from microtubules, also preventing further cleavage of Ci. In the complexes dissociated from the cytoskeleton, Ci is possibly modified into a nuclear form that positively transactivates target genes. This transactivation function would appear to be mediated by association of Ci, possibly free from the complex, with CBP, a coactivator of transcription (Akimaru et al., 1997).

Vertebrate Glis—Variation on the Same Theme?

Vertebrates have at least three *Gli* genes (e.g., Platt et al., 1997; Lee et al., 1997). Cell transfection and embryo injection assays show that endogenous Gli proteins are mostly cytoplasmic and partially colocalize with microtubules, paralleling the distribution of Ci (Lee et al., 1997). This contrasts with the nuclear localization of the glioma derived Gli1 suggesting that it may be a mutated protein.

In the early frog neural plate, *Gli1* is the only *Gli* gene expressed in midline cells becoming floor plate, located immediately overlying the *Shh*-expressing notochord. Later on *Gli1* is expressed in immediately adjacent cells that appear to become ventral neurons. In contrast, *Gli2* and *Gli3* are expressed throughout the neural plate with the exception of the midline, with *Gli3* showing a graded distribution with highest levels laterally (the prospective dorsal neural tube). In mice, however, they are coexpressed in the early neural plate. Together, these observations raise the question of whether the Gli genes are functionally redundant or have undergone diversification of functions following their multiplication.

Ectopic Shh signaling induces *Gli1* but represses *Gli3* (Marigo et al., 1996; Lee et al., 1997). Functional analysis in frog embryos show that Gli1, but not Gli3, can mimic the effects of ectopic Shh in the neural tube, inducing ectopic floor plate and ventral neuronal differentiation (Lee et al., 1997). Moreover, a Gli binding site in a floor plate enhancer of *HNF-3*β is required for expression in transgenic mice (Sasaki et al., 1997). Consistent with this, Gli1 has been found to be able to activate and Gli3 to repress transcription in vitro (Marine et al., 1997; Sasaki et al., 1997).

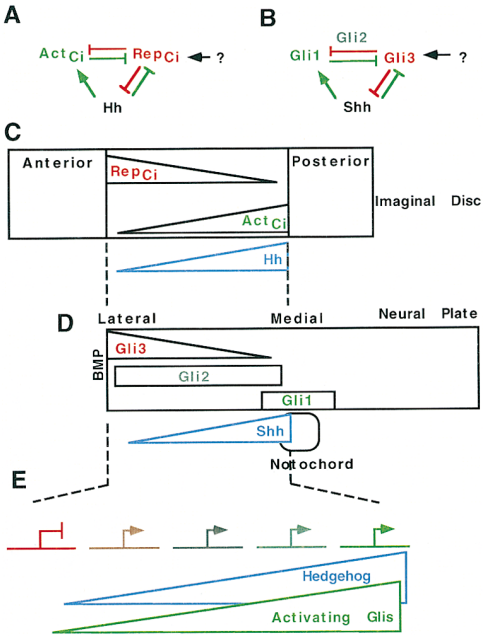

Figure 3. Interactions of Gli Proteins, Effects of Hedgehog Signaling, and Proposed Interpretation of a Hedgehog Gradient through Graded Levels of Activating Gli Function

(A) Critical interactions of Ci and Hh. Hh induces activity of Ci. In the absence of the repressive form of Ci, there is ectopic *Hh* expression. Hh signaling represses the formation of the repressive form. Active and repressive Ci compete for binding sites. The question mark and arrow to ^{Rep}Ci indicate the unknown mechanism that constitutively cleaves Ci in A cells.

(B) Critical interactions of Glis and Shh. Shh induces the expression of Gli1. In the absence of Gli3 there is ectopic *Shh* expression. Shh signaling represses the expression of *Gli3*. Gli1 and Gli3 compete for binding sites. Gli2 is proposed to balance the interactions of Gli1 and Gli3 and to have activating function, antagonizing Gli3, where Gli is not expressed. The question mark and arrow to Gli3 indicate signals that activate *Gli3* transcription, possible including BMPs. These interactions may be central in the patterning of many tissues and cell groups such as somites, limbs, face, and neural plate.

(C) Opposing distributions of activating and repressive forms of Ci resulting from a graded distribution of Hh.

(D) Expression patterns of *Gli* genes in the neural plate of the frog embryo that result, in part, from the actions of Shh. BMPs from the adjacent epidermal ectoderm may also contribute to *Gli3* expression.

(E) Proposed readout of the combination of active and repressive Gli proteins as a consequence of a Hh gradient. Distinct target genes could be induced at different thresholds of activating Gli function.

It is possible that Hedgehog signaling in all animals involves two antagonistic basic operations, one repressive and one activating (Figures 3A and 3B), acting in partially overlapping domains (Figures 3C and 3D). Flies have achieved this by processing their Gli protein, Ci, making a nuclear repressor form whereas the full-length protein, possibly after modification, becomes the activating nuclear form. Vertebrates may have solved this by gene duplication and functional divergence. Gli1 could be the equivalent of ^{Act}Ci^{N} and Gli3 the equivalent of ^{Rep}Ci$^{N\text{-}75}$. Further evidence for this parallel and a role for Gli3 in repressing *Shh* expression comes from the finding that loss of Ci in A cells and loss of Gli3 in mouse

Xt mutants (Masuya et al., 1995) result in the ectopic expression of Hh and Shh, respectively.

Graded Levels of Overall Gli Function May Interpret a Hedgehog Gradient

Different Gli proteins may compete for binding sites in vivo as all Gli family proteins can bind to the same canonical Gli binding site in vitro (e.g., Vortkamp et al., 1995). Such competition suggests a mechanism for the induction of different cell fates (Figure 3E). For example, in the wing imaginal disc these are A cells in which $^{Rep}Ci^{N\text{-}75}$ is active, boundary cells in which $^{Act}Ci^{N}$ is active, and P cells that lack Ci expression. However, it is also possible that in cells receiving attenuated levels of Hh signaling, the choice of fate depends on the ratio of activating to repressive nuclear Ci proteins as attenuated Hh signaling may not be able to completely repress cleavage. This competition for Gli/Ci binding sites would take place in boundary cells located distal from the boundary with P cells, where graded levels of nuclear/cytoplasmic Ci are detected.

In vertebrates, overlapping expression of *Gli* genes may result in a similar situation. In this case, the overlap of Gli2 with Gli1 and Gli3 is predicted to be critical in determining the overall "activating Gli" readout, although the role of Gli2 remains unclear as it could show partial redundancy with Gli3 (Mo et al., 1997).

These experiments and observations raise the possibility that competition for binding sites by opposing forms or activities of Gli proteins represent a molecular basis for the interpretation of the graded effects of Hh/Shh signaling (Figure 3E). Gli target gene regulation, however, is likely to be more complex. For example, Zic transcription factors (Aruga et al., 1994) containing a Gli-type zinc finger domain homologous to that of the fly gene *odd-paired*, also bind the canonical Gli target sites.

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