

TGF β induces SIK to negatively regulate type I receptor kinase signaling

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Signal transduction by transforming growth factor β (TGF β) coordinates physiological responses in diverse cell types. TGF β signals via type I and type II receptor serine/threonine kinases and intracellular Smad proteins that regulate transcription. Strength and duration of TGF β signaling is largely dependent on a negative-feedback program initiated during signal progression. We have identified an inducible gene target of TGF β /Smad signaling, the salt-inducible kinase (SIK), which negatively regulates signaling together with Smad7. SIK and Smad7

form a complex and cooperate to down-regulate the activated type I receptor ALK5. We further show that both the kinase and ubiquitin-associated domain of SIK are required for proper ALK5 degradation, with ubiquitin functioning to enhance SIK-mediated receptor degradation. Loss of endogenous SIK results in enhanced gene responses of the fibrotic and cytostatic programs of TGF β . We thus identify in SIK a negative regulator that controls TGF β receptor turnover and physiological signaling.

Introduction

The profound roles that TGF β plays during embryogenesis, adult tissue homeostasis, and disease pathogenesis necessitate deeper understanding of mechanisms that regulate this signaling pathway. Signaling occurs via the TGF β type II receptor (T β RII) that trans-phosphorylates T β RI, also known as activin receptor-like kinase (ALK) 5. Activated ALK5 phosphorylates receptor-regulated Smads (Smad2 and Smad3), promotes their association with Smad4, and leads to regulation of transcription (Feng and Derynck, 2005).

Smad7, a negative regulator of TGF β /Smad signaling, is an immediate early gene target of the pathway (for review see Itoh and ten Dijke, 2007). Smad7 binds to ALK5, competing with Smad2/3 phosphorylation and mediating receptor ubiquitination, mechanisms which link to the process of TGF β receptor internalization and lysosomal degradation (Di Guglielmo et al., 2003).

We have identified gene targets of Smad signaling (Kowanetz et al., 2004). A highly regulated gene, *SNF1LK* (*sucrose nonfermented 1-like kinase*) or *salt-inducible kinase 1* (*SIK1*, hereby abbreviated as SIK), encodes a serine/threonine kinase. SIK is one of 14 AMP-activated protein kinases that can be phosphorylated by the tumor suppressor LKB1 (Alessi et al., 2006). SIK expression is induced during cardiogenesis and skeletal muscle differentiation (Ruiz et al., 1994) or in adrenal glands, leading to steroidogenesis (Okamoto et al., 2004).

We uncover a new functional role of SIK in the TGF β pathway. SIK induction is necessary for physiological TGF β signaling. Ubiquitin promotes association of SIK to Smad7, which down-regulates ALK5. Thus, SIK defines a new negative-feedback loop initiated by incoming Smad signals.

Results and discussion

TGF β induces SIK in a Smad-dependent manner

We have reported Smad4-dependant gene profiles in response to TGF β 1 or bone morphogenetic protein (BMP) 7 using human

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Abbreviations used in this paper: ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; CA, constitutively active; GAPDH, glyceraldehyde-3'-phosphate dehydrogenase; PAI-1, plasminogen activator inhibitor 1; SIK, salt-inducible kinase; T β RII, TGF β type II receptor; TCL, total cell lysate; UBA, ubiquitin associated.

The online version of this paper contains supplemental material.

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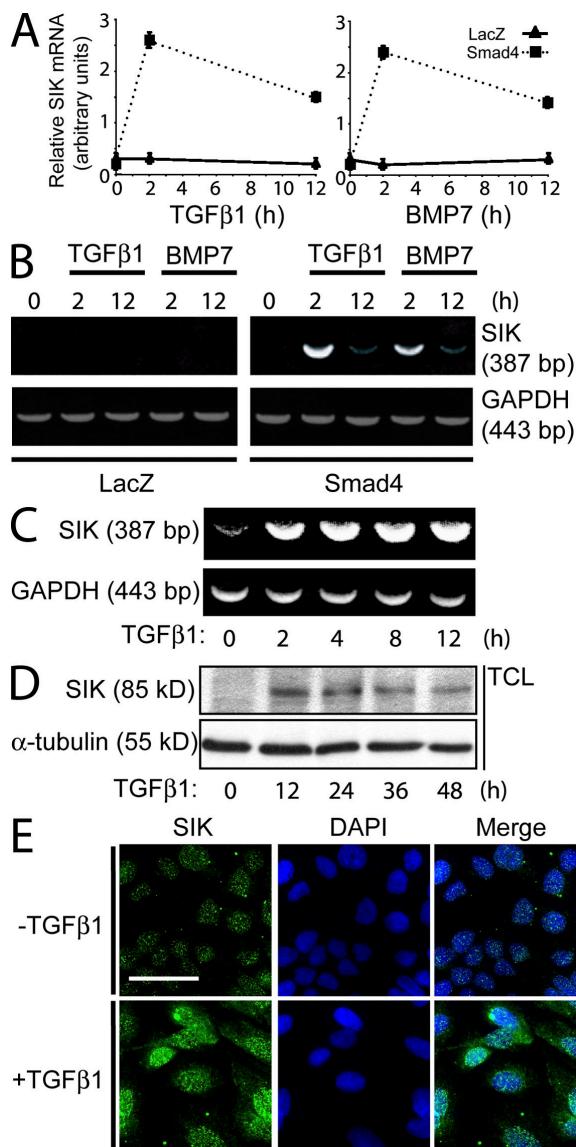


Figure 1. Smad signaling induces endogenous SIK. (A) cDNA microarray analysis of SIK mRNA in Smad4-deficient MDA-MB-468 cells after infection with adenovirus expressing LacZ or Smad4 and stimulation with 2 ng/ml TGF β 1 or 300 ng/ml BMP7. Normalized mean expression values with error bars from triplicate microarray expressions are shown (arbitrary units). (B and C) Semiquantitative RT-PCR analysis of SIK and GAPDH mRNA in MDA-MB-468 cells as in A (B) or in response to 2 ng/ml TGF β 1 in HaCaT cells (C). Amplified cDNA sizes are in bp. (D) SIK and α -tubulin loading control protein profiles in response to 5 ng/ml TGF β 1 in HaCaT cells. Protein sizes are in kilodaltons. (E) Induction of nuclear and cytoplasmic levels of endogenous SIK by 5 ng/ml TGF β 1 in HaCaT cells (bar, 10 μ m).

breast carcinoma MDA-MB-468 cells that lack both *Smad4* gene copies (Kowanetz et al., 2004). After reconstitution with Smad4, TGF β 1 or BMP7 rapidly induced SIK mRNA, followed by slow decrease (Fig. 1, A and B). In human HaCaT keratinocytes, TGF β 1 induced and sustained SIK mRNA and protein levels (Fig. 1, C and D; and Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200804107/DC1>). Endogenous TGF β 1-induced SIK protein showed punctate nuclear, cytoplasmic, and peripheral localization (Fig. 1 E). SIK represents a new gene target of TGF β /BMP7 Smad signaling. Interestingly, the

Caenorhabditis elegans orthologue of SIK, Kin-29, regulates chemosensory neuronal signaling and body size, a process which is dependent on TGF β /Smad (Lanjuin and Sengupta, 2002; Maduzia et al., 2005), which is in agreement with our data.

SIK down-regulates activated ALK5

Gain-of-function experiments with transfected SIK showed specific down-regulation of ALK5 after TGF β 1 stimulation, whereas minor effects were scored without stimulation (Fig. 2 A). SIK did not down-regulate Smad7 or GFP (Fig. S1 B), excluding translational inhibition or induction of proteolysis.

SIK affected ALK5 turnover as it reduced the half-life of a constitutively active (CA) ALK5 from 5.5 to 2.8 h without affecting a KR- (kinase-dead) ALK5 or an unrelated adaptor protein CIN85 (Fig. 2 B). Proteasomal (LLnL) and lysosomal (chloroquine) inhibitors stabilized the ALK5 levels in the presence of SIK (Fig. 2 C), suggesting both proteasomal and lysosomal mechanisms in ALK5 down-regulation by SIK.

SIK has an N-terminal kinase domain with lysine 56 binding to ATP and a central ubiquitin-associated (UBA) domain, which regulates conformation and kinase activity (Jaleel et al., 2006). A catalytically inactive (K56R) SIK or a deletion mutant lacking the UBA domain (Δ UBA) failed to down-regulate CA-ALK5 (Fig. 2 D). This suggests that both catalytic activity and UBA domain of SIK affect ALK5 turnover.

RNAi against SIK significantly enhanced endogenous ALK5 levels (Fig. 2 E). Increased presence of endogenous cell surface receptors measured with chemically cross-linked radioligand upon depletion of SIK further strengthened this evidence (Fig. 2 F, 0 h). Thus, endogenous SIK must regulate total ALK5 levels, which also affects cell surface receptor numbers, available for signaling. Receptor down-regulation was also slower after SIK RNAi. Significantly higher ligand-bound receptor levels were observed for up to 1 h of internalization compared with control (Fig. 2 F).

Because proteasomal and lysosomal inhibitors block ALK5 degradation (Fig. 2 C), we suggest that ALK5 turnover takes place in lysosomes. Proteasomes might promote trafficking to the lysosome, as has already been established for the EGF receptor (Longva et al., 2002; Alwan et al., 2003). How proteasomes regulate TGF- β receptor internalization and degradation remains unclear.

SIK associates and cooperates with Smad7 for ALK5 down-regulation

Down-regulation of ALK5 is mediated by Smad7 (for review see Itoh and ten Dijke, 2007). We reasoned that SIK could associate directly with ALK5 or Smad7. Coimmunoprecipitation assays showed that SIK formed complexes with both ALK5 and Smad7 (Fig. 3, A and B). To detect SIK-ALK5 complexes, we had to achieve low SIK expression and high CA-ALK5 levels, which resulted in active ALK5 signaling with small effects on receptor down-regulation. SIK bound stronger to the Smad7 linker and C-terminal (Mad homology 2) domains than to its N-terminal domain (Fig. 3 B). When endogenous human ALK5, Smad7, and SIK were immunoprecipitated from HaCaT lysates, SIK coprecipitated primarily with Smad7 and weakly with

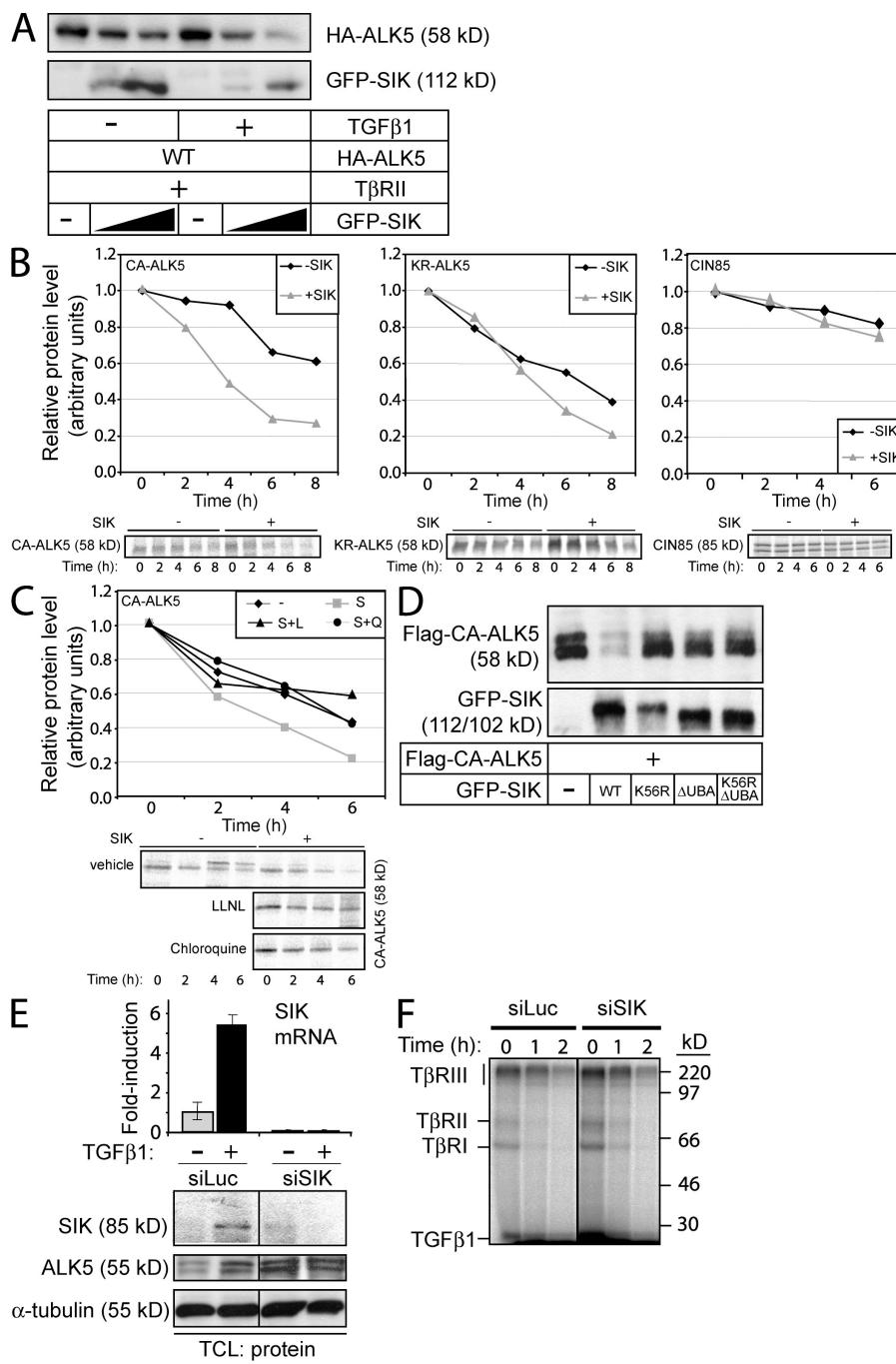


Figure 2. SIK down-regulates activated ALK5.

(A) Immunoblot of ALK5 expressed in COS1 cells together with T β RII and stimulation or not with 2 ng/ml TGF β 1 for 12 h in the absence or presence of increasing wild-type GFP-SIK amounts. (B and C) Pulse-chase analysis of CA-ALK5, KR-ALK5, and CIN85 in the absence (-) or presence (+) of cotransfected SIK (S). A 30-min pulse was followed by chase for the indicated times. Treatment with proteasomal (LLnL 200 μ M; S + L) or lysosomal (chloroquine 400 μ M; S + Q) inhibitor during the chase is shown in C. Radioactive quantitation of ALK5 or CIN85 protein is plotted relative to the pulse signal. Representative data from three independent repeats are shown. Protein autoradiograms are shown below each graph. (D) Immunoblot of CA-ALK5 in COS1 cells cotransfected with wild-type and mutant SIK constructs. (E, top) quantitative real-time RT-PCR analysis of SIK mRNA in HaCaT cells after transfection with control (siLuc) and specific (siSIK) siRNAs and 8 h of stimulation with 5 ng/ml TGF β 1. Mean fold induction of TGF β -stimulated SIK (black bars) relative to unstimulated levels (gray bars) are plotted, with standard errors determined from triplicate samples. (E, bottom) Immunoblot of endogenous SIK, ALK5, and α -tubulin loading control in HaCaT total cell lysates (TCL) prepared as per mRNA analysis. (F) Mv1Lu cells transfected with control (siLuc) or specific (siSIK) siRNA were incubated with radio-TGF β 1 before cross-linking and incubation at 37°C for the indicated time periods. Cell surface receptor autoradiograms show the three TGF β receptors (T β R) marked as type I, II, and III, and the unbound ligand (TGF β) migrating at the bottom. Protein sizes are in kilodaltons. Black lines indicate that intervening lanes have been spliced out.

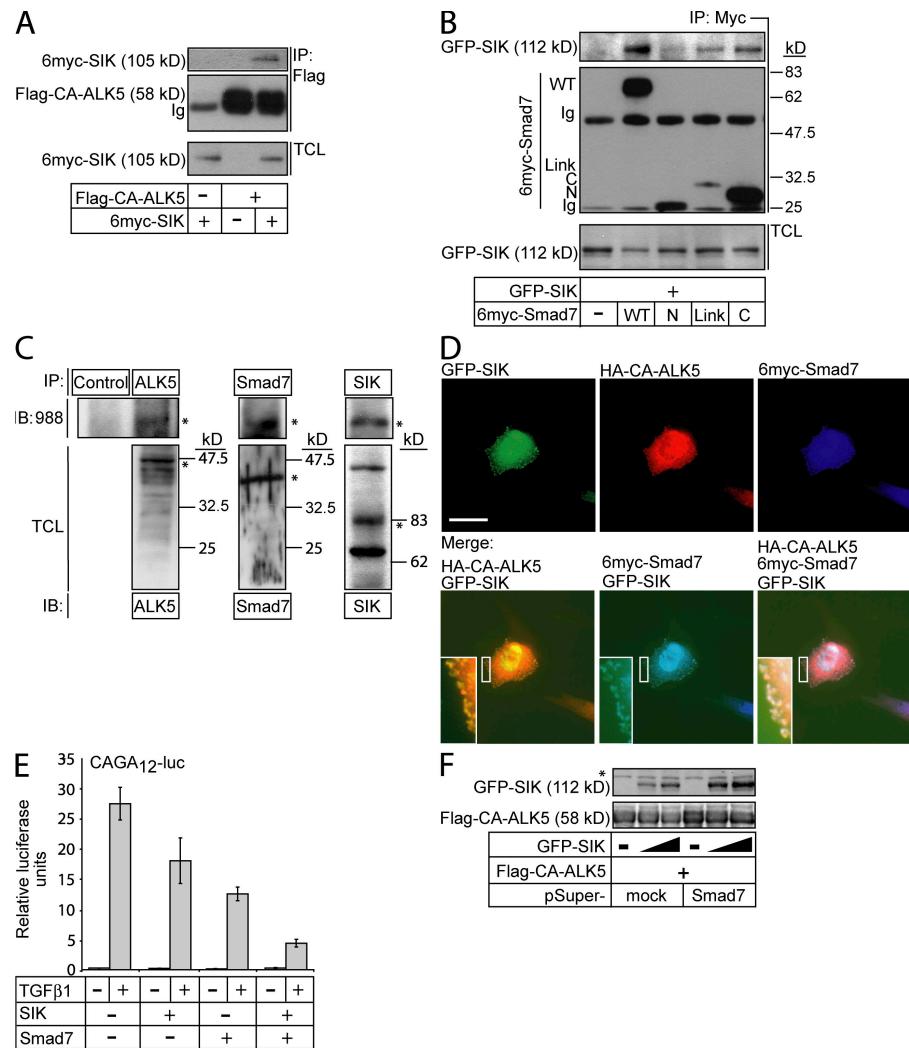
ALK5 (Fig. 3 C), confirming the evidence with transfected proteins. Thus, protein complexes between endogenous SIK, Smad7, and ALK5 occur in vivo.

Smad7 resides in the nucleus and moves to the cytoplasm in response to TGF β (for review see Itoh and ten Dijke, 2007). SIK also resides in both nucleus and cytoplasm, and its localization is regulated by hormones in adrenal cells or by the 14-3-3 adaptor (Okamoto et al., 2004; Al-Hakim et al., 2005). In transfected TGF β -sensitive Mv1Lu cells, SIK localized in nuclei and cytoplasm with pronounced punctate distribution proximal to the plasma membrane (Fig. 3 D), which is similar to endogenous SIK (Fig. 1 E). Colocalization of SIK, CA-ALK5, and Smad7 was primarily observed in peripheral

clusters (Fig. 3 D, insets). Thus, SIK, Smad7, and ALK5 must form complexes in cytoplasmic regions proximal to the plasma membrane.

To verify the cooperation of Smad7 and SIK in negatively regulating TGF β signaling, we measured the effects of SIK and Smad7 on the transcriptional induction of a Smad3/Smad4-specific promoter-reporter, CAGA₁₂, by TGF β 1 (Fig. 3 E). SIK enhanced the inhibitory effect of Smad7 by more than twofold. Knockdown of endogenous Smad7 stabilized and rescued CA-ALK5 down-regulation by SIK, even though Smad7 depletion led to increased SIK levels (Fig. 3 F). Thus, SIK mediates down-regulation of signaling ALK5 receptor in a Smad7-dependent manner.

Figure 3. SIK associates and cooperates with Smad7 to down-regulate ALK5. (A) Coimmunoprecipitation of CA-ALK5 with wild-type (WT) SIK and TCL controls for SIK. Low levels of SIK coexpressed with high levels of CA-ALK5 allowed detection of SIK-ALK5 complexes with only small effects on receptor down-regulation. (B) Coimmunoprecipitation of wild-type SIK with wild type and various Smad7 domains (C, C-terminal; Link, linker; N, N-terminal). TBL controls for SIK and immunoglobulin chains (Ig) are shown. (C) Coimmunoprecipitation of endogenous SIK with endogenous ALK5 and Smad7. HaCaT cell lysates, pretreated with 50 μ M MG132 and 5 ng/ml TGF β 1 overnight, were immunoprecipitated with anti-ALK5, anti-Smad7, and anti-SIK (988) antibodies, followed by immunoblotting with anti-SIK (988) antibody. TBL immunoblots with the three antibodies demonstrate specificity of each antiserum. Asterisks indicate specific protein bands and sizes are in kilodaltons. The top left panel (Mock and ALK5) was exposed four times longer than the top right panel (Smad7 and SIK). (D) Triple confocal immunofluorescence analysis of wild-type SIK, CA-ALK5, and wild-type Smad7 in transfected Mv1Lu cells. Insets show high magnifications of peripheral clusters just below the plasma membrane (bar, 10 μ m). (E) SIK represses synergistically with Smad7 CAGA₁₂ promoter induction by 0.5 ng/ml TGF β 1 for 6 h in HepG2 cells transfected with empty vector or Flag-SIK and Flag-Smad7. Normalized promoter activity is plotted as mean values from triplicate determinations with standard error bars. (F) Depletion of endogenous Smad7 stabilizes the CA-ALK5 receptor and wild-type SIK. Immunoblot of HEK293T cell extracts after transient transfection with shRNA vector pSuper-Smad7 or its empty (mock) version together with increasing amounts (triangles) of GFP-SIK and constant amount of CA-ALK5. The asterisks indicate nonspecific protein bands. Protein sizes are shown in kilodaltons.



Ubiquitin and the UBA domain modulate ALK5 regulation by SIK

We examined whether ubiquitin affected the ability of SIK to facilitate ALK5 degradation (Fig. 4 A). Ubiquitin significantly enhanced the potency of CA-ALK5 down-regulation by SIK. A K48R ubiquitin mutant completely blocked this down-regulation. This implies that polyubiquitin chain formation via lysine 48 affects ALK5 degradation, which is compatible with a proteasome-dependent mechanism and inhibition of ALK5 degradation by LLnL (Fig. 2 D).

We examined SIK localization in relation to ubiquitin in Mv1Lu cells (Fig. 4 B). SIK distributed in nuclei, cytoplasm, and prominent peripheral structures, SIK-K56R showed more cytoplasmic enrichment, and both colocalized with cytoplasmic ubiquitin clusters. SIK lacking its UBA domain showed no colocalization, as it distributed diffusely in the cytoplasm, suggesting that SIK-ubiquitin clusters depend on the integrity of the UBA domain. These punctate structures were also enriched in 19S and 20S proteasomes (unpublished data).

We then expressed SIK and ubiquitin in different pools of cells before immunoprecipitation to measure association of SIK with ubiquitinated proteins but not SIK ubiquitination, per se

(Fig. 4 C and Fig. S2 A, available at <http://www.jcb.org/cgi/content/full/jcb.200804107/DC1>). SIK readily coprecipitated ubiquitinated proteins of diverse sizes, confirming that they were distinct from ubiquitinated SIK. Wild-type and SIK-K56R bound to ubiquitinated proteins, whereas SIK-ΔUBA or a mutant lacking both kinase and UBA domains (ΔN) did not, suggesting that the SIK UBA domain is needed for binding to polyubiquitinated proteins (Fig. 4 C). C-terminal deletion of SIK, after its UBA domain, had no effect on binding ubiquitinated proteins (Fig. 4 C). Thus, SIK can associate with ubiquitinated proteins in cells.

Obvious ubiquitinated protein candidates to which SIK could bind were ALK5 and Smad7. Accordingly, the SIK-Smad7 complex was enhanced by coexpressing ubiquitin, whereas deletion of the UBA domain of SIK prohibited such enhancement (Fig. 4 D). A slow migrating SIK form was obvious in complex with Smad7 (Fig. 4 D, asterisk), which may represent ubiquitinated SIK. In contrast, neither ubiquitin nor UBA domain deletion had any effect on SIK-ALK5 complexes (Fig. S2 B). Ubiquitin therefore enhances association between SIK and Smad7, and the SIK UBA domain mediates formation of this ternary complex.

The SIK UBA domain was recently shown to not bind ubiquitin but to modulate SIK kinase activity in vitro (Jaleel et al., 2006).

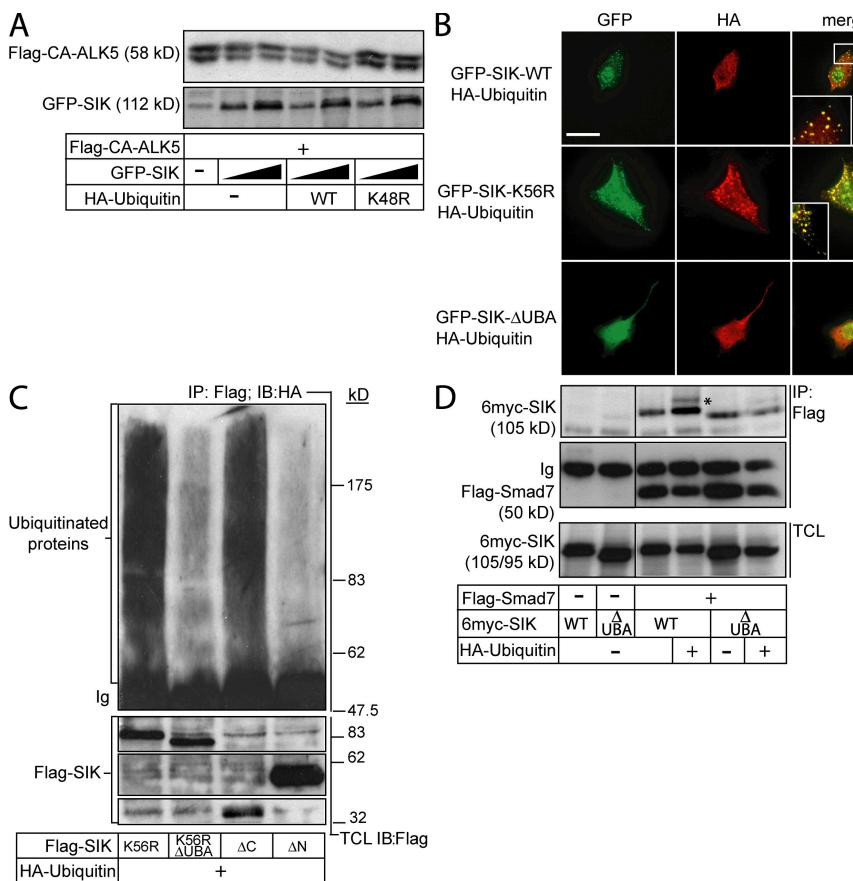


Figure 4. Role of ubiquitin and the UBA domain of SIK in ALK5 down-regulation. (A) Immunoblot of CA-ALK5 in the presence of increasing doses of WT GFP-SIK and coexpression of WT or K48R mutant ubiquitin. (B) Confocal immunofluorescence analysis of wild-type or mutant SIK proteins cotransfected with HA-ubiquitin in Mv1Lu cells. Insets show higher magnifications of peripheral clusters below the plasma membrane (bar, 10 μ m). (C) SIK lysate was mixed briefly with lysate for ubiquitinated proteins before immunoprecipitation. SIK mutant proteins were immunoprecipitated (anti-Flag) and ubiquitinated proteins are detected (anti-HA immunoblot). Total SIK levels (bottom) and immunoglobulin heavy chain (Ig) are shown. (D) Coimmunoprecipitation of wild-type Smad7 with wild-type myc-SIK or UBA deletion mutant in the absence or presence of ubiquitin. The asterisk indicates a slow migrating SIK species, possibly representing ubiquitinated SIK. TLC controls of SIK variants, the immunoglobulin heavy chain (Ig), and protein sizes in kilodaltons are shown. Black lines indicate that intervening lanes have been spliced out.

We also failed in demonstrating in vitro binding of recombinant mono- or oligoubiquitin chains to SIK (unpublished data). SIK immunopurified from mammalian cells clearly bound to ubiquitinated proteins via its UBA domain (Fig. 4 C and Fig. S2 A). This could reflect an in vivo modification or the requirement for another binding protein. Further studies will reveal the mechanism by which ubiquitin and the SIK UBA domain regulate its own function.

SIK negatively regulates TGF β signaling

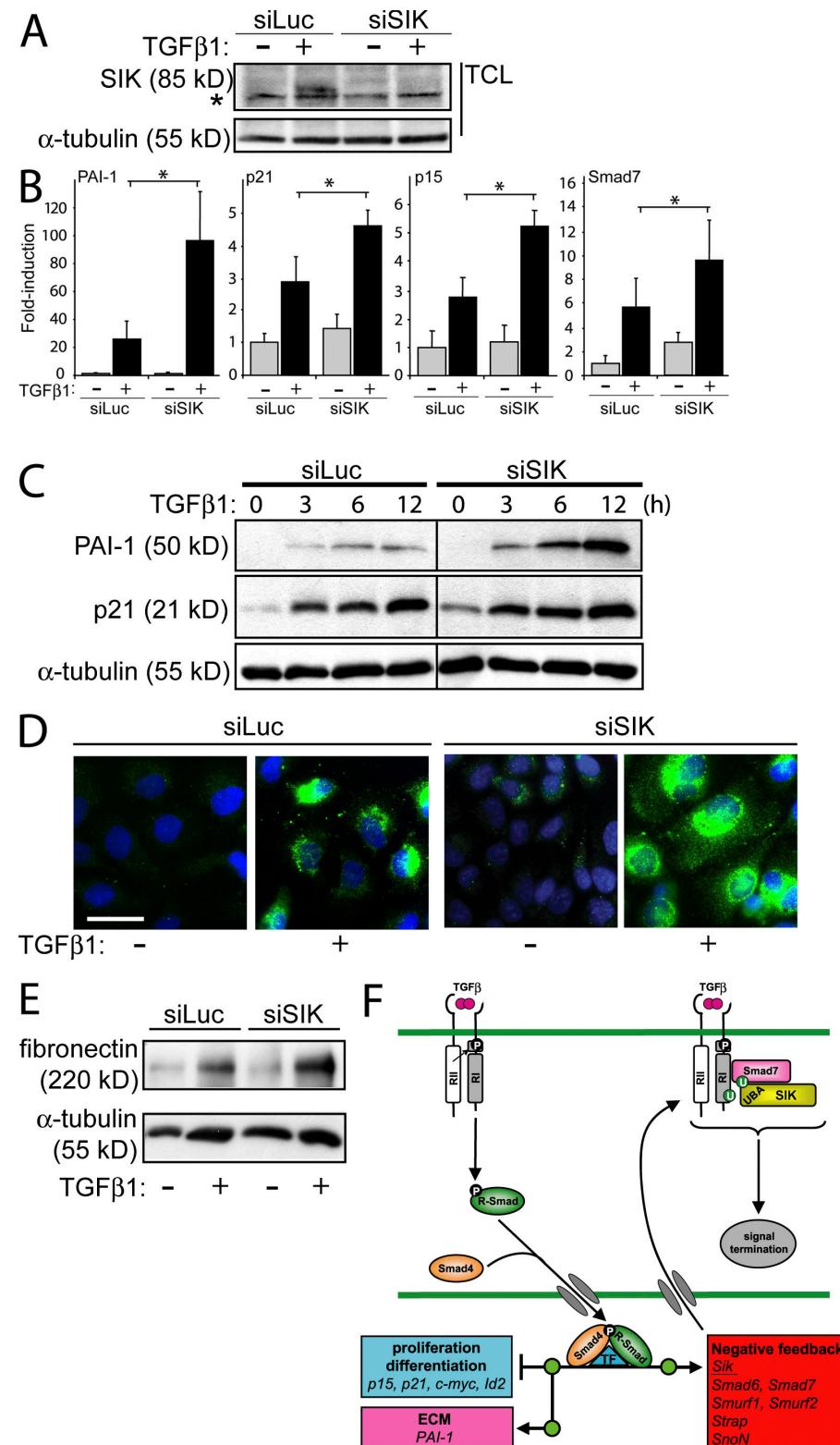
Stimulation of cells after endogenous SIK depletion enhanced the potency of TGF β signaling (Fig. 5, A and B). Signaling was assessed by measuring mRNA levels of well characterized TGF β gene targets, representing three distinct physiological programs: the fibrotic program (*plasminogen activator inhibitor 1* [*PAI-1*], TGF β inducible), the cytostatic program (cell cycle inhibitors *p21* and *p15*, TGF β inducible; transcriptional regulators *c-myc* and *Id2*, TGF β repressible), and the negative-feedback program (*Smad7*, TGF β -inducible; Fig. 5 B and Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200804107/DC1>). We also confirmed that SIK knockdown enhanced the magnitude of endogenous *PAI-1* and *p21* protein accumulation in response to TGF β 1 (Fig. 5 C). Finally, protein levels and pericellular localization of fibronectin, another TGF β gene target from the fibrotic program, augmented significantly after depleting SIK (Fig. 5, D and E). Thus, induction of SIK by TGF β represents a central step in negative regulation of TGF β signaling.

The identification of SIK as an immediate early gene induced by the Smad pathway led to important new insights into

how TGF β receptor signaling is negatively regulated (Fig. 5 F). SIK joins Smad7 in the negative-feedback loop of TGF β signaling (for review see Itoh and ten Dijke, 2007). SIK and Smad7 interact, and ubiquitin enhances their complex. SIK also binds to ALK5 and down-regulates the receptor in a Smad7-dependent manner. This model applies to various epithelial cell models that are highly sensitive to TGF β including keratinocytes and lung cells. Our study emphasizes the role of ubiquitin and proteasomes in regulation of TGF β signaling by SIK. Our data also support a role for the kinase activity of SIK (Fig. 2 D). This offers the first evidence for a kinase/ubiquitin binding protein being involved in the down-regulation of a signaling receptor. Identifying possible SIK substrates within the ALK5–Smad7 complex and explaining their functional role in regulation of TGF β signaling will be important. We provide evidence for lysosomal degradation of TGF β receptors (Fig. 2 C). This is a relatively unexplored step during TGF β receptor internalization. TGF β receptor ubiquitination and proteasomal degradation is often reported (for review see Itoh and ten Dijke, 2007). A lysosomal pathway of receptor-ligand degradation would better fit to the classical mechanisms of receptor internalization and degradation. During this process, ubiquitin and proteasomes might act as intermediate processors of TGF β receptor trafficking from the plasma membrane to the lysosome.

Evolutionary conservation of SIK from humans to *C. elegans*, and functional conservation of SIK as a regulator of homeostatic (this study) and developmental (Maduzia et al., 2005)

Figure 5. Endogenous SIK negatively regulates endogenous TGF β signaling. (A) Immunoblot of endogenous SIK and α -tubulin loading control in HaCaT TCL after transfection with control (siLuc) and specific (siSIK) siRNAs and 12 h of stimulation with 5 ng/ml TGF β 1. The asterisk marks a nonspecific protein band. (B) Quantitative real-time RT-PCR analysis in HaCaT cells treated with siRNAs as in A and stimulated with 5 ng/ml TGF β 1 for 6 h (PAI-1 and p21) or 8 h (Smad7 and p15). Mean fold induction of TGF β -stimulated mRNA levels (black bars) relative to unstimulated levels (gray bars) are plotted, with standard errors determined from triplicate or quadruplicate samples. Asterisks indicate significant difference (t test: $P < 0.05$) in mRNA levels in cells transfected with siLuc versus siSIK siRNA and stimulated with TGF β 1. (C) Immunoblot of endogenous PAI-1, p21, and α -tubulin loading control in HaCaT cells treated as in A and stimulated with 5 ng/ml TGF β 1 for the indicated times. Black lines indicate that intervening lanes have been spliced out. (D) Immunofluorescence analysis of endogenous fibronectin in HaCaT cells treated as in A and stimulated with 5 ng/ml TGF β 1 for 12 h [bar, 10 μ m]. (E) Immunoblot of endogenous fibronectin and α -tubulin loading control in the same experimental setup as in D. Protein sizes are shown in kilodaltons. (F) Model of SIK as a negative regulator of TGF β signaling. TGF β receptors activate nuclear Smad complexes with transcription factors (TF) to mediate gene responses (green circles). Genes of the cytostatic (proliferation), differentiation, ECM, and negative-feedback programs are listed. Negative-feedback loop protein products assemble the machinery that inhibits signaling. SIK associates with the T β RI and Smad7, promoting internalization and termination of receptor signaling.



pathways guided by TGF β signaling opens new territories for exploration of such pathways across species. Characterizing the role of the *Drosophila melanogaster* SIK orthologue will greatly benefit this cause.

Because SIK is activated by the tumor suppressor kinase LKB1 (Alessi et al., 2006), our study suggests a role of LKB1

in processes of TGF β signaling and receptor down-regulation. Thus, SIK being a component of the T β RI–Smad7 complex provides a nodal point for crosstalk of the TGF β pathway with LKB1 or AMP-activated protein kinase pathways. SIK therefore opens a new window for the elucidation of the process of down-regulation of serine/threonine kinase receptors.

Table I. Human oligonucleotide primers used for RT-PCR

Gene	Primer Sequence	Strand	Product size	Temperature	PCR cycle	Reference
SIK	5'-GTCGGGGCGCACCTTAGCA-3' 5'-CAGCCTTCGCCCAGTCTT-3'	+	387	bp 62	27	NM_173354
IDB2	5'-ACGACCCGATGAGCCTGCTA-3' 5'-TCCTGGAGCGCTGGTCTG-3'	+	213	55	24	Kowanetz et al. (2004)
CDKN1A (p21)	5'-CTGCCCAGCTCACCTCC-3' 5'-CAGGTCCACATGGCTTCCT-3'	+	123	57	28	Kowanetz et al. (2004)
c-MYC	5'-ACCCGGACGACGAGACCTCATCA-3' 5'-GGGGCTGGTGCAATTGGTTGTT-3'	+	683	63	23	Kowanetz et al. (2004)
MADH7 (Smad7)	5'-CAGATCCCAACTCTCTG-3' 5'-GTGAAAGATGACCTCCAGCC-3'	+	533	53	27	Kowanetz et al. (2004)
GAPDH	5'-ATCACTGCCACCCAGAAGAC-3' 5'-ATGAGGTCCACCCACCTGTT-3'	+	443	57	22	Kowanetz et al. (2004)
		-				

A previously published reference or the NCBI database accession number is indicated.

Materials and methods

Reagents

HaCaT keratinocytes, MDA-MB-468, HEK293T and HepG2 carcinoma, COS1 and Mv1Lu cells, and culture conditions were as previously described (Kowanetz et al., 2004; Morén et al., 2005). Recombinant TGF- β 1 was obtained from PeproTech. Anti- β -tubulin (T8535) and anti-Flag (M5) antibodies were obtained from Sigma-Aldrich; anti-GFP (A11122) from Invitrogen; anti-PAI-1 and anti-p21 (Cip1/WAF1) from BD Biosciences; anti-HA (Y-11), anti-T β RI (V-22), and anti- α -tubulin (TU-02) from Santa Cruz Biotechnology, Inc.; rabbit polyclonal anti-human SIK (988, raised against peptide CEEQDTQESLPSSTGRR mapping C-terminally from the UBA domain), anti-Smad7, and mouse monoclonal anti-myc (9E10) were made in house; rabbit polyclonal anti-CIN85(CT) was from I. Dikic (Goethe University, Frankfurt, Germany); secondary antibodies coupled to HRP from GE Healthcare; secondary antibodies coupled to FITC and TRITC from Dako; Alexa Fluor 546 from Invitrogen; and AMCA7 (7-amino-4-methylcoumarin-3-acetic acid) from Jackson ImmunoResearch Laboratories.

Human SIK-specific siRNA, ON-TARGET plus SMARTpool reagent L-003959-00, and control siRNA against the luciferase reporter vector pGL2 (X65324) were from Thermo Fisher Scientific. An EST clone BG719047 of human SIK complete cDNA was obtained from H. Takemori and M. Okamoto (National Institute of Biomedical Innovation, Osaka, Japan). The cDNA was fused to a Flag, 6myc, and GFP creating pcDNA3-Flag-hSIK, pcDNA3-6myc-hSIK, and pEGFP-hSIK. The SIK ATP binding site mutant K56R was created by the Quick-Change mutagenesis kit (Stratagene). Deletion mutants SIK- Δ UBA, SIK (K56R)- Δ UBA, SIK- Δ C, and SIK- Δ N were created using a PCR-based strategy and were subcloned in pcDNA3-Flag and pEGFP. DNA constructs were sequence verified.

Vectors pcDNA3-ALK5(wt)-HA (wild-type receptor with C-terminal hemagglutinin tag), pcDNA3-ALK5(CA)-HA (CA T204D mutant receptor), pcDNA3-ALK5(KR)-HA (kinase dead ATP-binding site mutant receptor), pcDNA3-Flag-Smad7, pcDNA3-6myc-Smad7, pcDNA3-6myc-Smad7 deletion mutants (N, Linker, C), pcDNA3-HA-ubiquitin, and pcDNA3-HA-ubiquitin(K48R) and gene reporters pGL3-CAGA₁₂-luc and pCMV- β gal were previously described (Morén et al., 2005); pcDNA3-CIN85 was from I. Dikic (Goethe University, Frankfurt, Germany); pcDNA3-T β RI(wt)-Flag and pcDNA3-T β RI(wt)-Flag were from E.B. Leaf (Mayo Clinic, Rochester, MN); pSuper-Smad7 expressing a short hairpin RNA against mouse and human Smad7 mRNAs was made by ligating the Smad7-specific hairpin (5'-GATCCCCGAGGCTGTGTCTGTGAATT CAAGAGATTCACAG-CAACACAGCCTTTGGAAA-3'; underlined sequences show the hairpin stem) to pSuper (a gift from R. Agami, Netherlands Cancer Institute, Amsterdam, Netherlands; Brummelkamp et al., 2002).

Transient transfections

HaCaT cells were transfected with siRNAs via Lipofectamine 2000 (Invitrogen) or SilentFect (Bio-Rad Laboratories), COS1 and Mv1Lu cells via Lipofectamine, and HEK293T and HepG2 cells via calcium phosphate as previously described (Kowanetz et al., 2004). 24 h after transfection, cells were stimulated with TGF- β 1 as indicated in the figures.

RT-PCR

Total HaCaT or MDA-MB-468 RNA was analyzed by quantitative or semi-quantitative RT-PCR as previously described (Niimi et al., 2007). PCR primers are listed in Tables I and II, and glyceraldehyde-3'-phosphate dehydrogenase (GAPDH) was the reference gene. A two-tailed paired Student's *t* test (significance at *P* < 0.05) was used to compare TGF- β 1-inducible

Table II. Human oligonucleotide primers used for quantitative real-time RT-PCR

Gene	Primer Sequence	Strand	Product size	Temperature	Reference
SIK	5'-CAACCTGGGGCACTACGATGAGCA-3' 5'-GGCGCACTGGGCACTCCGATACT-3'	+	167	bp 60	NM_173354
CDKN1B (p15)	5'-TGGACCTGGTGGCTACGAAT-3'(+) 5'-AGGGCCTAACGGTGGTCA-3'(-)	+	150	60	Niimi et al. (2007)
CDKN1A (p21)	5'-CTGCCCAGCTCACCTCC-3'(+) 5'-CAGGTCCACATGGCTTCCT-3'(-)	+	123	60	Niimi et al. (2007)
PAI-1	5'-GAGACAGGCAAGCTCGGATT-3' 5'-GCCCTCCAAAGTGCATTAC-3'	+	101	60	ENST00000223095
MADH7 (Smad7)	5'-ACCCGATGGATTCTCAAACC-3' 5'-GCCAGATAATTGGTCCCCCT-3'	+	85	60	ENST00000262158
GAPDH	5'-GGAGTCAACGGATTGGTCGTA-3'(+) 5'-GGCAACATAATCCACTTACCAAGAGT-3'(-)	+	78	60	Niimi et al. (2007)
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A previously published reference, the NCBI database accession number, or the Ensembl database transcript (ENST) number is indicated.

levels after control or SIK-specific knockdown in triplicate (*PAI-1*, *p21*, and *p15*) or quadruplicate (*Smad7*) determinations.

Promoter-reporter assays

HepG2 cells transfected with the Smad3-responsive reporter pGL3-CAGA₁₂-luc for 36 h before stimulation with TGF- β 1 for 6 h were analyzed with the enhanced luciferase assay kit (BD Biosciences). Normalized promoter activity is plotted as mean values from triplicate determinations with standard deviations.

Immunoblotting, immunoprecipitation, and ubiquitination assays

SDS-PAGE, immunoblot, coimmunoprecipitation, pulldown, and ubiquitination analysis was as previously described (Kowanetz et al., 2004; Morén et al., 2005). For efficient detection of endogenous SIK, HaCaT cells were pretreated with 50 μ M MG132 (EMD) for 6 h at 37°C. For pulse-chase analysis, a 30 min pulse with ³⁵S-methionine/cysteine was followed by a chase, as indicated in the Figures and as previously described (Morén et al., 2005). Cells treated with proteasomal inhibitor, calpain inhibitor I (N-Acetyl-Leu-Leu-norleucinal, LLnL; Roche) or lysosomal inhibitor chloroquine (Sigma-Aldrich) were also analyzed. To measure association between SIK and ubiquitinated proteins, HEK293T cells were transfected with Flag-SIK in one plate and HA-ubiquitin in another and treated with proteasomal inhibitors, MG132, or LLnL. Individual cell lysates were combined before immunoprecipitation.

Confocal microscopy

Approximately 70% confluent transfected Mv1Lu monolayers were analyzed by immunofluorescence 24 h after transfection as previously described (Kowanetz et al., 2004). Nuclei were counterstained with DAPI or propidium iodide. A confocal microscope (Axiovert 200 M; Carl Zeiss, Inc.) equipped with LSM 510 laser was used with the 63 \times /0.75 NA objective lens (Carl Zeiss, Inc.) and photographing at ambient temperature in the presence of immersion oil. Images acquired with a charge-coupled device camera (C4742-95; Hamamatsu Photonics) and the acquisition software QED Camera Plug-in v.1.1.6 (QED Imaging Inc.) were reduced in memory content using Photoshop 6.0 (Adobe), without additional processing.

Receptor affinity cross-linking

Mv1Lu cells incubated on ice with iodinated TGF- β 1 were cross-linked, as previously described (Moustakas et al., 1993), and shifted to 37°C, as indicated in the figure, before SDS-PAGE and autoradiography.

Online supplemental material

Fig. S1 shows endogenous SIK protein levels in response to TGF β 1 and the lack of effect of SIK expression on GFP or Smad7 levels. Fig. S2 shows interaction of SIK with ubiquitinated proteins and interaction between SIK and ALK5 in presence of ubiquitin. Fig. S3 shows mRNA profiles of *Smad7*, *p21*, *c-myc*, and *Id2* during a time course in response to TGF β 1 in control cells or after SIK knockdown. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200804107/DC1>.

We thank R. Agami, I. Dikic, E.B. Leof, M. Okamoto and H. Takemori for valuable reagents and A. Morén and U. Valcourt for assistance and suggestions.

The work was supported by the Ludwig Institute for Cancer Research, the Swedish Cancer Society (project number 4855-B03-01XAC), the Natural Sciences Foundation of Sweden (project number K2004-32XD-14936-01A), and the Strategic Japanese-Swedish Cooperative Program "Multidisciplinary BIO" (project number 26575-1) supported by the Swedish Agency for Innovation Systems and the Swedish Foundation for Strategic Research.

The authors declare no competing interests.

Submitted: 18 April 2008

Accepted: 25 July 2008

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