

Review

The Role of PRAJA and ELF in TGF- β Signaling and Gastric Cancer

Lopa Mishra^{1, 2,*}

Varalakshmi Katuri¹

Stephen Evans³

¹Laboratory of GI Developmental Biology; Department of Surgery, Medicine and The Lombardi Comprehensive Cancer Center; Georgetown University; Washington, DC USA

²Veterans Affairs Medical Center; Washington, DC USA

³Departments of Surgery; Georgetown University; Washington, DC USA

*Correspondence to: Dr. Lopa Mishra; Georgetown University; Med/Dent Bldg, NW 212; 3900 Reservoir Rd, NW; Washington DC 20007 USA; Tel.: 202.687.5707; Fax: 202.687.0992; Email: lm229@georgetown.edu

Received 07/11/05; Accepted 07/13/05

Previously published online as a *Cancer Biology & Therapy* E-publication: <http://www.landesbioscience.com/journals/cbt/abstract.php?id=2015>

KEY WORDS

ELF, PRAJA, TGF- β , ubiquitination, gastric cancer

ACKNOWLEDGEMENTS

This work was supported by Sally Funderberg award, NIH grants RO1 DK56111 (L.M.), RO1 CA106614 (L.M.), and VA Merit Award (L.M.) The authors wish to thank Tiffany Blake and Vidhya Murugesan for critical review of the manuscript.

ABSTRACT

Emerging research has shown that the transforming growth factor-beta (TGF- β) pathway plays a key role in the suppression of gastric carcinoma. Biological signals for TGF- β are transduced through transmembrane serine/threonine kinase receptors, which in turn signal to Smad proteins. Inactivation of the TGF- β pathway often occurs in malignancies of the gastrointestinal system, including gastric cancer. Yet, only a fraction of sporadic gastric tumors exhibit inactivating mutations in early stages of cancer formation, suggesting that other mechanisms play a critical role in the inactivation of this pathway. Smad4, a tumor suppressor, is often mutated in human gastrointestinal cancers. The mechanism of Smad4 inactivation, however, remains uncertain and could be mediated through E3-mediated ubiquitination of Smad4/adaptor protein complexes. The regulation of the TGF- β pathway through a PRAJA, a RING finger (RING-H2) protein, and ELF, a β -Spectrin adaptor protein, both which were originally identified in endodermal stem/progenitor cells committed to foregut lineage, could play a pivotal role in gastric carcinogenesis. PRAJA, which functions as an E3 ligase, interacts with ELF in a TGF- β -dependent manner in gastric cancer cell lines. PRAJA is increased five-fold in human gastric cancers, and inactivates ELF. This is particularly significant since ELF, a Smad4 adaptor protein, possesses potent anti-oncogenic activity and is frequently seen to be inactivated in carcinogenic gastric cells. Strikingly, PRAJA manifests substantial E3-dependent ubiquitination of ELF and Smad3, but not Smad4. The alteration of ELF and/or Smad4 expression and function in the TGF- β signaling pathway may be induced by enhancement of ELF degradation, which is mediated by the high level expression of PRAJA in gastrointestinal cancers. These studies reveal a mechanism for gastric tumorigenesis whereby defects in adaptor proteins for Smads, such as ELF, can undergo degradation by PRAJA, through the ubiquitin-mediated pathway.

Gastric cancer remains the second most common cancer worldwide¹⁻³ and the five-year survival is less than 20%.¹ Most cases are detected in advanced stages, which contribute to these low survival rates. However, the factors that govern progression from gastric epithelial cell hyperplasia through dysplasia to in situ carcinoma and invasive disease are poorly understood and possibly involve multiple molecules, including E-cadherin, CD44, c-erbB2, p53, tie-1, c-met, MKK4, k-sam, and more recently, Smad4.¹⁻⁵ Only a fraction of sporadic gastric tumors exhibit inactivating mutations in the early stages of cancer formation, suggesting that other mechanisms play a critical role in the inactivation of this pathway.⁶ Allelic loss of chromosome 18q has been noted in intestinal-type gastric adenocarcinomas, and the inactivation of Smad4, a tumor suppressor gene located in chromosome 18q21.1, appears to play a significant role in gastric tumorigenesis.^{7,8} Microsatellite instability (MIS) and associated modification of the TGF- β Receptor II, IGFR II, BAX, E2F-4, hMSH3, and hMDH6 genes are found in a subset of gastric carcinomas.⁹ Importantly, recent studies elucidating the role of TGF- β are now shedding light on the molecular pathogenesis of gastric cancer. TGF- β is a multifunctional growth factor that has significant regulatory effects and seems to play a role in regulation of gut development. It is noted that in TGF- β 1 null animals, there is stimulation of gastric epithelial proliferation leading to epithelial hyperplasia.¹⁰ Many of the effects of TGF- β are mediated by physical interactions of the Smad proteins with various transcription factors such as RUNX (Fig. 1).

Most importantly, recent studies by have firmly established a mouse model for gastric cancer in the Smad4 heterozygous mice allowing for the analysis of the TGF- β signaling pathway in gastric carcinogenesis. Smad4 heterozygous mice develop cancers in the gastric fundus and antrum when they are over 11 months old, and in the duodenum and cecum in older animals at a lower frequency.^{8,11,12} With increasing age, polyps in the antrum show sequential changes from hyperplasia to dysplasia, carcinoma in situ, and finally invasion. These alterations initiate a dramatic expansion of the gastric epithelium where Smad4 is expressed. Loss of the remaining Smad4 wild type allele is detected only in later stages

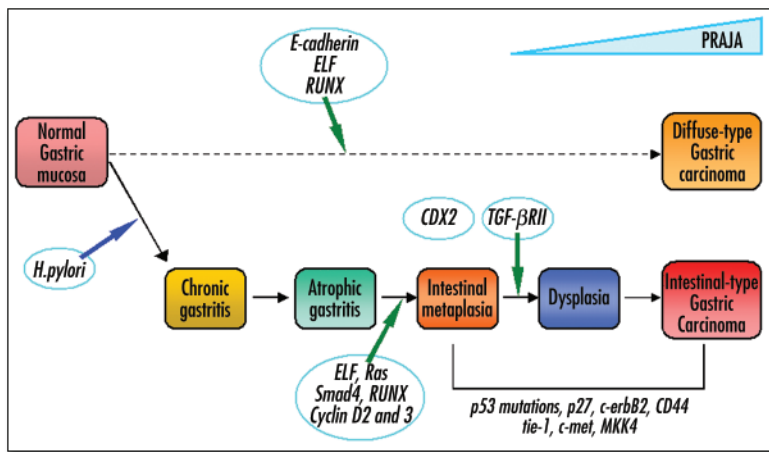


Figure 1. Schematic diagram shows progression of gastric adenocarcinoma. Gastric cancer has been histologically classified into two main types, intestinal and diffuse. Defects in E-cadherin ELF, RUNX function are specifically associated with diffuse-type gastric cancer, which probably develops through a shorter, unidentified sequence of events from gastric epithelial cells. On the other hand, the carcinogenic pathway of intestinal-type gastric carcinomas seems to be more complicated, and involves well-characterized sequential stages. *H. pylori* infection induces a transition from normal mucosa to chronic superficial gastritis, which then leads to atrophic gastritis. As a consequence of inflammation and regeneration, the gastric mucosa can undergo intestinal metaplasia dysplasia and eventually gastric adenocarcinoma. ELF, Ras, Smad4, RUNX and CDX2, etc., are one of the most likely candidates linked with the induction of intestinal metaplasia. p53, p27, c-erb2, etc., alterations could be involved in development of both intestinal- and diffuse-type gastric cancer.

of tumor progression, suggesting that haploinsufficiency of Smad4 is sufficient for tumor formation. Smad 2, 3, 4 and adaptor proteins such as ELF, a β -Spectrin adaptor protein, are crucial for gastrointestinal development.^{13,14} Most interestingly 90% (18/20) of *elf^{+/+}/Smad4^{+/-}* heterozygous mutants develop an exacerbated phenotype of earlier gastric hyperplasia, ectasia, foveolar gland dysplasia, and hamartomas with obstructing tumors at the antrum and pylorus. In patients with TGF- β 1 positive tumors, survival rate was significantly better in patients with preserved Smad4 expression, than those with reduced expression. As a correlary, it appears that Smad4 expression, particularly in the TGF- β pathway, is a sensitive predictor of outcome for patients with advanced gastric cancer.¹¹ Additional studies have demonstrated that Smad4 functions as a tumor suppressor on the gastrointestinal tract and similar studies have shown that Smad3 expression may have a critical role in tumor suppression in the early stages of gastric carcinogenesis.^{15,16} Hence, gene dosage is critically important in this protein family as haploinsufficiency phenotypes result in gastric cancer, holoprosencephaly, liver hypoplasia as well as colon carcinoma.^{13,17-20} Importantly, other mechanisms, such as targeted protein proteolysis, have now been shown to play an important role in the regulation of protein levels.²¹

UBIQUITINATION IN TGF- β , E3 LIGASES

Biological functions of many proteins are altered by their covalent attachment to polypeptide modifiers inducing targeted protein proteolysis.²² The best-known example of this type of modification is ubiquitination.²³ Ubiquitin is an abundant 76-amino acid polypeptide that can be covalently conjugated to specific proteins by the formation of an isopeptide bond between its carboxyl terminus

and the amino group of a lysine residue of the target protein.^{24,25} Ubiquitin-dependent protein degradation is involved in the regulation of various cellular processes, including cell cycle progression, signal transduction, transcription, DNA repair, and protein quality control.²⁶⁻²⁸ Recent studies have revealed the ability of Smads to interact with multiple components of the 26S proteasome system before and after Smad activation.²⁹

The selectivity of ubiquitination is largely mediated by the recognition of substrates by E3 ligases—posttranslational modifications, such as phosphorylation of the substrate, can regulate this interaction. Despite the large number of substrates, relatively few E3s have been characterized on a molecular level.³⁰ E3s with known amino acid sequences include the N-end rule E3s of yeast and mammals and members of the HECT (homologous to E6-AP C terminus) family.³¹ Mammalian HECT E3s include E6 AP, which targets p53 for ubiquitination in the presence of human papillomavirus E6,³² and Nedd4, which ubiquitinates epithelial sodium channel subunits.^{33,34} Other E3s include Mdm2, which catalyzes both its own ubiquitination and that of p53,^{26,35} the anaphase-promoting complex (APC); and other F box and cullin-containing complexes whose substrates include Sic1p, G₁ cyclins, inhibitor of nuclear factor κ B (I κ B), and β -catenin.²⁷ Interestingly, PRAJA may play a pivotal role in TGF- β tumor suppression of gastric cancer. RING E3s, which mediate ubiquitination, represent the largest E3 family known to date and include the c-Cbl proto-oncogene product, the multisubunit SCF and APC cell cycle-regulatory complexes, the Mdm2 proto-oncogene product that regulates the p53 tumor suppressor, BRCA1, and members of the IAP family of antiapoptotic proteins.³⁶

PRAJA

PRAJA, a RING finger (RING-H2) protein first isolated from foregut endodermal stem/progenitor cells, plays a vital role in various cell processes including foregut lineage maintenance and TGF- β /BMP regulated function as an E3 ligase. Praja open reading frame (ORF) comprises 1188 bases encoding a 395 amino acid proteins of ~60 kD, and is involved in cell proliferation, apoptosis, juxtaposition, and architecture (also in Sanskrit, birth, development and offspring).³⁷ PRAJA exhibits extensive homology to RING finger proteins at the C terminus end between residues 347 and 673.³⁷ Computer analysis of this domain demonstrates that it contains a perfect C₃H₂C₃ Zn binding motif or RING-H2 finger found in many regulatory proteins, as well as several human proto-oncogene products such as promyelocytic leukemia protein (PML), ret finger protein (RFP), BRCA1 and inhibitors of apoptosis (IAP).³⁸ RINGs, defined by eight cysteines and histidines that coordinate two zinc ions, vary substantially in length and composition. RINGs have cysteines in the first three and last three coordination sites and a His in the fourth site. Additionally, proteins bearing this motif have either a Cys [C₃HC₄ RING (RING-HC)] or a His [C₃H₂C₃ RING (RING H2)] in the fifth position. The first, second, fifth, and sixth cysteines/histidines coordinate one cation, and the third, fourth, seventh, and eighth coordinate the second.³⁹ As determined with consensus sequences, PRAJA falls into the RING H2 category, with the amino acids indicated predicted to be coordination sites.

The RING finger protein PRAJA is regulated by TGF- β and facilitates the ubiquitination of ELF, a tumor suppressor adaptor protein involved in TGF- β signaling. In addition, it is possible that, in vivo, changes in conformation, alterations induced by phosphorylation, and changes in intermolecular associations such as dimerization, may influence the capacity of RING finger proteins to function with E2s in ubiquitination. Recent studies also show that PRAJA ubiquitinates BMP regulated proteins, Dlxin-1 and Msx2, and thus regulates the transcriptional function of homeodomain protein DLX5 through Dlxin-1 via an ubiquitin-dependent degradation pathway.⁴⁰ Importantly, this observation confirms the role of PRAJA in the TGF- β /BMP pathway. Adhering to these attributes, the compact RING module of PRAJA could confer an additional regulatory role on a wide array of TGF- β signaling proteins that associate with it and may have differential effects in determining ELF fate.

The importance of RING finger domain in ubiquitination is further supported by the fact that in vivo assays with the RING finger mutant PRAJA (Δ -PRAJA) abolish ubiquitination of ELF. RING mutations of BRCA1 are associated with familial carcinomas. Strikingly, PRAJA manifests substantial E3-dependent ubiquitination of Smad4 adaptor protein ELF, but not Smad4, while still exhibiting ubiquitinated Smad3 conjugates. This finding suggests a role for PRAJA in the membrane localization of the ELF and the Smad signaling complex. Furthermore, in the absence of a substrate, PRAJA has the capacity to ubiquitinate itself. This ability is consistent with recent observations that multiple, otherwise unrelated RING finger proteins, including Mdm2, AO7, Siah1 and kf 1, have the inherent capacity to ubiquitinate themselves.⁴¹

ROLE OF ELF IN TGF- β SIGNALING

ELF was originally identified as a key protein involved in endodermal stem/progenitor cells committed to foregut lineage.^{42,43} Furthermore, ELF, as a β -Spectrin and a major dynamic scaffolding protein, is important for the generation of functionally distinct membranes, protein sorting, cell adhesion and the development of a polarized differentiated epithelial cell. As an adaptor protein, ELF also plays a key role in the transmission of TGF- β mediated transcriptional response through Smads. *Elf*^{-/-} deficient mice have disrupted TGF- β signaling because of an alteration of ELF interactions with Smad3 and Smad4.¹³ Immunohistochemical labeling for ELF in normal gastric tissues of wild-type mice shows strong expression in epithelial cells: in adult stomach, ELF expression is greater in the stem cell zone that gives rise to parietal cells and also more prominent in surface mucous cells than in chief cells.

An examination of *Elf*^{-/-} mice for tumor development reveals that 40.0% *Elf*^{-/-} (8/20) developed tumors of varying etiology.⁶ This tumor incidence is comparable to that seen in the *Smad4*^{+/-} mice (45%, 9/20). Most interestingly, 90% (18/20) of the *Elf*^{-/-}/*Smad4*^{+/-} heterozygous mutants develop an exacerbated phenotype of earlier gastric hyperplasia, ectasia, foveolar gland dysplasia, hamartomas with obstructing tumors at the antrum and pylorus. Abnormal mitosis, apoptosis and glandular dilatation are seen in the polyps and hamartomas, suggesting a cooperative interaction between ELF and Smad4, which leads to enhanced tumorigenesis. The gastric mucosa of these double heterozygotes is three to four times as thick as that of wild-type mucosa, and was also two to three times thicker than the *Elf*^{-/-} or *Smad4*^{+/-} mutant antral mucosa. This suggests that disruption of *Elf*, in addition to disruption of *Smad4*, results in hyperplasia of the gastric mucosa, suggesting that gastric epithelial

cell proliferation was stimulated by the disruption of *Elf*. Loss of response to TGF- β signaling alters apoptosis in many cells including gastric epithelial cells. In mouse models, suppressed apoptosis indicates that *Elf* may be important in TGF- β induction of apoptosis in gastric epithelial cells and that it may contribute to the epithelial cell hyperplasia in the *Elf*^{-/-}/*Smad4*^{+/-} glandular stomach. Little or no ELF expression is detected in *Smad4*^{+/-} and *Elf*^{-/-}/*Smad4*^{+/-} mutant tumor tissues with the decreased levels of Smad4 when compare to the control.

Paradoxically, the TGF- β pathway activity is also associated with increased oncogenicity in advanced human tumors, promoting invasion and motility, as well as indirect effects on angiogenesis and immune surveillance.^{44,45} For instance, TGF- β mediated repression of E-cadherin with loss of E-cadherin expression results in the translocation of β -catenin from cell-cell contacts to the cytoplasm and the induction of epithelial-mesenchymal transitions, leading to an invasive phenotype.⁴⁶ On the other hand, Smad4-induces E-cadherin with recruitment of catenins to the plasma membrane.⁴⁷ Aberrant distribution of non-erythroid β -Spectrins, in association with loss of membranous E-cadherin, has been described in high-grade carcinomas with poor prognosis.⁴⁸ Genetic alterations leading to a loss of genes encoding E-cadherin¹, as well as silencing of RUNX,⁴⁹ have also been demonstrated to be of key importance in the suppression of gastric cancers. However, a majority of sporadic cancers do not display E-cadherin mutations or epigenetic manifestations of either E-cadherin or RUNX.^{50,51} Runt domain transcription factors are also important targets of TGF- β super family signaling and appear to play an important role in mammalian development. RUNX3 has been intensively studied as a runt transcription factor and it has been shown that the tumorigenicity of human gastric cancer cell lines is inversely related to their level of RUNX3 expression suggesting that this transcriptional factor may be related to the genesis and progression of early human gastric cancer.⁵² RUNX proteins themselves form complexes with Smad2 and Smad3 that then transmit TGF- β /activin signals.⁵³

Mouse models demonstrate that the TGF- β signaling pathway is significant in the formation of gastrointestinal cancers. Gastric cells with intact TGF- β 1 signaling molecules show ELF-Smad3-Smad4 interactions upon TGF- β 1 stimulation. Interestingly, ELF-deficient gastric cancer cells do not respond to TGF- β 1 stimulation, suggesting that ELF plays a critical role in gastric cancer suppression. Importantly, loss of ELF results in aberrant localization of Smad3, Smad4 and E-cadherin in gastric cancer cells. Our studies show that tumors from *Elf*^{-/-} and *Elf*^{-/-}/*Smad4*^{+/-} mutant mice reveal dramatic changes in the distribution of E-cadherin in gastric cells. In normal control cells, E-cadherin is expressed at cell-cell contact sites. In contrast, *Elf* mutants as well as the ELF-deficient human gastric cancer cells exhibit abnormal E-cadherin expression and also E-cadherin- β -catenin dependent epithelial cell-cell adhesion is disrupted in *Elf*^{-/-}/*Smad4*^{+/-} mutant gastric epithelial cells. ELF plays critical role in cell-cell adhesion which is mediated by TGF- β signaling. A full length ELF cDNA clone was constructed that encoded the N-terminal actin and membrane binding domain; as well as the C-terminal domain that includes the ankyrin binding region; active phosphorylation sites at serine residues; and a hinge region regulating oligomer formation.^{11,42,43,54} Transient transfection of full length *Elf* restored E-cadherin expression in ELF mutants was observed, as well as in ELF-deficient human gastric cancer cell lines (HS746T), but not Smad3 or Smad4.⁵⁵

ELF binding to Smad4 is observed upon TGF- β 1 stimulation, thus raising the possibility that ELF is a co-adaptor protein involved

in Smad4 localization and activation. α - and β -Spectrins have been found in multiple vertebrate tissues such as polarized epithelial cells in the gut and kidney.⁵⁶ ELF plays critical role in Smad4 localization upon TGF- β stimulation, which suggests that these molecules interact and translocate to the cell nucleus. Interestingly, spectrins have been found to be expressed in nuclei and bear a carboxy-terminus bipartite nuclear localizing signal.⁵⁷ Therefore, it is conceivable that ELF binds to Smad4 in the cytoplasm upon TGF- β 1 activation, and assists in transporting the protein into the nucleus.

Modulation of adaptor proteins through degradation could thus result in escape from TGF- β growth suppression. Recently, disruption of the adaptor function of ELF has been shown to lead to tumorigenesis. Like *Smad4*^{+/-} mice, *elf*^{+/-} heterozygotes develop normally without apparent defects. An examination of *elf*^{+/-} and *elf*^{+/-}/*Smad4*^{+/-} mice for tumor development revealed that 40% *elf*^{+/-} (8/20) mice developed tumors of varying etiology. This tumor incidence was comparable to that seen in the *Smad4*^{+/-} mice (45%, 9/20). Most interestingly, 90% (18/20) of *elf*^{+/-}/*Smad4*^{+/-} heterozygous mutants developed an exacerbated phenotype of earlier gastric hyperplasia, ectasia, foveolar gland dysplasia, hamartomas with obstructing tumors at the antrum and pylorus. Regulation of ELF abundance therefore is likely to be important in tumorigenesis. Yet, while these studies highlight the critical role for adaptor proteins such as ELF in this signaling pathway, mutational inactivation of this pathway in gastric cancers is uncommon suggesting that other mechanisms, such as protein modification of Smads, ELF, and SARA through ubiquitination, are responsible for gastric tumorigenesis. A similar model for PRAJA modulation of ELF/Smads is shown in Figure 2. Smad proteins are globular proteins with two conserved domains, the Mad Homology 1 (MH1) and MH2 domains, separated by a middle linker region. Recent studies have also demonstrated that most of the tumor derived mutations map to the MH2 domain, which is involved in receptor recognition, interactions with transcription factors and homo and hetero-oligomerization among Smads. The MH2 domain of Smad2 or Smad3 is also crucial for interacting with an 85 residue Smad-binding domain (SBD) in SARA.⁵⁸ It will be interesting to determine the specific domains of ELF that interact with the Smad, MH1 or MH2 domains. Smad interactions will be pivotal for future development of therapeutic drugs aimed at enhancing ELF-Smad interactions, potentially suppressing poor prognostic cancers such as gastric carcinoma.

PRAJA-ELF INTERACTIONS IN TGF- β SIGNALING AND GASTRIC CANCER

Mechanisms such as targeted protein proteolysis through ubiquitination are now considered to play a major role in regulating protein levels in TGF- β signaling and disease.^{21,37,41,59,60} Most interestingly, our analysis has shown that PRAJA, a RING-H2 E3 ligase targeting ELF ubiquitination, likely represents a major mechanism for controlling the ELF/Smad cellular response to TGF- β signaling. This modulation of ELF/Smad interactions adds a further dimension to gastric cancer formation.

PRAJA, which is over expressed in human gastric cancers, ubiquitinates ELF and Smad3 but not Smad4 in a TGF- β dependent manner. PRAJA E3 ligase activity regulates TGF- β signaling by controlling ELF abundance through ubiquitin mediated degradation. Inhibition of the antiproliferative effect of TGF- β is often associated with progression of tumorigenesis in human cancers.⁶¹ Thus PRAJA represents one such mechanism for the TGF- β inactivation of gastric

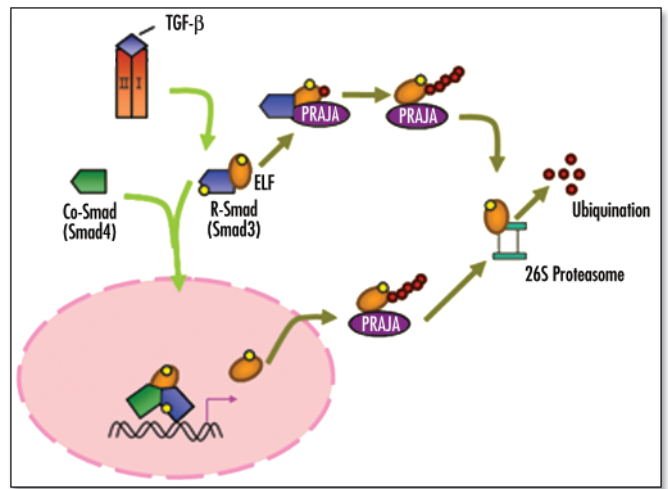


Figure 2. A hypothetical schematic diagram showing PRAJA and ELF association. After activation of TGF- β Type II and Type I receptor, TGF- β stimulates ELF/Smad3 association in a phosphorylation dependent manner, followed by association with PRAJA. Ubiquitinated ELF is then targeted by the 26S proteasome. Arrows indicate the signal flow and are color coded light green for Smad activation and formation of a transcriptional complex, olive green for Ubiquitination of ELF through PRAJA. The yellow circles indicate activated phosphate groups and the red circles indicate ubiquitin molecules.

carcinogenesis. The importance of RING finger domain in ubiquitination is further supported by the fact that in vivo assays with the RING finger mutant PRAJA (Δ -PRAJA) abolish ubiquitination of ELF. Strikingly, PRAJA manifests substantial E3-dependent ubiquitination of ELF, but not Smad4, while still exhibiting ubiquitinated Smad3 conjugates. This finding suggests a role for PRAJA in the membrane localization of the ELF and the Smad signaling complex thus potentially controlling ELF/Smad activation by the TGF- β Type I receptor. Furthermore, it is observed that in the absence of a substrate, PRAJA has the capacity to ubiquitinate itself. This ability is consistent with recent observations that multiple, otherwise unrelated RING finger proteins, including Mdm2, AO7, Siah1 and klf 1, have the inherent capacity to ubiquitinate themselves.⁴¹ RING mutations of BRCA1 are associated with familial carcinomas.⁶² A potential model for RING-mediated E3 activity is one in which the RING and surrounding regions not only associate with E2-ubiquitin but also provide a favorable environment for the transfer of ubiquitin from E2 to an available lysine. Such a mechanism is analogous to the mechanism in models for the function of N end rule E3s.⁶³

Polyubiquitination thus involves the 26S proteasome, which is composed of two major components, the 19S cap and the 20S core protein complexes.⁶⁴ The 19S complex is involved in the recognition of polyubiquitinated substrates, assists in the unfolding of the target protein for entry into the opening of the cylinder, and cleaves off the polyubiquitinated chain from the substrates. Only small peptides and unfolded elongated proteins then enter into the openings in the cylindrical 20S core complex, where proteolytic activities reside within the hollow transverse cavity.⁶⁴ A group of enzymes known as deubiquitinating enzymes or ubiquitin-specific peptidases can also be involved in modulating the extent of ubiquitination by removing ubiquitin moieties.⁶⁵ These enzymes also serve to generate mature monoubiquitin from preubiquitin forms, to break down polyubiquitin chains liberated by the proteasome, and regenerate free ubiquitin that can readily form adducts with abundant intracellular nucleophiles

such as amines and glutathione. PRAJA, like Cbl and Mdm2, may function as a RING E3 ligase involved in both mono- and poly-ubiquitination of ELF, and monoubiquitination of Smad3.

These findings suggest that PRAJA could be a key mediator of ELF and Smad3 degradation and that endogenous levels of ELF are dynamically regulated through a possible ELF PRAJA feedback loop via the ubiquitin-mediated proteasomal pathway (Figure 2). The differential activity of PRAJA, both as an essential regulatory protein in hepatocyte development and as an ubiquitinase of ELF, an important tumor suppressor adaptor protein, inhibits apoptosis, and is possibly dictated by its own intracellular concentration. The hypothesis thus is that low levels of PRAJA maintained in wild-type gastric cells are likely to play important regulatory roles for normal development. However, when PRAJA activities are high, PRAJA-mediated ubiquitination induces ELF degradation, abrogating the ELF Smad TGF- β signaling pathway, thereby leading to tumorigenesis (Fig. 2).

Together, these studies suggest a model in which PRAJA interactions with ELF, Smad3 and Smad4 are crucial for gastro-intestinal epithelial cell growth and differentiation. A model representing PRAJA as a potential regulatory protein in TGF- β signaling pathway is shown in Figure 2. In brief, we propose that, upon TGF- β induction, receptor I (I) is activated by receptor II (II). This is followed by ELF phosphorylation and subsequently the complex is formed between ELF and Smad3. Thereafter PRAJA emerges as an E3 ubiquitin ligase that attaches ubiquitin molecules to ELF and localizes the complex towards the plasma membrane (Figure 2). At the same time ubiquitin chains elongate and ELF becomes displaced from the signaling pathway and then becomes a target for 26S proteasome and is destroyed. In normal circumstances, Smad4, Smad3 and phosphorylated ELF form a complex that maintains the signaling pathway for the normal growth of the cells. Indeed, disruption of this complex within TGF- β signaling is maybe a common cause of cancer formations in the gastrointestinal tract, particularly in foregut organs such as the stomach, liver and pancreas.

Multiple other cancers derived from meso-endodermally derived epithelium are associated with TGF- β /BMP pathway inactivation, where it may regulate progenitor cell fate.^{13,18,66,67} Indeed, the functions of TGF- β are complex and extend beyond their role in the inhibition of cell growth. TGF- β also induces the growth of mesenchymal cells, alters synthesis of extracellular matrix components, and metalloproteases, which is involved in cell invasion.^{13,44,59,67-69} TGF- β signals modulate the immune response to tumors and are thought to play a role in tumor angiogenesis.⁷⁰ The development of gastric tumors in *elf^{fl/fl}/Smad4^{+/-}* mutants indicates a crucial role for ELF, a β -spectrin, which acts as an essential adaptor protein for the proper transmission of signals generated by the TGF- β pathway. Research has clearly indicated that loss of expression of ELF through PRAJA could play an important role in the development of gastric tumors and that further exploration of this interaction could hold the key to unlocking the mechanisms of one of the most lethal forms of cancer.

References

- Guilford P, Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, Reeve AE. E-cadherin germline mutations in familial gastric cancer. *Nature* 1998; 392:402-5.
- Powell SM, Harper JC, Hamilton SR, Robinson CR, Cummings OW. Inactivation of *smad4* in gastric carcinomas. *Cancer Res* 1997; 57:4221-4.
- Ross JS, McKenna BJ. The *her-2/neu* oncogene in tumors of the gastrointestinal tract. *Cancer Invest* 2001; 19:554-68.
- Yokozaki H. Molecular characteristics of eight gastric cancer cell lines established in japan. *Pathol Int* 2000; 50:767-77.

- Lin W, Kao HW, Robinson D, Kung HJ, Wu CW, Chen HC. Tyrosine kinases and gastric cancer. *Oncogene* 2000; 19:5680-9.
- Katuri V TY, Culling L, Jogunoori W, Deng CX, Rashid A, Sidawy A, Evans S, Mishra B, Mishra L. Critical interactions between *tgf-b* signaling/elf, and e-cadherin/ b-catenin mediated tumor suppression. *Oncogene* 2005; Accepted Pending Revision.
- Takaku K, Oshima M, Miyoshi H, Matsui M, Seldin ME, Taketo MM. Intestinal tumorigenesis in compound mutant mice of both *dpc4* (*smad4*) and *apc* genes. *Cell* 1998; 92:645-56.
- Xu X, Brodie SG, Yang X, Im YH, Parks WT, Chen L, Zhou YX, Weinstein M, Kim SJ, Deng CX. Haploid loss of the tumor suppressor *smad4/dpc4* initiates gastric polyposis and cancer in mice. *Oncogene* 2000; 19:1868-74.
- El-Rifai W, Powell SM. Molecular biology of gastric cancer. *Semin Radiat Oncol* 2002; 12:128-40.
- Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SM, Lawler J, Hynes RO, Boivin GP, Bouck N. Thrombospondin-1 is a major activator of *tgf-beta1* in vivo. *Cell* 1998; 93:1159-70.
- Xiangming C, Natsugoe S, Takao S, Hokita S, Ishigami S, Tanabe G, Baba M, Kuroshima K, Aikou T. Preserved *smad4* expression in the transforming growth factor beta signaling pathway is a favorable prognostic factor in patients with advanced gastric cancer. *Clin Cancer Res* 2001; 7:277-82.
- Taketo MM, Takaku K. Gastro-intestinal tumorigenesis in *smad4* mutant mice. *Cytokine Growth Factor Rev* 2000; 11:147-57.
- Tang Y, Katuri V, Dillner A, Mishra B, Deng CX, Mishra L. Disruption of transforming growth factor-beta signaling in *elf* beta-spectrin-deficient mice. *Science* 2003; 299:574-7.
- Weinstein M, Monga SP, Liu Y, Brodie SG, Tang Y, Li C, Mishra L, Deng CX. Smad proteins and hepatocyte growth factor control parallel regulatory pathways that converge on beta1-integrin to promote normal liver development. *Mol Cell Biol* 2001; 21:5122-31.
- Han SU, Kim HT, Seong do H, Kim YS, Park YS, Bang YJ, Yang HK, Kim SJ. Loss of the *smad3* expression increases susceptibility to tumorigenicity in human gastric cancer. *Oncogene* 2004; 23:1333-41.
- Lin W, Kao HW, Robinson D, Kung HJ, Wu CW, Chen HC. Tyrosine kinases and gastric cancer. *Oncogene* 2000; 19:5680-9.
- Shi Y, Massague J. Mechanisms of *tgf-beta* signaling from cell membrane to the nucleus. *Cell* 2003; 113:685.
- Siegel PM, Massague J. Cytostatic and apoptotic actions of *tgf-beta* in homeostasis and cancer. *Nat Rev Cancer* 2003; 3:807-21.
- Weinstein M, Yang X, Deng C. Functions of mammalian *smad* genes as revealed by targeted gene disruption in mice. *Cytokine Growth Factor Rev* 2000; 11:49-58.
- Tang B, Vu M, Booker T, Santner SJ, Miller FR, Anver MR, Wakefield LM. Tgf-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest* 2003; 112:1116-24.
- Xu J, Attisano L. Mutations in the tumor suppressors *smad2* and *smad4* inactivate transforming growth factor beta signaling by targeting smads to the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A* 2000; 97:4820-5.
- Schwartz DC, Hochstrasser M. A superfamily of protein tags: Ubiquitin, sumo and related modifiers. *Trends Biochem Sci* 2003; 28:321-8.
- Li X, Makela S, Streng T, Santti R, Poutanen M. Phenotype characteristics of transgenic male mice expressing human aromatase under ubiquitin c promoter. *J Steroid Biochem Mol Biol* 2003; 86:469-76.
- Janse DM, Crosas B, Finley D, Church GM. Localization to the proteasome is sufficient for degradation. *J Biol Chem* 2004; 279:21415-20. Epub 2004 Mar 23.
- Wang X, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M. The origin and liver repopulating capacity of murine oval cells. *Proc Natl Acad Sci USA* 2003; 100:11881-8.
- Koepp DM, Harper JW, Elledge SJ. How the cyclin became a cyclin: Regulated proteolysis in the cell cycle. *Cell* 1999; 97:431-4.
- Laney JD, Hochstrasser M. Substrate targeting in the ubiquitin system. *Cell* 1999; 97:427-30.
- Hicke L, Dunn R. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol* 2003; 19:141-72.
- Izzi L, Attisano L. Regulation of the *tgfbeta* signalling pathway by ubiquitin-mediated degradation. *Oncogene* 2004; 23:2071-8.
- Staub O, Dho S, Henry P, Correa J, Ishikawa T, McGlade J, Rotin D. Ww domains of *nedd4* bind to the proline-rich py motifs in the epithelial *na+* channel deleted in liddle's syndrome. *EMBO J* 1996; 15:2371-80.
- Dinudom A, Harvey KE, Komwatana P, Young JA, Kumar S, Cook DI. *Nedd4* mediates control of an epithelial *na+* channel in salivary duct cells by cytosolic *na+*. *Proc Natl Acad Sci USA* 1998; 95:7169-73.
- Honda R, Yasuda H. Association of p19(*arf*) with *mdm2* inhibits ubiquitin ligase activity of *mdm2* for tumor suppressor p53. *EMBO J* 1999; 18:22-7.
- Liu W, Sato Y, Hosoda Y, Hirasawa K, Hanai H. Effects of higenamine on regulation of ion transport in guinea pig distal colon. *Jpn J Pharmacol* 2000; 84:244-51.
- Zhu Y, Paszty C, Turetsky T, Tsai S, Kuypers FA, Lee G, Cooper P, Gallagher PG, Stevens ME, Rubin E, Mohandas N, Mentzer WC. Stomatocytosis is absent in "stomatatin"-deficient murine red blood cells. *Blood* 1999; 93:2404-10.
- Bonni S, Wang HR, Causing CG, Kavsak P, Stroschein SL, Luo K, Wrana JL. Tgf-beta induces assembly of a *smad2-smurf2* ubiquitin ligase complex that targets *snor* for degradation. *Nat Cell Biol* 2001; 3:587-95.
- Sun Y. Targeting e3 ubiquitin ligases for cancer therapy. *Cancer Biol Ther* 2003; 2:623-9.

37. Mishra L, Tully RE, Monga SP, Yu P, Cai T, Makalowski W, Mezey E, Pavan WJ, Mishra B, Praja1, a novel gene encoding a ring-h2 motif in mouse development. *Oncogene* 1997; 15:2361-8.
38. Schwede T, Kopp J, Guex N, Peitsch MC. Swiss-model: An automated protein homology-modeling server. *Nucleic Acids Res* 2003; 31:3381-5.
39. Saurin AJ, Borden KL, Boddy MN, Freemont PS. Does this have a familiar ring? *Trends Biochem Sci* 1996; 21:208-14.
40. Sasaki A, Masuda Y, Iwai K, Ikeda K, Watanabe K. A ring finger protein praja1 regulates dlx5-dependent transcription through its ubiquitin ligase activity for the dlx/msx-interacting mage/necdin family protein, dlx1-1. *J Biol Chem* 2002; 277:22541-6. Epub 2002 Apr 16.
41. Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM. Ring fingers mediate ubiquitin-conjugating enzyme (e2)-dependent ubiquitination. *Proc Natl Acad Sci USA* 1999; 96:11364-9.
42. Mishra L, Cai T, Levine A, Weng D, Mezey E, Mishra B, Gearhart J. Identification of elf1, a beta-spectrin, in early mouse liver development. *Int J Dev Biol* 1998; 42:221-4.
43. Mishra L, Cai T, Yu P, Monga SP, Mishra B. Elf3 encodes a novel 200-kd beta-spectrin: Role in liver development. *Oncogene* 1999; 18:353-64.
44. Sporn MB, Roberts AB. The transforming growth factor-betas: Past, present, and future. *Ann N Y Acad Sci* 1990; 593:1-6.
45. Kretzschmar M, Doody J, Timokhina I, Massague J. A mechanism of repression of tgfbeta/ smad signaling by oncogenic ras. *Genes Dev* 1999; 13:804-16.
46. Peinado H, Quintanilla M, Cano A. Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: Mechanisms for epithelial mesenchymal transitions. *J Biol Chem* 2003; 278:21113-23. Epub 2003 Mar 28.
47. Muller N, Reinacher-Schick A, Baldus S, van Hengel J, Bex G, Baar A, van Roy F, Schmiegel W, Schwarte-Waldhoff I. Smad4 induces the tumor suppressor e-cadherin and p-cadherin in colon carcinoma cells. *Oncogene* 2002; 21:6049-58.
48. Sormunen RT, Leong AS, Vaaraniemi JP, Fernando SS, Eskelinen SM. Immunolocalization of the fodrin, e-cadherin, and beta-catenin adhesion complex in infiltrating ductal carcinoma of the breast-comparison with an in vitro model. *J Pathol* 1999; 187:416-23.
49. Li CC, Xu B, Hirokawa M, Qian Z, Yoshimoto K, Horiguchi H, Tashiro T, Sano T. Alterations of e-cadherin, alpha-catenin and beta-catenin expression in neuroendocrine tumors of the gastrointestinal tract. *Virchows Arch* 2002; 440:145-54.
50. Kusano M, Kakiuchi H, Mihara M, Itoh F, Adachi Y, Ohara M, Hosokawa M, Imai K. Absence of microsatellite instability and germline mutations of e-cadherin, apc and p53 genes in japanese familial gastric cancer. *Tumour Biol* 2001; 22:262-8.
51. Avizienyte E, Launonen V, Salovaara R, Kiviluoto T, Aaltonen LA. E-cadherin is not frequently mutated in hereditary gastric cancer. *J Med Genet* 2001; 38:49-52.
52. Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB, Kim HM, Kim WJ, Yamamoto H, Yamashita N, Yano T, Ikeda T, Itohara S, Inazawa J, Abe T, Hagiwara A, Yamagishi H, Ooe A, Kaneda A, Sugimura T, Ushijima T, Bae SC, Ito Y. Causal relationship between the loss of runx3 expression and gastric cancer. *Cell* 2002; 109:113-24.
53. Hanai J, Chen LF, Kanno T, Ohtani-Fujita N, Kim WY, Guo WH, Imamura T, Ishidou Y, Fukuchi M, Shi MJ, Stavnezer J, Kawabata M, Miyazono K, Ito Y. Interaction and functional cooperation of pebp2/cbf with smads. Synergistic induction of the immunoglobulin germline alpha promoter. *J Biol Chem* 1999; 274:31577-82.
54. Miyaki M, Iijima T, Konishi M, Sakai K, Ishii A, Yasuno M, Hishima T, Koike M, Shitara N, Iwama T, Utsunomiya J, Kuroki T, Mori T. Higher frequency of smad4 gene mutation in human colorectal cancer with distant metastasis. *Oncogene* 1999; 18:3098-103.
55. Katuri VTY, Marshall B, Rashid A, Jogunoori W, Volpe EA, Sidawy AN, Evans S, Blay J, Gallicano GI, Mishra L, Mishra B. Inactivation of elf/tgf-beta signaling in human gastrointestinal cancer. *Oncogene* 2005; In Press.
56. Ma Y, Zimmer WE, Riederer BM, Bloom ML, Barker JE, Goodman SM, Goodman SR. The complete amino acid sequence for brain beta spectrin (beta fodrin): Relationship to globin sequences. *Brain Res Mol Brain Res* 1993; 18:87-99.
57. Zhang Q, Skepper JN, Yang F, Davies JD, Hegyi L, Roberts RG, Weissberg PL, Ellis JA, Shanahan CM. Nesprins: A novel family of spectrin-repeat-containing proteins that localize to the nuclear membrane in multiple tissues. *J Cell Sci* 2001; 114:4485-98.
58. Wu G, Chen YG, Ozdamar B, Gyuricza CA, Chong PA, Wrana JL, Massague J, Shi Y. Structural basis of smad2 recognition by the smad anchor for receptor activation. *Science* 2000; 287:92-7.
59. Derynck R, Zhang YE. Smad-dependent and smad-independent pathways in tgfbeta family signalling. *Nature* 2003; 425:577-84.
60. Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem* 1998; 67:425-79.
61. Markowitz SD, Roberts AB. Tumor suppressor activity of the tgfbeta pathway in human cancers. *Cytokine Growth Factor Rev* 1996; 7:93-102.
62. Brzovic PS, Meza J, King MC, Klevit RE. The cancer-predisposing mutation c61g disrupts homodimer formation in the nh2-terminal brca1 ring finger domain. *J Biol Chem* 1998; 273:7795-9.
63. Ohta T, Michel JJ, Schottelius AJ, Xiong Y. Roc1, a homolog of apc11, represents a family of cullin partners with an associated ubiquitin ligase activity. *Mol Cell* 1999; 3:535-41.
64. Hochstrasser M. Ubiquitin-dependent protein degradation. *Annu Rev Genet* 1996; 30:405-39.
65. Burrows JF, McGrattan MJ, Rasclé A, Humbert M, Baek KH, Johnston JA. Dub-3, a cytokine-inducible deubiquitinating enzyme that blocks proliferation. *J Biol Chem* 2004; 279:13993-4000. Epub 2003 Dec 29.
66. Tang Y, McKinnon ML, Leong LM, Rusholme SA, Wang S, Akhurst RJ. Genetic modifiers interact with maternal determinants in vascular development of tgfb1(-/-) mice. *Hum Mol Genet* 2003; 12:1579-89.
67. Souchelnytskyi S, Moustakas A, Heldin CH. Tgf-beta signaling from a three-dimensional perspective: Insight into selection of partners. *Trends Cell Biol* 2002; 12:304-7.
68. Heldin CH, Miyazono K, ten Dijke P. Tgf-beta signalling from cell membrane to nucleus through smad proteins. *Nature* 1997; 390:465-71.
69. Itoh F, Adachi Y, Imai K. [matrix metalloproteinases in human gastrointestinal carcinomas]. *Nippon Shokakibyō Gakkai Zasshi* 2003; 100:152-60.
70. Weiser TS, Guo ZS, Ohnmacht GA, Parkhurst ML, Tong-On P, Marincola FM, Fischette MR, Yu X, Chen GA, Hong JA, Stewart JH, Nguyen DM, Rosenberg SA, Schrumpp DS. Sequential 5-aza-2 deoxycytidine-depsipeptide fr901228 treatment induces apoptosis preferentially in cancer cells and facilitates their recognition by cytolytic t lymphocytes specific for ny-eso-1. *J Immunother* 2001; 24:151-61.