Journal of Cell Science 112, 4485-4489 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
JCS4698

COMMENTARY

Integrin-linked kinase and PINCH: partners in regulation of cell-extracellular matrix interaction and signal transduction

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Published on WWW 30 November 1999

SUMMARY

Integrin-linked kinase (ILK) is a focal adhesion serine/threonine protein kinase that is emerging as a key signaling protein functioning at one of the early convergence points of integrin- and growth factor-signaling pathways. ILK binds to PINCH through the N-terminal ankyrin (ANK) repeat domain and the PINCH binding is crucial for focal adhesion localization of ILK. The ILK-PINCH interaction also connects ILK to Nck-2, an SH2–SH3-containing adaptor protein that interacts with components of growth factor and small GTPase signaling pathways. The kinase activity of ILK is regulated by both cell adhesion and growth factors in a phosphoinositide 3-kinase (PI3K)-dependent manner. ILK phosphorylates downstream targets such as protein kinase B (PKB, also known as Akt) and glycogen synthase kinase 3 (GSK-3) and regulates their activities. Overexpression of ILK in epithelial cells leads to striking morphological changes mimicking epithelial-mesenchymal transition, including upregulation of integrin-mediated fibronectin matrix assembly and downregulation of cell-cell adhesions. Furthermore, ILK regulates nuclear translocation of β-catenin and gene expression, and promotes cell cycle progression and tumor formation. Recent genetic studies in *Drosophila melanogaster* and *Caenorhabditis elegans* have shown that lack of expression of ILK or PINCH results in phenotypes resembling those of integrin-null mutants, which demonstrates that ILK and PINCH are indispensable for integrin function during embryonic development.

Key words: ILK, PINCH, Integrin, Nck-2, Signaling

INTRODUCTION

Cellular behavior is determined by environment cues, including both the extracellular matrix (ECM) and soluble factors such as growth factors. Signals from the ECM and growth factors are transmitted into cells through different types of transmembrane receptor but converge downstream at multiple points and thus regulate common cellular processes, such as cell proliferation, survival, differentiation and migration. Integrin-linked kinase (ILK) and PINCH are two widely expressed focal adhesion proteins that form a complex in cells and the ILK-PINCH complex is emerging as a key signaling complex that functions at one of the early convergence points of cell-adhesion- and growth-factor-trigged signal transduction. Here, I discuss the structures and functions of ILK and PINCH, and attempt to identify areas that will be critical for understanding the mechanisms by which ILK and PINCH function in cell adhesion and signal transduction.

STRUCTURES AND MOLECULAR ACTIVITIES OF ILK AND PINCH

Human integrin-linked kinase was initially identified by Hannigan et al. (1996) from a yeast two-hybrid screen of human cDNA library, using a β1 integrin cytoplasmic domain containing sequence as bait. Subsequently, the murine homologue was cloned (Li et al., 1997) and the human and mouse ILKs found to be 99% identical (Hannigan et al., 1996; Li et al., 1997). Both human and mouse ILK proteins contain 452 amino acid residues with an apparent molecular mass of 59K. Examination of the ILK protein sequences reveals three structurally distinct domains (Fig. 1). At the N-terminus of ILK lie four ankyrin (ANK)-repeats. Although the structure of the ILK ANK-repeat domain has not been determined, it is probably a cupped-hand structure consisting of an antiparallel β-sheet (fingers) and α-helix bundles (palm) that are perpendicular to the plane of the β-sheet. This prediction is based on recently solved structures of several ANK-repeat-containing proteins (Sedgwick and Smerdon, 1999). Immediately C-terminal to the ANK repeat domain is a pleckstrin homology (PH)-like motif that typically is involved in phosphoinoside lipid binding. The ILK C-terminal sequence exhibits significant homology to other protein kinase catalytic domains and thus is predicted to fold into the bi-lobate structure that is characteristic of kinase domains. A closer examination of the ILK C-terminal sequence, however, reveals

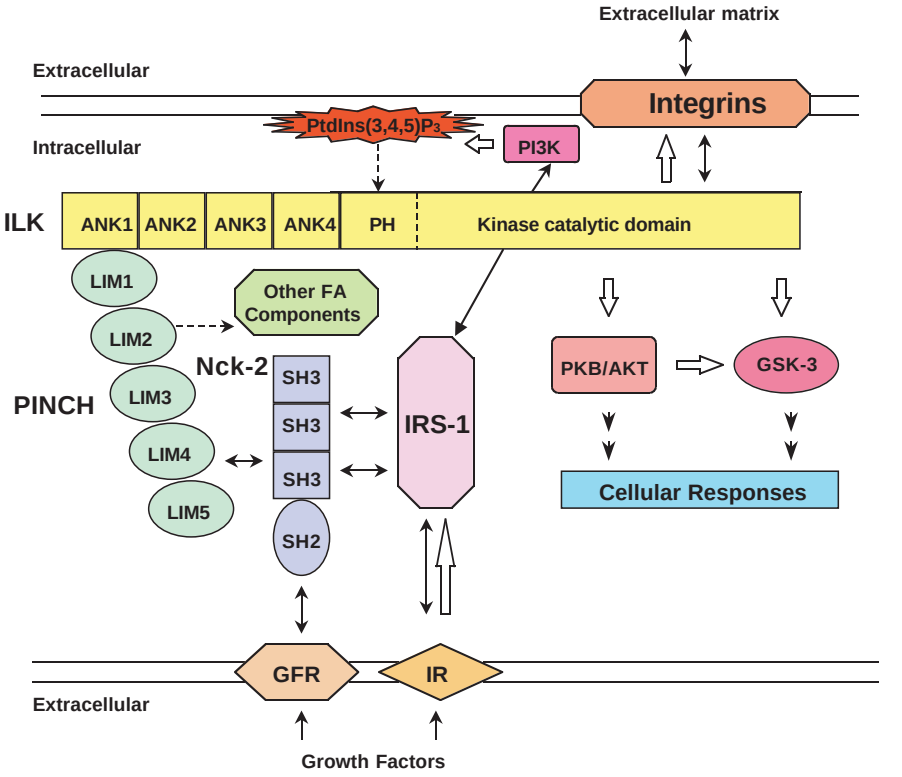

Fig. 1. Domain structures, molecular activities and hypothetical signaling mechanism of ILK and PINCH. Domain structures of ILK, PINCH and Nck-2, and molecular interactions or reactions mediated by the ILK and PINCH domains are depicted. A model of the ILK-PINCH signaling mechanism is proposed here based on experimental data discussed in the text. In this model, the ILK ANK-repeat domain binds to PINCH, which, together with other components of focal adhesions, such as integrins, mediates the localization and clustering of ILK at focal adhesions in response to cell adhesion. Additionally, PINCH might mediate interactions with other proteins, such as Nck-2, which associates with ligand-activated growth factor receptor kinases or intracellular components of growth factor signaling pathways, such as IRS-1. This would result in assembly of supramolecular signaling complexes containing ILK, PINCH, Nck-2 and other regulators or substrates of ILK. The PH-like domain of ILK, through an interaction with PtdIns(3,4,5)P_3, is involved in PI3K-activated upregulation of ILK activity induced either by cell adhesion to fibronectin or by insulin stimulation. The kinase domain of ILK, upon stimulation by PtdIns(3,4,5)P_3, catalyzes serine/threonine phosphorylation of downstream targets including integrins, PKB/Akt and/or GSK-3, which leads to modulation of cell-ECM interaction, gene expression or cell survival. Double solid arrows, protein-protein interactions; open arrows, phosphorylation reactions; dashed arrows, putative intermolecular interactions; FA, focal adhesion; GFR, growth factor receptor; IR, insulin receptor; IRS1, insulin receptor substrate 1; PKB, protein kinase B; ILK, integrin-linked kinase; PI3K, phosphoinositide 3-kinase; PH, pleckstrin homology-like motif; GSK-3, glycogen synthase kinase 3; ANK1-4, ankyrin repeats 1-4, LIM1-5, LIM domains 1-5. The PINCH-binding activity requires all four ANK repeats of ILK. The PH-like motif partially overlaps with the ILK catalytic domain and a dashed line is used to reflect this fact. The interaction between Nck-2 and IRS-1 is mediated primarily by the second and the third SH3 domains of Nck-2 (Tu et al., 1998). Drawings of the molecules are for illustration only and are not to scale.

that ILK lacks several sequences that are conserved in other protein kinase catalytic domains (Hannigan et al., 1996). Studies of the three-dimensional structure of the ILK C-terminal domain should shed light on the catalytic mechanism of ILK.

Recent studies have provided evidence for the functions of each of the three ILK domains. ILK immune complexes derived from mammalian cells, which contain native mammalian ILK and potentially other protein kinases associated with ILK, can catalyze serine/threonine phosphorylation of an artificial substrate (myelin basic protein), a synthetic peptide containing the β1 integrin cytoplasmic sequence, as well as several endogenous mammalian proteins (Hannigan et al., 1996). Furthermore, a purified recombinant GST-ILK fusion protein can directly phosphorylate myelin basic protein and several physiologically relevant substrates, including protein kinase B (PKB, also known as Akt), glycogen synthase kinase 3 (GSK-3), and ILK itself (autophosphorylation) in vitro (Delcommenne et al., 1998; Hannigan et al., 1996). Substitution of Glu359 (within the catalytic subdomain VIII) with a lysine residues inhibits the kinase activity (Novak et al., 1998; Wu et al., 1998). These data suggest that ILK is a bona fide protein kinase. The ILK-catalyzed phosphorylation of PKB/Akt and GSK-3 is particularly interesting. ILK can phosphorylate PKB/Akt at Ser473 (Delcommenne et al., 1998), one of the two phosphorylation sites that are critical for activation of PKB/Akt. Overexpression of a kinase-inactive form of ILK, which presumably functions as a dominant negative inhibitor of the endogenous ILK, inhibited phosphorylation of PKB/Akt at Ser473 in vivo (Delcommenne et al., 1998). This suggests that ILK plays a role in activation of PKB/Akt in vivo. ILK can also phosphorylate GSK-3 in vitro, and inhibits GSK-3 activity in vivo (Delcommenne et al., 1998) either by activation of PKB/Akt or by direct phosphorylation of GSK-3.

Yeast two-hybrid and co-immunprecipitation studies have indicated that the ILK C-terminal kinase domain, in addition to catalyzing protein phosphorylation, can mediate interaction with the β1 integrins (Hannigan et al., 1996). ILK can also associate with integrins of other β subfamilies, such as β3 and/or β5 integrins (Hannigan et al., 1996). It is not known, however, whether the ILK-integrin interaction is regulated in vivo. Given that the relatively short β1 integrin cytoplasmic domain is a binding or phosphorylation target of several cytoskeletal and signaling proteins, and that ILK kinase activity is regulated by integrin-mediated cell adhesion to fibronectin (Delcommenne et al., 1998), the ILK-integrin interaction is probably a regulated one. Elucidating the molecular basis of the ILK-integrin interaction and identifying the factors that influence it will facilitate our understanding of the role of this interaction in ILK signaling.

Recombinant and mammalian ILK normally exists in a

relatively low-kinase-activity-state. The in vitro kinase activity of purified recombinant ILK protein can be stimulated by phosphatidylinositol 3,4,5-trisphosphate (PtsIns $(3,4,5)P_3$; Delcommenne et al., 1998), presumably through direct binding of the 3-phosphoinoside lipid to the PH motif of ILK. Cellular ILK activity is transiently upregulated by cell adhesion to fibronectin or by treatment with insulin (Delcommenne et al., 1998). Inhibition of phosphoinositide 3-kinase (PI3K) with Wortmannin or Ly294002 inhibited the cell adhesion- or insulin-induced ILK activation. This is consistent with regulation of ILK activity by PtsIns $(3,4,5)P_3$ in vivo. Furthermore, overexpression of a constitutively active catalytic subunit (P110) of PI3K activated ILK in vivo (Delcommenne et al., 1998). These studies strongly suggest that the PH motif of ILK participates in regulation of ILK kinase activity in response to cell adhesion and insulin. A prediction from these data is that mutations at the critical residues within the ILK PH motif should ablate cell adhesion- or insulin-stimulated ILK activation in vivo. The 3-phosphoinoside-lipid-dependent activation of ILK probably plays a role in physiological regulation of PKB/Akt and GSK-3 and, consequently, of the cellular processes mediated by PKB/Akt or GSK-3, including gene expression and cell survival.

The N-terminal ANK-repeat domain directly binds to PINCH, an intracellular protein containing five LIM domains (Fig. 1). The LIM domain is a protein-binding motif consisting of a cysteine-rich consensus sequence of approximately 50 residues that form two separate zinc fingers (Dawid et al., 1998; Jurata and Gill, 1998; Yao et al., 1999). PINCH was initially identified by Rearden (1994) from screening a human cDNA library with antibodies recognizing senescent erythrocytes. Yeast two-hybrid screens using the N-terminal ANK-repeat domain of ILK as bait reveal that PINCH binds to ILK (Tu et al., 1999). The interaction between ILK and PINCH occurs in mammalian cells as well as in vitro (Tu et al., 1999). A series of mutational studies have defined the structural basis underlying the ILK-PINCH interaction. The PINCH-binding activity requires all four ANK repeats of ILK (Tu et al., 1999; Li et al., 1999). Only the second zinc finger of the first LIM domain of PINCH is required for the interaction (Li et al., 1999), however. In spite of the overall similarity between the three-dimensional structures of the LIM domains, this LIM-ANK-repeats-mediated recognition system is highly specific. Neither the other PINCH LIM domains (LIM2, LIM3, LIM4, or LIM5) nor LIM domains from other proteins (e.g. those of paxillin or zyxin) recognize the ILK ANK repeats (Tu et al., 1999).

Unlike ILK, PINCH does not contain a catalytic domain, and thus the primary role of PINCH is to mediate intermolecular interactions. Tu et al. recently cloned a novel PINCH-binding protein termed Nck-2 from a yeast two-hybrid screen of a human cDNA library based on a specific interaction with the PINCH LIM4 domain (Tu et al., 1998). The interaction between PINCH and Nck-2 has been confirmed by independent biochemical binding assays (Tu et al., 1998). Chen et al. (1998) and Braverman and Quilliam (1999) have independently cloned an identical protein (termed as Nckβ or Grb4) in cDNA library screens based on sequence homology. Nck-2 comprises primarily three N-terminal SH3 domains and one C-terminal SH2 domain (Fig. 1). Nck-2 is structurally related to Nck-1 (68% identical at the protein level), an adaptor protein that was initially identified from screening a human melanoma cDNA (Lehmann et al., 1990). Nck-1 has been implicated in regulation of fundamental cellular processes including cell proliferation, migration and cytoskeleton organization (McCarty, 1998). Nck-2 and Nck-1 are encoded by different genes but are co-expressed in many different types of cell (Chen et al., 1998; Tu et al., 1998; Braverman and Quilliam, 1999). Intriguingly, although Nck-2 and Nck-1 recognize several common protein targets, including the EGF receptor, the PDGF receptor, IRS-1, $p21^{cdc42/rac}$-activated kinase and Sos that play crucial roles in growth factor- and small GTPase-signaling, the PINCH-binding activity is specific to Nck-2 (Tu et al., 1998). Thus, Nck-2 might play a unique role in connecting the growth factor signaling pathway with the PINCH-ILK complex and thus the integrin-signaling pathway.

The PINCH-Nck-2 interaction is mediated by a single PINCH LIM domain (LIM4) and the third SH3 region of Nck-2 (Tu et al., 1998). This SH3-LIM recognition system is distinguishable from the traditional SH3-PxxP recognition system. Substitution of the highly conserved tryptophan residue located immediately C-terminal to the n-Src loop in the third SH3 domain of Nck-2 with a lysine residue, which eliminates the binding of the SH3 domain to proline-rich sites located in other Nck-2-binding proteins (Y. Tu and C. Wu, unpublished observations), did not inhibit the PINCH-binding (Tu et al., 1998). The specificity of the PINCH-binding activity is conferred at least in part by Nck-2 residues 176-194, which differ significantly from the corresponding Nck-1 residues (Tu et al., 1998).

Given that there are five LIM domains in PINCH, and each LIM domain could potentially mediate two independent protein-protein interactions, additional PINCH-binding proteins probably exist. Identification and characterization of proteins that interact with other PINCH LIM domains will likely provide new insights into the mechanism by which PINCH and ILK function in cells.

CELLULAR FUNCTIONS OF ILK AND PINCH

We have recently demonstrated that ILK is a constituent of cell-matrix focal adhesions (Li et al., 1999, in this issue), which are major sites at which cells attach to the ECM and signals are transduced bidirectionally between the ECM and intracellular signaling network. Intriguingly, although β1 integrins are localized in epithelial cell-cell adhesions as well as cell-ECM focal adhesions, ILK is largely absent from epithelial cell-cell adhesions (Li et al., 1999). Overexpression of ILK in IEC-18 epithelial cells induced striking morphological changes mimicking epithelial-mesenchymal transition (Novak et al., 1998; Wu et al., 1998). One of the cellular processes that is regulated by ILK in the epithelial cells is fibronectin matrix assembly, an important physiological process that is controlled by multiple factors, including integrins and cytoskeleton (Wu et al., 1995). Overexpression of ILK in epithelial cells dramatically stimulated fibronectin matrix assembly and this upregulation depends on, among other things, the catalytic activity of ILK (Wu et al., 1998). The cell surface fibronectin-binding activity appears to increase in cells overexpressing ILK (Wu et al., 1998), which suggests that ILK plays a role in regulation of ‘inside-out’ integrin signaling.

A closer examination of the integrin activation state in cells that have different levels of ILK expression or activity should provide valuable information on the mechanism by which ILK regulates fibronectin matrix assembly. A reduction of cell-cell adhesion accompanies the increase in fibronectin matrix assembly in epithelial cells overexpressing ILK. The former is probably caused, at least in part, by a reduction in E-cadherin expression (Novak et al., 1998; Wu et al., 1998).

Cell survival and cell growth are other cellular processes that are regulated by ILK and they probably are connected to alterations in fibronectin matrix assembly in certain types of cell. The requirement for anchorage to the ECM for normal epithelial cell survival and growth is well established (Assoian and Zhu, 1997; Frisch and Ruoslahti, 1997; Giancotti and Mainiero, 1994; Schwartz, 1997). Overexpression of ILK in IEC-18 cells allows them to grow in soft agar (Hannigan et al., 1996; Radeva et al., 1997; Wu et al., 1998) and therefore ILK can promote anchorage-independent cell growth. ILK-stimulated anchorage-independent cell growth is distinguishable from that promoted by Ha-Ras in that the former can be partially inhibited by N-terminal fragments of fibronectin (Wu et al., 1998), which inhibit fibronectin matrix assembly. However, although these data imply that fibronectin matrix assembly plays an important role in regulation of cell survival and growth, the effect of ILK on anchorage-independent cell growth might not mediated entirely by upregulation of fibronectin matrix assembly. Overexpression of ILK increased the expression or activity of several key components of the cell cycle machinery, including cyclin A, cyclin D1, and CDK4 proteins, and reduced the inhibitory activity of p27 (Radeva et al., 1997). Thus, ILK might also regulate cell growth and survival by controlling gene expression and post-translational regulation.

Regulation of gene expression by both the ECM and growth factors is well documented. ILK seems to play an important role in ECM- and growth factor-regulated gene expression. Two transcription factors, LEF-1 and AP-1, that are known to be regulated by extracellular factors, can be activated by ILK (Novak et al., 1998; Troussard et al., 1999). Overexpression of ILK promotes translocation of β-catenin to the nucleus and formation of β-catenin-LEF-1 complexes (Novak et al., 1998), and therefore could influence the expression of LEF-1/β-catenin-responsive genes. Although the specific genes that are down- or up-regulated by ILK through this pathway have yet to be determined, they might include E-cadherin and genes involved in regulation of cell cycle. In a recent study, Troussard et al. (1999) have shown that activation of ILK, either by stable or transient overexpression, increases AP-1 activity. Activation of AP-1 by ILK appears to be achieved through inhibition of GSK-3, which is a substrate of ILK, because overexpression of an active GSK-3 overrode the ILK-induced activation of AP-1 (Troussard et al., 1999).

What are the cellular functions of PINCH? A major role of PINCH is probably regulation of ILK functions, particularly subcellular compartmentation of ILK. ILK is targeted to and clustered at focal adhesions upon cell adhesion to the ECM (Li et al., 1999). Ablation of the PINCH-binding activity by mutagenesis eliminated the ability of ILK to localize to focal adhesions (Li et al., 1999). Thus, PINCH might play an essential role in localization of ILK to focal adhesions. Additional functions of PINCH probably exist. For example, PINCH could mediate the formation of complexes between ILK and Nck-2 (Tu et al., 1999), and consequently, could provide a physical connection between the integrin/ILK signaling pathway and growth factor- or small GTPase-signaling pathways. Increasing knowledge of the molecular properties of PINCH, ILK and Nck-2 should allow us to uncover additional functions of PINCH in the near future.

The biochemical and cellular studies discussed above suggest a model of ILK signaling in which each of the three domains of ILK plays a crucial but distinct role (Fig. 1).

ROLES OF ILK AND PINCH IN DEVELOPMENT AND DISEASE

The structures of ILK and PINCH are well conserved across species from human and mouse to *Drosophila melanogaster* and *Caenorhabditis elegans*, which is consistent with a role for ILK and PINCH in fundamental cellular processes. Recent genetic studies in *C. elegans* and *Drosophila* have shed light on the roles of PINCH and ILK in development. The *C. elegans* homologue of mammalian PINCH is encoded by *unc-97*, a gene that is crucial for muscle attachment and movement (Hobert et al., 1999). The C-PINCH gene (*unc-97*) is expressed in developing embryos (beginning around 300 minutes of development) as well as in adult animals. Studies using a green florescent protein (GFP)-tagged C-PINCH (UNC-97) revealed that the GFP-UNC-97 was located in body-wall muscles and concentrated at the focal adhesion-like muscle attachment sites (dense bodies) where β-integrin/PAT-3 is clustered. In *Drosophila*, homologues of PINCH and integrin also co-localize in developing embryos (Hobert et al., 1999). Inhibition of *unc-97* expression causes an embryonic-arrest phenotype termed Pat (Paralyzed and Arrested elongation at the Two-fold stage) that is similar to the loss-of-function phenotype of β-integrin/*pat-3*, vinculin/*deb-1* and perlecan/*unc-52* (Hobert et al., 1999). In another recent study, Mackinnon and Williams found that another *pat* gene, *pat-4*, encodes the *C. elegans* homologue of ILK, and a null mutation in ILK/*pat-4* causes a Pat phenotype that resembles those of animals in which β-integrin or PINCH is mutated (A. C. Mackinnon and B. Williams, personal communication). The studies in these model systems illustrate the importance of PINCH and ILK in embryonic development. They are highly consistent with the biochemical and cellular studies discussed above, and together they provide strong evidence for a crucial role of the ILK-PINCH complex in integrin function. Although mutations in genes that encode human and mouse PINCH and ILK have not yet been reported, given the crucial roles of PINCH/*unc-97* and ILK/*pat-4* in *C. elegans* and *Drosophila* development, and the important molecular and cellular activities displayed by mammalian PINCH and ILK, loss-of-function mutations in the human and mouse PINCH and ILK genes will probably have severe developmental consequences.

Because ILK and PINCH participate in fundamental cellular processes such as cell-ECM interactions and intracellular signal transduction pathways regulating cell survival and proliferation, relatively modest changes, such as alterations in expression or activities, might contribute to pathogenesis of human diseases such as cancer and complications in diabetes that involve alterations in cell proliferation and cell-ECM

interactions. The gene encoding human ILK is located at chromosome 11p15.5-p15.4 (Hannigan et al., 1997), a region containing several breakpoints associated with the Beckwith-Wiedemann syndrome, which is a genetic disorder characterized by excessive growth and predisposition to Wilms' tumor. Clinical studies using a polyclonal anti-ILK antibody suggest that ILK is overexpressed in several human tumors, including Ewing's sarcoma, primitive neuroectodermal tumor and medulloblastoma (Chung et al., 1998). Studies using a transgenic mouse model system indicate that ILK expression in vivo can be up-regulated by constitutively active ErbB-2 (Xie et al., 1998), a member of the EGF receptor family that is implicated in tumorigenesis. Furthermore, overexpression of ILK in epithelial cells promoted tumorgenicity in vivo (Wu et al., 1998). Recent studies have shown that ILK plays an important role in PTEN-dependent cell cycle regulation and survival through the PKB/Akt pathway (S. Dedhar, personal communication), and therefore is probably involved in the tumor formation promoted by PTEN-inactivation mutations.

I thank Shoukat Dedhar (Jack Bell Research Centre and University of British Columbia) and Benjamin Williams (University of Illinois at Urbana-Champaign) for sharing results before publication, and Jun Qin (Cleveland Clinic) for valuable discussions. This work is supported by National Institutes of Health grant DK54639, research project grant # 98-220-01-CSM from the American Cancer Society, and the V foundation for Cancer Research. C.W. is a V Foundation Scholar.

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