MODULATION OF CELL SIGNALING BY TOMOREGULINS IN EMBRYOGENESIS AND CANCER

by

PAUL WILLIAM HARMS

CHENBEI CHANG, COMMITTEE CHAIR
STUART J. FRANK
LOUIS B. JUSTEMENT
FANG-TSYR LIN
ROSA A. SERRA

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

BIRMINGHAM, ALABAMA

2006
MODULATION OF CELL SIGNALING BY TOMOREGULINS IN EMBRYOGENESIS AND CANCER

PAUL WILLIAM HARMS

DEPARTMENT OF CELL BIOLOGY

ABSTRACT

Growth factor signals often regulate similar cellular processes both during embryogenesis and in adult homeostasis. Stringent control of these signals ensures proper embryonic development and correct cell physiology in mature individuals. Recently, a family of two members of Tomoregulin (or transmembrane protein with epidermal growth factor-like and two follistatin domains [TMEFF]) was found to interact with transforming growth factor-β (TGF-β) and ErbB signaling pathways, both of which are implicated in development and in cancer biology in the adult. The function as well as the mechanisms of TMEFFs in modulating these two signals has not been elucidated in detail. In this dissertation, I investigate the activities of TMEFFs in modulating signaling by the of TGF-β and ErbB pathways in a vertebrate model and in mammalian cell culture, respectively. In the first part, I showed that TMEFF1 selectively inhibits nodal but not activin signals in early Xenopus embryos through direct interaction with the nodal coreceptor Cripto. Accordingly, Cripto rescues the inhibition of the nodal signaling by TMEFF1 in Xenopus ectodermal explants. Furthermore, I showed that the Cripto-FRL1-Cryptic (CFC) domain in Cripto, which is essential for its binding to the type I nodal receptor ALK4, is also important for its interaction with TMEFF1. This study demonstrates for the first time that nodal signaling can be regulated by a novel mechanism of blocking the Cripto coreceptor. In the second part, I uncovered a novel association between both TMEFFs and all four ErbB family members in
coimmunoprecipitation assays. The epidermal growth factor-like domain of TMEFF1 was dispensable for its association with ErbB4, suggesting that TMEFFs bind ErbB receptors in a manner unlike orthodox ErbB ligands. Functional analyses of the consequence of TMEFF/ErbB interaction revealed that TMEFFs do not affect ErbB signaling in tumor cell lines despite the direct physical association. TMEFFs may thus have subtle effects on ErbB-regulated processes in a cell type- and/or environment-dependent manner. In summary, my work identifies direct associations of TMEFFs with Cripto and ErbB receptor tyrosine kinases and suggests novel roles for TMEFFs in modulating TGF-β and ErbB signaling during embryonic development and tumorigenesis.
ACKNOWLEDGMENTS

My dissertation work has been a richly rewarding experience, and I would like to express my profound appreciation for all those whose encouragement, advice, and assistance made it possible. First, I would like to acknowledge my deep gratitude for the patience, insight, and guidance of my advisor, Dr. Chenbei Chang. Working with Dr. Chang has been an exceptional learning experience; she has always been available to provide feedback and instruction, while challenging me to develop skills in independent planning and critical analysis. I am fortunate to have a mentor dedicated to training her students in every area important for our future success as well-rounded investigators. I am grateful to the current and former members of the Chang lab: Shuyi Nie, Dr. Gustavo Bonacci, and Jason Fletcher. It has been an honor and a pleasure to work with them; they have displayed great generosity with respect to suggestions and assistance. In addition, Jason Fletcher, our former technician, provided extensive help in culturing several of the inducible cell lines used in this study. I would like to thank the members of my thesis advisory committee (Drs. Stuart Frank, Louis Justement, Fannie [Fang-Tsyr] Lin, and Rosa Serra) for their perceptive questions and thoughtful advice.

I would like to extend thanks to a number of individuals for providing indispensable materials and technical assistance for my project. I would like to thank Dr. Fannie Lin and her student, Jocelyn (Yun-Ju) Lai, for their generosity in providing the protocols, expertise, and resources necessary for my examination of cell signaling in
tumor cell lines. I am very grateful for Dr. Xinbin Chen and members of his lab, especially Kelly Harms and Dr. Jin Zhang, for providing essential advice and resources toward the development of inducible cell lines, luciferase assays, and study of cell proliferation. In addition, Dr. Chen provided the U118 glioma cells used in this study. I thank Drs. Monica Richert and Daniel Welch for their gift of the T47D cell line used in this study. I also thank Drs. Mark Stonecypher and Steven Carroll for technical advice on immunoprecipitation of ErbB receptors. I am grateful for the advice of Gordon Meares with respect to blotting for phospho-Akt. In addition to those listed above, I would like to broadly thank all the students, faculty, and others in the Department of Cell Biology, for their friendship and their kindness in providing advice and resources. I thank Drs. Tracy Keller, Malcolm Whitman, Eva Reissman, Michael Shen, Hiroshi Hamada, and Makoto Asashima for kindly providing plasmids used in this study. I would also like to acknowledge the Medical Scientist Training Program for giving career advice and partial financial support during my Ph.D. training.

I am grateful for all of my friends and mentors who have supported my efforts toward a career in research over the years. In particular, I would like to thank Dr. Sidney Fleischer, for his thoughtful guidance and for the opportunity to do undergraduate research in his lab. I also thank Dr. Hongbo Xin, then in Dr. Fleischer’s lab, for providing my initial training in the fundamentals of bench research.

Finally and most importantly, I would like to thank my family, without whose love and support I would not have been able to achieve this goal. I would like to express my profound appreciation for my wife, Kelly Harms, who has been a vital source of strength and encouragement during my graduate studies. I also thank my parents and
brother, for all the ways they have supported me and encouraged my interest in the sciences throughout my life.
TABLE OF CONTENTS

ABSTRACT ............................................................................................................................ iii

ACKNOWLEDGMENTS ........................................................................................................ v

LIST OF FIGURES ............................................................................................................... x

CHAPTER

1 INTRODUCTION .............................................................................................................. 1

TMEFF Regulation of TGF-β Family Signaling................................................................. 3
  Nodal Signaling Mediates Embryonic Patterning......................................................... 3
  Mechanism and Regulation of Nodal Signaling ........................................................... 5
  Regulation of Nodal Signaling by TMEFF1 ................................................................. 9
  TMEFF Inhibition of BMP Signaling ........................................................................... 10
  Remaining Questions Regarding TMEFF1 Regulation of TGF-β Signaling .............. 10
TMEFFs and Cancer ....................................................................................................... 11
  TMEFFs and ErbB Signaling ...................................................................................... 12
  Summary of Findings ............................................................................................... 16

2 TOMOREGULIN-1 (TMEFF1) INHIBITS NODAL SIGNALING THROUGH DIRECT BINDING TO THE NODAL CORECEPTOR CRIPTO ................................................................. 17

3 A NOVEL INTERACTION BETWEEN TOMOREGULINS AND ERBB RECEPTORS .......................................................................................................................... 41

4 SUMMARY AND DISCUSSION .................................................................................... 73

  TMEFF1 Inhibits Nodal Signaling Through Direct Binding to the Nodal Coreceptor Cripto ......................................................................................................................... 74
  TMEFFs and Cell Proliferation .................................................................................... 78
  TMEFFs and ErbBs .................................................................................................... 78
  TMEFFs and FGF Signaling .................................................................................... 82

  Concluding Remarks ................................................................................................ 84
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>TMEFF domain structure</td>
</tr>
<tr>
<td>2</td>
<td>TGF-β family signal transduction</td>
</tr>
</tbody>
</table>

# TOMOREGULIN-1 (TMEFF1) INHIBITS NODAL SIGNALING THROUGH DIRECT BINDING TO THE NODAL CORECEPTOR CRIPTO |

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TMEFF1, a nodal inhibitor, binds directly to the nodal coreceptor Cripto, but does not associate with either nodal or ALK4</td>
</tr>
<tr>
<td>S1</td>
<td>FRL1, the <em>Xenopus</em> Cripto family member, binds to both ALK4 and TMEFF1</td>
</tr>
<tr>
<td>2</td>
<td>Inhibition of nodal activity by TMEFF1 is cell-autonomous and is rescued by Cripto</td>
</tr>
<tr>
<td>3</td>
<td>Cripto and TMEFF1 associate in CHO cells, and this association is attenuated by deletion of the CFC domain of Cripto</td>
</tr>
</tbody>
</table>

# A NOVEL INTERACTION BETWEEN TOMOREGULINS AND ERBB RECEPTORS |

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TMEFFs do not modulate glioma proliferation</td>
</tr>
<tr>
<td>2</td>
<td>TMEFF1/HA does not modulate breast carcinoma proliferation</td>
</tr>
<tr>
<td>3</td>
<td>TMEFFs associate with ErbB receptors</td>
</tr>
<tr>
<td>4</td>
<td>TMEFF residues 236-310 influence TMEFF/ErbB association</td>
</tr>
<tr>
<td>5</td>
<td>TMEFFs do not modulate ErbB signaling in glioma cells</td>
</tr>
<tr>
<td>6</td>
<td>TMEFF1 does not alter the proliferative response to EGF or HRG in T47D-2T breast carcinoma cells</td>
</tr>
<tr>
<td>7</td>
<td>TMEFF1 may not alter the proliferative response to FGF signaling in T47D-2T breast carcinoma cells</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Tomoregulin-1, or transmembrane protein with epidermal growth factor (EGF)-like and two follistatin domains (TMEFF1) was first described as a protein expressed in the hypothalamo-pituitary axis of *Xenopus laevis* (Eib and Martens 1996). A close relative, TMEFF2 (Horie et al. 2000) (also known as tomoregulin-2 (Uchida et al. 1999), TENB2 (Glynne-Jones et al. 2001), TPEF (Liang et al. 2000), or HPP1 (Young et al. 2001) has since been identified. TMEFF1 and TMEFF2 share 47% amino acid identity and an identical domain structure (Figure 1). TMEFF genes are present throughout the vertebrate lineage; homologs of TMEFF1 and/or -2 have been described in humans, rodents, and newts (Eib et al. 1998; Morais da Silva et al. 2001; Siegel et al. 2002), and a predicted TMEFF homolog is present in zebrafish (locus BC098543). TMEFF homologs are well conserved through vertebrate evolution, with 80% similarity between human, mouse, and *Xenopus* TMEFF1, and 99% similarity between human and mouse TMEFF2 (no TMEFF2 homolog has been identified in *Xenopus*). However, BLAST searches fail to identify potential TMEFF homologs in invertebrates.

Expression patterns for TMEFFs suggest roles in embryogenesis and central nervous system (CNS) function. In humans, TMEFF1 is expressed in the embryo as well as in adult heart, brain, and skeletal muscle (Gery et al. 2003), while TMEFF2 is expressed in the brain, prostate, and certain parts of the gastrointestinal tract.
Figure 1. TMEFF domain structure. (A) Schematic of TMEFF domains. (B) Comparison of TMEFF1 and TMEFF2 amino acid sequences across species. Yellow = residues identical in all sequences. Blue = residues identical in three or more sequences. Green = similar residues. Dashed lines = glycosaminoglycan attachment motifs. Asterisks = putative G-protein activating motifs. Alignment was generated by Vector NTI. N = N-terminus, SP = signal peptide, FS = follistatin domain, EGF = epidermal growth factor-like domain, TM = transmembrane domain, CT = cytoplasmic tail, C = C-terminus.
(Uchida et al. 1999; Horie et al. 2000; Young et al. 2001). In mouse and *Xenopus*, TMEFFs are predominantly expressed in the embryo and in the adult CNS (Eib and Mar- tens 1996; Eib et al. 2000).

**TMEFF Regulation of TGF-β Family Signaling**

TMEFFs possess two follistatin-like (FS) modules. FS modules derive their name from a cysteine-rich domain repeated three times in the activin inhibitor follistatin. FS modules are present in many secreted proteins, including several proteins which inhibit signaling by the transforming growth factor β (TGF-β) family of cytokines (reviewed in Chang 2006 [in press]). In line with this, TMEFF1 overexpression inhibits signaling by the TGF-β family members nodal, Vg1, and bone morphogenetic protein (BMP) 2 (Chang et al. 2003).

**Nodal Signaling Mediates Embryonic Patterning**

Nodals are TGF-β ligands conserved throughout the chordate lineage (for reviews, see Schier and Shen 2000; Whitman 2001; Schier 2003). In *Xenopus*, six nodal homologs are present (*Xenopus* nodal-related [Xnr] 1-6) (Jones et al. 1995; Smith et al. 1995; Joseph and Melton 1997; Takahashi et al. 2000). Zebrafish possess three nodal factors: squint, cyclops, and southpaw (Feldman et al. 1998; Rebagliati et al. 1998; Long et al. 2003). In mammals, only a single nodal gene is present. Despite this variability in the number of nodal family members, experiments examining loss of nodal signaling have revealed that nodals play essential roles in embryogenesis which are highly conserved through vertebrate evolution (Whitman 2001).
Vertebrate embryos, along with those of all other triploblastic metazoans, form three distinct germ layers during gastrulation: ectoderm, mesoderm, and endoderm (reviewed in Gilbert 1991). Mesoderm and endoderm share common features, and may be referred to as mesendoderm prior to their separation late in gastrulation. Formation of a three-layered embryo facilitates coordinated organization of a complex body plan by allowing for instructive signaling between adjacent layers. In addition, each germ layer directly contributes to a distinct set of tissues. Specifically, ectoderm will give rise to nervous tissue, epidermis, sensory placodes, and tissues derived from neural crest cells. Mesoderm gives rise to the musculoskeletal system, hematopoietic system, heart, kidney, and smooth muscle of the gut. Endoderm is the major contributor to the digestive tract and its associated organs, including the lungs and liver (Gilbert 1991). Proper formation and patterning of these germ layers is crucial for embryonic viability.

Loss-of-function and overexpression studies reveal that nodal signaling plays a critical and highly conserved role in the formation of mesendoderm in vertebrate embryos (Schier 2003). Zebrafish with inactivating mutations in the nodal homologs cyclops and squint fail to develop head and trunk mesoderm and endoderm (Schier 2003). Mouse embryos with nodal mutations also display defects in mesendoderm (Schier 2003). Inhibition of nodal signaling in frog embryos leads to impairment in mesendoderm formation (Agius et al. 2000). Conversely, misexpression of nodal in the animal cap of the *Xenopus* embryo (which is fated to become ectoderm) leads to mesendodermal induction (Schier and Shen 2000; Whitman 2001).

In addition to mesendodermal induction, nodal also plays roles in left-right asymmetry (Schier 2003), anterior/posterior positioning in the mouse (Ding et al. 1998),
and patterning of the zebrafish CNS ventral midline (Rebagliati et al. 1998). Due to the embryonic lethality of loss of nodal function, postnatal roles for nodal have not yet been demonstrated.

Mechanism and Regulation of Nodal Signaling

Since the discovery of TGF-β itself, the TGF-β superfamily has expanded to include over 30 members in mammals, including BMPs, activins, nodal, myostatin, and anti-Mullerian hormone (for reviews, see Massague 1998; Massague and Chen 2000; Miyazawa et al. 2002; Shi and Massague 2003). Despite the diversity within the superfamily, TGF-β ligands signal to cells via a highly conserved scheme (Figure 2A) (Massague and Chen 2000). The ligand associates with type I and type II serine/threonine kinase receptors. Different TGF-β ligands utilize distinct and/or overlapping sets of type I and type II receptors (Figure 2B). For example, BMPs bind the type I receptors activin receptor-like kinase (ALK) 2/3/6 and the type II receptors BMPRII or ActRIIA/B, while nodal/activin/Vg1 bind the type I receptor ALK4 and the type II receptors ActRIIA/B. Upon ligand binding, the constitutively active type II receptor associates with and activates the type I receptor by phosphorylation. The activated type I receptor then phosphorylates receptor Smads (R-Smads), which are considered the major cytoplasmic signal transducers for TGF-β signaling. Different TGF-β family members utilize distinct R-Smads; for example, BMPs make use of Smads 1/5/8, while TGF-β/activin/nodal typically utilize Smads 2/3. Phospho-Smads form a hetero-oligomeric complex with Smad4 and translocate to the nucleus where, in cooperation with cofactors, the Smad complex regulates transcription of target genes.
Figure 2. TGF-β family signal transduction. (A) Schematic diagram of TGF-β family signal transduction. (B) Examples of receptor complexes utilized by selected TGF-β family ligands.
In addition to type I (ALK4) and type II (ActRIIA/B) receptors, nodal also requires the presence of a coreceptor of the EGF-Cripto/Frizzled/Cryptic (EGF-CFC) family for the formation of a functional nodal/ALK4/ActRII signaling complex (Gritsman et al. 1999; Reissmann et al. 2001; Yeo and Whitman 2001; Bianco et al. 2002; Yan et al. 2002). Nodal may also signal via the type I receptor ALK7; in this context an EGF-CFC coreceptor potentiates nodal/ALK7 signaling but is not strictly required (Reissmann et al. 2001). EGF-CFC family proteins contain an EGF-like motif important for nodal recruitment and a conserved CFC domain which mediates association with ALK4 (Yeo and Whitman 2001). Mammals possess two EGF-CFC orthologs ( Cripto and Cryptic) and zebrafish possess a single EGF-CFC gene (One-eyed pinhead), while Xenopus possesses three (FRL1/XCR1, XCR2, and XCR3) (Whitman 2001; Adamson et al. 2002; Dorey and Hill 2006; Onuma et al. 2006). Other TGF-β family members in addition to nodal utilize EGF-CFC coreceptors, including Vg1/GDF1 (Cheng et al. 2003), GDF3 (Chen et al. 2006), and derriere (Dorey and Hill 2006; Onuma et al. 2006).

Due to the central roles of nodal in embryonic patterning, proper spatiotemporal control of nodal activity is critical. The magnitude and range of TGF-β superfamily signaling may be regulated at every step of the signaling process (Massague and Chen 2000). TGF-β family members are synthesized in a precursor form which requires cleavage of the prodomain by proprotein convertases for release of the active mature ligand (Joseph and Melton 1997). A number of soluble factors directly antagonize nodal signaling by interaction with nodal or its receptors; these include Lefty, the DAN family members Coco and Cerberus, and BMPs (Chang 2006 [in press]). Lefty/antivin, a divergent member of the TGF-β superfamily, limits the extent of nodal signaling during mesendo-
derm induction and left/right patterning. Lefty antagonizes nodal signaling by binding the EGF-CFC coreceptor to block the nodal/coreceptor interaction (Chen and Shen 2004; Cheng et al. 2004). In *Xenopus*, the DAN family members Cerberus and Coco antagonize nodal signaling by direct binding to the nodal ligand (Piccolo et al. 1999; Bell et al. 2003). Other DAN family members which inhibit nodal signaling include Caronte in the chick; Cer-1 and Cerl-2/Dante in the mouse; and Charon in zebrafish (Chang 2006 [in press]). Other members of the TGF-β family may directly modulate nodal signaling. Specifically, BMP heterodimerization with nodal results in inhibition of both nodal and BMP signaling (Yeo and Whitman 2001). In addition, engineered nodal prodomain cleavage mutants (such as the cleavage mutant for *Xenopus* nodal-related 2 [cmXnr2]) block nodal and BMP signaling by heterodimerizing with wild-type nodal or BMP (Yeo and Whitman 2001; Eimon and Harland 2002; Onuma et al. 2005).

In addition to soluble antagonists, nodal signaling may also be regulated by proteins located at the cell surface or within the cell. Nodal/activin signaling is antagonized by the transmembrane protein Nicalin and its binding partner Nomo via an undetermined mechanism (Haffner et al. 2004). In zebrafish, the cytoplasmic protein Dapper-2 inhibits nodal signaling by promoting degradation of the nodal receptor ALK4 (Zhang et al. 2004b). Other mechanisms by which TGF-β (and hence nodal) signal transduction might be negatively regulated in the cytoplasm include Smad degradation, blocking Smad/receptor association, and interference with Smad trafficking to the nucleus. Regulatory factors also modify the transcriptional activity of Smad/cofactor complexes in the nucleus. For instance, DRAP1 antagonizes nodal signaling by inhibiting DNA binding by
FoxH1, a winged-helix transcription factor which cooperates with Smads to mediate the positive feedback response to nodal signaling (Iratni et al. 2002).

**Regulation of Nodal Signaling by TMEFF1**

At the beginning of this work, *Xenopus* TMEFF1 (X7365) was found to be a novel inhibitor of nodal signaling in the animal cap assay (Chang et al. 2003). In the *Xenopus* animal cap (or ectodermal explant) assay, the ability of a signaling molecule to induce mesendoderm from ectoderm is assayed. Specifically, mRNA encoding a mesendoderm inducer such as nodal is injected into a two- or four-cell stage *Xenopus* embryo. The animal region of the embryo (which is fated to become ectoderm and does not normally express mesendodermal markers) is removed at blastula stage and incubated to gastrula stage. Induction of mesendodermal markers is assessed by RT-PCR. TMEFF1 inhibited mesendodermal marker induction by nodal and Vg1, but not activin, in animal caps. Consistent with the animal cap results, overexpression of TMEFF1 in the dorsal region of the embryo results in reduction of anterior structures (Chang et al. 2003). This phenotype is reminiscent to that induced by the nodal cleavage mutant cmXnr2, which inhibits both nodal and BMP signaling (Chang 2006 [in press]). In animal caps, the cytoplasmic domain of TMEFF1 was dispensable for TMEFF inhibition of nodal signaling, but the transmembrane domain was required, suggesting that TMEFF1 inhibits nodal signaling by acting at the cell surface or within the plasma membrane (Chang et al. 2003).
**TMEFF Inhibition of BMP Signaling**

In addition to inhibition of nodal signaling, TMEFF also suppresses BMP signaling in the *Xenopus* embryo. TMEFF1 blocks mesoderm induction by BMP in animal caps, and may inhibit BMP signaling to induce cement gland markers (Chang et al. 2003). However, TMEFF1 does not affect epidermal induction by BMP, suggesting that it is not a strong inhibitor of BMP signaling (Chang et al. 2003). The mechanism by which TMEFF1 inhibits BMP signaling is unknown. Deletion analyses show that the cytoplasmic tail of TMEFF1 is critical for its inhibition of BMP signaling, but dispensable for inhibition of nodal signaling; this suggests that TMEFF inhibits nodal and BMP via distinct mechanisms (Chang et al. 2003).

**Remaining Questions Regarding TMEFF1 Regulation of TGF-β Signaling**

More detailed understanding of the manner in which TMEFF1 inhibits signaling by Nodal, Vg1, and BMP will refine our knowledge of how these TGF-β family signals are spatiotemporally regulated to pattern the vertebrate embryo. Here, we identified the mechanism by which TMEFF1 suppresses nodal signaling (see Chapter 2). Several questions remain regarding the regulation of TGF-β family signaling by TMEFF1. For example, although the cytoplasmic tail of TMEFF1 is required for the inhibition of BMP signaling, the precise mechanism remains unclear. Also, whether TMEFF affects signaling by other TGF-β family members remains unknown. In addition, it is not known whether inhibition of nodal/Vg1 and BMP signals by TMEFF1 is conserved from *Xenopus* to mammals. While both mammals and *Xenopus* possess TMEFF1, mammals also possess a TMEFF2 gene which is expressed in both the embryo and adult (Horie et al. 2000; Liang
et al. 2000; Glynne-Jones et al. 2001; Kanemoto et al. 2001; Young et al. 2001). It remains unknown whether TMEFF2 is also capable of inhibiting nodal/Vg1 signaling. Finally, further study is needed to define how regulation of TGF-β family signals by endogenous TMEFFs contributes to proper embryonic patterning, postnatal homeostasis, and the promotion or suppression of tumorigenesis.

TMEFFs and Cancer

In addition to functions in embryogenesis, TMEFFs might also impact the development or progression of cancers. Expression of TMEFFs is altered in dysplasias and neoplasms in several tissues, suggesting that TMEFFs might play roles in tumor suppression in certain tissues. TMEFF2 expression is silenced by promoter hypermethylation in a subset of hyperplastic colonic polyps, colon carcinomas, and gastric adenocarcinomas (Young et al. 2001; Shibata et al. 2002). Hypermethylation of the TMEFF2 promoter has also been demonstrated in cancers of the esophagus, small intestine, gall bladder, lung, breast, bladder, and prostate (Liang et al. 2000; Geddert et al. 2004; Takahashi et al. 2004; Suzuki et al. 2005; Brucher et al. 2006a), although accompanying changes in TMEFF2 expression were not examined in these studies. TMEFF1 expression is decreased in brain tumors through an undetermined mechanism (Gery et al. 2003). Consistent with a role in tumor suppression, TMEFFs can negatively regulate cell proliferation, since forced overexpression of TMEFF1 in glioma cells or TMEFF2 in prostate cancer cells results in decreased proliferation (Gery et al. 2002; Gery et al. 2003). More thorough understanding of the molecular roles TMEFFs play in cell functions may identify novel targets for cancer therapies.
The structure of TMEFFs suggests mechanisms by which they may regulate proliferation and other cellular functions. TMEFFs possess FS modules, which may participate in inhibition of signaling by FGF or VEGF (Kupprion et al. 1998; Yan and Sage 1999; Chlenski et al. 2004) or antagonize signaling by factors in the TGF-β family. The FS modules of TMEFFs flank glycosaminoglycan attachment motifs (see Figure 1B). In line with this, one group has reported a high molecular-weight band of TMEFF2 which is reduced in size by chondroitinase ABC, indicating that a subset of TMEFF2 exists in the form of chondroitin or dermatan sulfate proteoglycan and hence may play roles in the extracellular matrix (Glynne-Jones et al. 2001). The highly conserved cytoplasmic tail of TMEFFs possesses a putative activating motif for heterotrimeric G-proteins (Figure 1B), suggesting TMEFFs may signal as cell surface receptors for unidentified ligands (Eib and Martens 1996). TMEFFs also possess an EGF-like domain typical of ligands for the ErbB family of receptor tyrosine kinases. Consistent with this, TMEFF2 activates ErbB4 signaling (Uchida et al. 1999). However, further investigation is needed to define the relationship between TMEFFs and the proliferative response to growth factor signaling.

**TMEFFs and ErbB Signaling**

The ErbB family of receptor tyrosine kinases has four members in mammals: epidermal growth factor receptor (EGFR) (also known as ErbB1), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4) (for reviews, see Hackel et al. 1999; Olayioye et al. 2000; Yarden 2001; Yarden and Sliwkowski 2001). ErbB receptors have well-described roles in embryonic development, postnatal homeostasis, and malignancy (Yarden 2001). ErbB receptors consist of an extracellular region, which contains sites for ligand binding
and receptor dimerization; a single-pass transmembrane domain; and a cytoplasmic region which contains a tyrosine kinase domain and effector docking sites (Olayioye et al. 2000). Signaling by ErbB receptors follows the typical paradigm for receptor tyrosine kinases: ligand binding stimulates receptor dimerization and activation, which is followed by activation of cytoplasmic signaling cascades. Ligands in the EGF family of growth factors, comprising at least ten members, associate with the extracellular domain of ErbB receptors to trigger receptor signaling. Ligands bind ErbB family receptors with distinct specificities; for example, EGF binds only EGFR/ErbB1 with high affinity, while the beta-1 isoform of neuregulin (NRG1-β1) binds ErbB3 and ErbB4 (Yarden and Sliwkowski 2001). Ligand binding, which stimulates homo- or heterodimerization of ErbB family members, results in receptor activation and tyrosine phosphorylation. Cytoplasmic effector proteins are then recruited to specific phosphotyrosine residues on the activated receptors. Recruited proteins include enzymes with signaling activity (e.g. phospholipase Cγ, phosphoinositide-3-kinase (PI3K), and Src), adaptor proteins which link ErbBs to signaling cascades (e.g. Shc, Grb2, Grb7, and Nck) (Hackel et al. 1999), and negative regulatory proteins which mediate receptor dephosphorylation, internalization and/or proteolysis. Different ErbB receptors recruit overlapping but distinct sets of cytoplasmic signaling molecules, so the nature of the signal generated is dependent upon the ligand and the ErbB family members present in the dimer complex (Sweeney et al. 2000; Yarden and Sliwkowski 2001). Signaling outputs include proliferation, survival, chemotaxis, differentiation, or apoptosis, depending upon ligand/receptor complex, cell type, and context (Yarden 2001).
In addition to conventional ErbB ligands, a variety of factors activate ErbB receptors without directly binding as ligands. ErbB activation in this context refers to phosphorylation of effector docking sites, with or without accompanying activation of receptor kinase activity. Examples of molecules which stimulate transactivation of EGFR and/or other ErbB receptors include integrins (Moro et al. 1998), E-cadherin (Pece and Gutkind 2000), the growth factor Cripto (Bianco et al. 1999), and G-protein coupled receptor ligands such as lysophosphatidic acid (reviewed in Wu and Cunnick 2002). Such molecules have been hypothesized to transactivate ErbBs via non-receptor tyrosine kinases or by stimulating local release of ErbB ligands such as heparin-binding EGF-like growth factor (Wu and Cunnick 2002). Via transactivation, ErbB receptors participate in a broad range of signaling networks.

Numerous lines of evidence indicate that dysregulated ErbB signaling is a factor in the progression of human cancers. ErbB receptor tyrosine kinases were implicated in cancer in the early 1980s, when v-ErbB, an EGFR homolog, was found to be essential for transformation by the avian erythroblastosis virus (reviewed in Boerner et al. 2003). Mutation or overexpression of ErbB receptors has prognostic value in a variety of tumors. For instance, EGFR overexpression and/or activating mutations occur in cancers of the brain, breast, lung, bladder, kidney and prostate (Yarden and Sliwkowski 2001). EGFR overexpression correlates with shorter survival in gliomas and high-grade breast cancers (Yarden and Sliwkowski 2001). ErbB2 overexpression also occurs in many cancers, and is associated with increased tumor size and grade in breast cancer (Yarden and Sliwkowski 2001). Evidence suggests that improper ErbB signaling directly contributes to cancer progression by promoting tumor cell characteristics such as proliferation, trans-
formed growth, and chemotaxis. In light of this, several ErbB inhibitors are currently in clinical use as cancer therapeutics (Hynes and Lane 2005). In contrast to the causative nature of ErbB1/2 in tumorigenesis, the role of ErbB4 in tumorigenesis is less clear. ErbB4 expression is associated with more differentiated tumors (Carpenter 2003), and may promote differentiation in cultured breast carcinoma cells (Sartor et al. 2001). However, ErbB4 overexpression in thyroid cancer or childhood medulloblastoma is associated with a poor prognosis (Gullick 2003).

TMEFFs may signal as ErbB ligands. TMEFFs possess an EGF domain which conforms to the consensus sequence for ErbB-activating ligands (CX_7 CX_2-3 GX CX_10-13 CX_3 YX GX RCX_L), with the exception of the arginine, which is highly important for ligand-receptor affinity in EGF and NRG (Carpenter and Cohen 1990; Engler et al. 1990; Hommel et al. 1991; Eib and Martens 1996; Jones et al. 1998). Similar to ErbB ligands, TMEFF2 undergoes proteolytic cleavage to release a soluble ectodomain (Lin et al. 2003). In MKN28 gastric cancer cells, which express all four ErbB receptors, treatment with soluble TMEFF2 ectodomain stimulates phosphorylation of ErbB4 but not ErbB1/2/3 (Uchida et al. 1999). Numerous questions remain with regard to TMEFF activation of ErbBs. It is unknown whether TMEFF2 directly binds and activates ErbB4, or if TMEFF2-stimulated ErbB4 phosphorylation is due to transactivation by an unidentified receptor. In addition, the activation of ErbBs by TMEFF1 has not been examined. Functional consequences of TMEFF2/ErbB4 signaling remain undetermined, although it is tempting to speculate that TMEFF2 may transmit or modulate antiproliferative signals via ErbB4.
Summary of Findings

In these studies, we have addressed mechanistic roles for TMEFF in regulating cell signaling in the *Xenopus* embryo and in human tumor cells. We demonstrate that TMEFF inhibits nodal signaling by association with the coreceptor Cripto in *Xenopus* embryos and mammalian cells. We also examine mechanistic roles for TMEFF in cell proliferation. We have identified a novel interaction between TMEFF and ErbB receptor tyrosine kinases, but the significance of this association is unclear as TMEFF overexpression fails to modulate ErbB activation. In addition, we demonstrate that TMEFF does not play clear roles in modulating the proliferative response to FGF in breast cancer cells.
CHAPTER 2

TOMOREGULIN-1 (TMEFF1) INHIBITS NODAL SIGNALING THROUGH DIRECT BINDING TO THE NODAL CORECEPTOR CRIPTO

by

PAUL HARMS AND CHENBEI CHANG


Copyright
2006
by
Cold Spring Harbor Laboratory Press
Used by permission

Format adapted for dissertation
CHAPTER 2

TOMOREGULIN-1 (TMEFF1) INHIBITS NODAL SIGNALING THROUGH DIRECT BINDING TO THE NODAL CORECEPTOR CRIPTO

Abstract

Transforming growth factor-β (TGF-β) signals regulate multiple processes during development and in adult. We recently showed that tomoregulin-1 (TMEFF1), a transmembrane protein, selectively inhibits nodal but not activin in early Xenopus embryos. Here we report that TMEFF1 binds to the nodal coreceptor Cripto, but does not associate with either nodal or the type I ALK (activin receptor-like kinase) 4 receptor in coimmunoprecipitation assays. The inhibition of the nodal signaling by TMEFF1 in Xenopus ectodermal explants is rescued with wild-type but not mutant forms of Cripto. Furthermore, we show that the Cripto-FRL1-Cryptic (CFC) domain in Cripto, which is essential for its binding to ALK4, is also important for its interaction with TMEFF1. Our results demonstrate for the first time that nodal signaling can be regulated by a novel mechanism of blocking the Cripto coreceptor.

Introduction

Members of the transforming growth factor-β (TGF-β) family play pivotal roles in both invertebrate and vertebrate embryogenesis (for reviews, see Harland and Gerhart 1997; Schier and Shen 1999; Whitman 2001). During early vertebrate development, the activin/nodal/Vg1/GDF1 subfamily of ligands is involved in induction and patterning of
the mesodermal and endodermal germ layers. Subsequently, these ligands participate in regulation of left-right axis formation. Removal of gene functions in the activin/nodal/Vg1/GDF1 pathways by mutations, gene knockout, or dominant negative components leads to aberrant development of vertebrate embryos that are defective in mesendodermal tissues and have left-right laterality defect (Harland and Gerhart 1997; Schier and Shen 1999; Whitman 2001).

Like other TGF-β ligands, activin/nodal/Vg1/GDF1 signals through two types of transmembrane serine/threonine kinase receptors. On ligand binding, the constitutively active type II receptor forms a complex with the type I receptor ALK (activin receptor-like kinase) 4 and 7, and activates the type I receptor through phosphorylation. The activated ALK then phosphorylates the cytoplasmic signal transducers Smad2 and Smad3, which form a hexameric complex with the common Smad, Smad4, and translocate into the nucleus to regulate gene expression in conjunction with other transcription factors (for reviews, see Massague 1998; Shi and Massague 2003). All of these ligands use the same type I receptor ALK4 and the type II receptors ActRIIA/IIB; however, nodal and Vg1/GDF1, but not activin, also require a membrane-associated EGF-CFC protein belonging to the Cripto family as a coreceptor in their signaling transduction (Gritsman et al. 1999; Reissmann et al. 2001; Yeo and Whitman 2001; Bianco et al. 2002; Yan et al. 2002; Cheng et al. 2003). Mutation in the Cripto family member One-eyed pinhead (Oep) in zebrafish leads to defective nodal/Vg1/GDF1 signaling, so that the resulting embryos mimic those with the mutations in the nodal ligands (Gritsman et al. 1999; Cheng et al. 2003). In mouse, nodal mutants display several defects in common with the mutants of Cripto or the related factor Cryptic (Conlon et al. 1994; Lowe et al. 2001; Brennan et al.
Biochemical evidence suggests that Cripto members bind directly to nodal/Vg1/GDF1 ligands as well as the ALK4 receptor, thus facilitating the assembly of a functional receptor complex at the membrane (Reissmann et al. 2001; Yeo and Whitman 2001; Bianco et al. 2002; Yan et al. 2002; Cheng et al. 2003). Although Cripto members are not required for activin signaling, overexpression of Cripto does influence the activin activity by repressing the signal pathway (Gray et al. 2003). These results imply that Cripto may have a function in fine-tuning the signals through different TGF-β ligands in vivo.

TGF-β signals are regulated by multiple factors at different levels (for reviews, see Massague and Chen 2000; Shi and Massague 2003). While secreted factors modulate the binding of the TGF-β ligands to their cognate receptors, cytoplasmic and nuclear proteins regulate localization, degradation, and modification of the Smad signal transducers as well as the interaction of the Smads with other proteins, such as nuclear transcription factors. At the membrane level, TGF-β signaling can also be regulated either positively or negatively by receptor-like proteins, such as the type III TGF-β receptor betaglycan or the naturally occurring truncated receptor BAMBI (Lopez-Casillas et al. 1993; Onichtchouk et al. 1999). Recently, we showed that a transmembrane protein tomoregulin-1 (TMEFF1) can modulate signals through different TGF-β ligands in early Xenopus embryos (Chang et al. 2003). Although TMEFF1 has no effect on mesendodermal induction by activin in Xenopus ectodermal explants (animal caps), it inhibits both nodal and Vg1 activities in this assay (Chang et al. 2003). TMEFF1 contains two follistatin modules and an epidermal growth factor (EGF) motif in its extracellular domain, and a short conserved cytoplasmic tail following the transmembrane region (Eib and Martens 1996). Deletion
analysis showed that the cytoplasmic domain of TMEFF1 is dispensable for its nodal inhibitory activity, a result that suggests that TMEFF1 blocks nodal signaling at the ligand or at the receptor level (Chang et al. 2003). To understand further the mechanism by which TMEFF1 inhibits nodal, we undertook biochemical studies. Here we report that TMEFF1 binds directly to the Cripto coreceptor, but does not interact with either nodal or the ALK4 type I receptor in coimmunoprecipitation assays. The inhibition of the nodal signaling by TMEFF1 is rescued with wild-type but not mutant forms of Cripto. Furthermore, we show that the Cripto-FRL1-Cryptic (CFC) domain in Cripto is important for the interaction of the two proteins. Our data thus demonstrate for the first time that, in addition to the extracellular, cytoplasmic, and nuclear regulation, nodal signaling can be modulated at the membrane by a nonreceptor protein, TMEFF1. Our discovery that Cripto interacts with TMEFF1 may also help to shed light on the studies of nodal-independent functions of Cripto in other cellular contexts.

Results and Discussion

TMEFF1 Binds Directly to Cripto, But Does Not Associate with Nodal or ALK4

TMEFF1, a follistatin module-containing protein, selectively inhibits nodal and Vg1 but not activin (Chang et al. 2003). Because follistatin and the follistatin-related gene (FLRG) have been shown to inhibit activin through direct binding to the ligand (Kogawa et al. 1991; Schneyer et al. 2001; Tsuchida et al. 2001; Bartholin et al. 2002), it is possible that TMEFF1 uses a similar mechanism by which it selectively interacts with and blocks nodal but not activin. To test this possibility, we performed a biochemical study to assay for binding of nodal by TMEFF1. We first constructed a Flag-tagged TMEFF1
(TMEFF1-F) and examined its activity. A chimeric nodal ligand AXnr1-HA, consisting of the prodomain of activin and the HA-tagged mature region of Xnr1 (Xenopus nodal-related 1; Piccolo et al. 1999), was used in both the activity and the binding assays. As shown in Figure 1A, similar to the wild-type TMEFF1, TMEFF1-F blocks the induction of mesendodermal markers by AXnr1 in Xenopus animal caps (Fig. 1A, cf. lanes 3 and 5). The result suggests that TMEFF1-F retained its nodal inhibitory activity. When TMEFF1-F was coexpressed with AXnr1-HA in early Xenopus embryos and the embryonic extract was examined by a coimmunoprecipitation assay at early gastrula stages, we found that no TMEFF1-F was coprecipitated with AXnr1-HA by the anti-HA antibody; similarly, AXnr1-HA was not precipitated with TMEFF1-F by the anti-Flag antibody (Fig. 1B). The result implies that TMEFF1 may not bind to nodal. To confirm that our immunoprecipitation assay worked well, we analyzed the binding of nodal to Cripto under the same conditions, because it has been shown that Xenopus Xnr1 interacts with Cripto directly (Reissmann et al. 2001). As shown in Figure 1B, Cripto was coprecipitated with AXnr1 when a Flag-tagged Cripto (Cripto-F) was coexpressed with the HA-tagged AXnr1; the reverse coimmunoprecipitation also showed that AXnr1 was coprecipitated with Cripto (Fig. 1B). Our data thus demonstrate that, unlike Cripto, which binds to nodal but not activin (Reissmann et al. 2001; Gray et al. 2003), TMEFF1 does not selectively bind to nodal to inhibit its signaling. Our finding also helps to explain an earlier observation (Chang et al. 2003) that the soluble extracellular domain of TMEFF1, unlike the TMEFF1 mutant that lacks the cytoplasmic domain, does not block nodal signaling. This result, combined with the current finding, suggests that TMEFF1 may block nodal signaling at the membrane level.
Figure 1. TMEFF1, a nodal inhibitor, binds directly to the nodal coreceptor Cripto, but does not associate with either nodal or ALK4. (A) The Flag- and the HA-tagged TMEFF1 inhibit nodal activity in Xenopus animal caps. (B) TMEFF1, unlike Cripto, does not bind to nodal. (C) TMEFF1 does not associate with the ALK4 receptor. (D) TMEFF1 binds directly to the Cripto coreceptor. (E) Binding of TMEFF1 to Cripto reduces the association of ALK4 with Cripto. In panels B-D, the RNAs encoding the tagged proteins (2 ng Cripto-F, 3 ng TMEFF1-HA/F, 3 ng ALK4-HA, and 4 ng AXnr1-HA) were injected into the animal poles of two-cell-stage embryos. In panel E, the doses of RNAs used are 1 ng Cripto-F; 2 ng ALK4-HA; and 2, 4, and 6 ng TMEFF1-HA. GFP RNA was used to keep the total amount of injected RNA constant. Protein extract made from early gastrula embryos was split into equal halves and subjected to IP with either anti-HA or anti-Flag antibodies. Western blot was performed with anti-HA or anti-Flag antibodies, as indicated. (IP) Immunoprecipitation; (IB) immunoblotting.
In addition to the type I receptor ALK4 and the type II receptors ActRIIA/IIB, which are shared among activin, nodal, and Vg1/GDF1 pathways, a membrane-associated EGF-CFC Cripto family protein is also required for nodal and Vg1/GDF1 signaling (Gritsman et al. 1999; Reissmann et al. 2001; Yeo and Whitman 2001; Bianco et al. 2002; Yan et al. 2002; Cheng et al. 2003). The selective inhibition of nodal and Vg1 but not activin by TMEFF1 may therefore result from the failure to form a functional receptor complex between Cripto and ALK4 in the presence of TMEFF1. This may occur if TMEFF1 directly associates with either the ALK4 receptor or the Cripto coreceptor. To examine whether either of these nodal receptors indeed interacts with TMEFF1, we performed coimmunoprecipitation experiments. We first assayed for potential binding of TMEFF1 to ALK4, using TMEFF1-F and an HA-tagged ALK4, which has been shown to be active in *Xenopus* embryos (Chang et al. 1997). As a positive control for binding to ALK4 (Yeo and Whitman 2001), we also examined the interaction of Cripto-F with ALK4-HA in parallel. Whereas we observed that Cripto coprecipitated with ALK4 and vice versa, we could not detect association of TMEFF1 with ALK4 under the same conditions (Fig. 1C). Although our results do not exclude the possibility that there may be a weak interaction between TMEFF1 and ALK4 that we cannot detect in our coimmunoprecipitation assays, our data suggest that TMEFF1 may not block nodal signaling by binding to the ALK4 receptor.

We next examined whether TMEFF1 could bind to the Cripto coreceptor. For this purpose, we constructed an HA-tagged TMEFF1. Functional analysis in *Xenopus* animal caps showed that, similar to TMEFF1-F, TMEFF1-HA retained its nodal inhibitory activity (Fig. 1A). When Cripto-F was coexpressed with TMEFF1-HA in early *Xenopus* em-
bryos, we found that TMEFF1 was coprecipitated with Cripto by anti-Flag antibody; similarly, Cripto was coprecipitated with TMEFF1 by anti-HA antibody (Fig. 1D). Cripto or TMEFF1 alone cannot be precipitated by the antibodies that recognize the other epitope, suggesting that the coimmunoprecipitation is specific. In addition, we observed that TMEFF1 interacts directly with the *Xenopus* Cripto member FRL1 in our coimmunoprecipitation experiment (Supplementary Fig. 1). When compared with the positive control of Cripto binding to ALK4 (Yeo and Whitman 2001), we found that less protein was coprecipitated in the case of Cripto binding to TMEFF1 (Fig. 1D). Because no chemical cross-linker was used during coimmunoprecipitation, the result may imply that either the interaction of Cripto and TMEFF1 is weaker than that of Cripto and ALK4, or the protein complex is less stable. The discovery that TMEFF1 associates directly with Cripto but not nodal or ALK4, combined with the observation that the cytoplasmic domain is dispensable for the nodal inhibitory activity of TMEFF1 (Chang et al. 2003), strongly suggests that TMEFF1 inhibits nodal signaling through direct interaction with the Cripto coreceptor.

Because Cripto binds to both ALK4 and TMEFF1, it is possible that the two latter proteins compete for binding to available Cripto, and the exclusion of ALK4 from the Cripto complex leads to the inhibition of nodal signaling. To test this hypothesis, we performed a binding competition assay. Cripto-F was coexpressed with ALK4-HA in the absence or presence of increasing amounts of TMEFF1-HA in early *Xenopus* embryos, and protein extract from injected embryos at gastrula stages was immunoprecipitated with an anti-Flag antibody. As shown in Figure 1E, whereas the level of ALK4-HA expression was constant in all samples, the amount of ALK4-HA coprecipitated with Cripto-F
Supplementary Figure 1. FRL1, the Xenopus Cripto family member, binds to both ALK4 and TMEFF1. The Flag-tagged FRL1 (FRL1-F) was constructed by PCR the FRL1 coding sequence with the primers FRL1-N(SpeI): GGACTAGTACCATGCAGTTTTTAAGA TTCTTGCC, and FRL1-C(AscI): AGGCGCGCAAGTCCAAA TTCAGATGCCA, and inserting the digested PCR product into the SpeI and AscI sites of pCS2+Cripto-3Flag vector. RNAs encoding FRL1-F (2 ng), ALK4-HA (2 ng) and TMEFF1-HA (3 ng) was injected alone or in combination into the animal poles of two-cell stage frog embryos. Proteins from injected embryos were extracted at early gastrula stages and split to immunoprecipitate with either anti-HA or anti-Flag antibodies. Western blot was performed following SDS-PAGE protein separation. ECL kit (Amersham) was used to detect the precipitated proteins. (IP) Immunoprecipitation; (IB) immunoblotting.
declined gradually in the presence of increasing doses of TMEFF1 (Fig. 1E, cf. lanes 2-4 and 1). The data suggest that TMEFF1 may inhibit nodal signaling by an interaction with Cripto that prevents the access of ALK4 to Cripto.

**Inhibition of Nodal Signal by TMEFF1 Is Cell-Autonomous and Is Rescued by Wild-Type but Not Mutant Forms of Cripto**

Our biochemical data suggest that TMEFF1 may block nodal pathway through its interaction with Cripto. If this is true, then, unlike secreted nodal antagonists, TMEFF1 may only inhibit nodal signaling in a cell-autonomous manner. To test this, we performed a cell-mixing experiment. Animal caps expressing AXnr1 were dissociated at blastula stages and mixed with dissociated ectodermal cells that expressed Cerberus or TMEFF1. The mixed cells were reaggregated immediately and incubated to early gastrula stages for reverse transcriptase PCR (RT-PCR) assay (Fig. 2A). In this experiment, the secreted nodal inhibitor Cerberus was able to block nodal activity in a non-cell-autonomous fashion, so that the mesendodermal marker induction by AXnr1 was either completely blocked or greatly reduced (Fig. 2A). In contrast, though TMEFF1 inhibits nodal when coexpressed with AXnr1 in animal caps, it could not confer its nodal inhibitory activity to neighboring cells, so that the markers induced by AXnr1 were still present in the cell-mixing experiment (Fig. 2A, lane 4). The results suggest that TMEFF1 blocks nodal pathway cell autonomously.

If TMEFF1 inhibits nodal activities through its interaction with Cripto, then overexpression of Cripto may overcome the inhibition and rescue the nodal signaling.
Figure 2. Inhibition of nodal activity by TMEFF1 is cell-autonomous and is rescued by Cripto. (A) Unlike Cerberus, TMEFF1 inhibits nodal in a cell-autonomous fashion. (B,C) Nodal activity inhibited by TMEFF1 is rescued by wild-type (B) but not the mutant forms (C) of Cripto. RNAs encoding AXnr1 (0.2 ng), TMEFF1 (1 ng), Cerberus (1 ng), and wild-type or the mutant forms of Cripto (0.5-1 ng) were injected into the animal poles of two-cell-stage embryos. Animal caps were explanted at blastula stages and incubated to early gastrula before total RNA was extracted for RT-PCR assay. For the cell-mixing experiment in A, animal caps were dissociated, mixed, and reaggregated immediately as indicated, and the reaggregated cell mixtures were processed at gastrula stages, similar to other samples.
We thus examined this possibility in animal cap assays. As described previously, TMEFF1 inhibited mesendodermal marker induction by AXnr1 (Fig. 2B, cf. lanes 6 and 4). When Cripto was coinjected with AXnr1 and TMEFF1, the transcription of marker genes was restored (Fig. 2B, cf. lanes 7 and 6). In addition, a constitutively active ALK4, which could stimulate nodal signaling independent of Cripto (Gritsman et al. 1999), also rescued the marker induction by AXnr1 (data not shown). These results therefore support our model that TMEFF1 inhibits nodal signaling through blocking the activation of the nodal receptor complex.

To further analyze whether the functional rescue by Cripto is specific, we attempted to rescue the nodal activity with several Cripto mutants. Cripto family proteins contain two homologous regions among all members, a divergent EGF domain and a conserved CFC motif (Schier and Shen 1999; Shen and Schier 2000; Whitman 2001). It has been shown that both domains are required for nodal signaling. Deletion of the EGF domain or point mutation of conserved residues in either domain leads to defective nodal signaling in early *Xenopus* embryos (Yeo and Whitman 2001). In our assays, when we coexpressed AXnr1 and TMEFF1 with Cripto mutants that lack the EGF or the CFC domain, or contain point mutations in these domains, we found that the nodal signaling could not be rescued (Fig. 2C; data not shown). These results demonstrate that rescue of TMEFF1's effect on nodal signaling requires a wild-type Cripto.

*The CFC Domain in Cripto Is Important for Its Interaction with TMEFF1*

Cripto binds to ALK4 through its conserved CFC motif, and the EGF domain may be involved in its binding to nodal (Yeo and Whitman 2001; Yan et al. 2002). The inter-
action of TMEFF1 with Cripto may potentially mask either the EGF or the CFC motif so that Cripto cannot associate with either nodal or ALK4 to form a functional ligand/receptor complex. To address which domain is involved in the binding of Cripto to TMEFF1, we analyzed the interaction of TMEFF1 with the Cripto deletion mutants. For this experiment, we used the cell culture system. As shown in Figure 3A, similar to the situation in early *Xenopus* embryos, TMEFF1-HA was coprecipitated with Cripto-F when the plasmids encoding these genes were cotransfected into Chinese hamster ovary (CHO) cells, suggesting that the two proteins also interact in mammalian cell culture (Fig. 3A). When we used Flag-tagged CriptoΔEGF, which lacks the EGF domain (Fig. 3B; Yeo and Whitman 2001), we observed that the level of coprecipitated TMEFF1 was comparable to that when wild-type Cripto was used (Fig. 3C). In contrast, when we coexpressed TMEFF1 with CriptoΔCFC, which lacks the CFC motif (Fig. 3B), we observed a consistent reduction of the coprecipitated TMEFF1 (Fig. 3C). The data reveal that the CFC domain, which is critical in physical interaction with ALK4, is also important in binding of Cripto to TMEFF1. Two conserved residues in the CFC domain are essential for Cripto binding to ALK4; when these residues are mutated, the resulting mCFC mutant no longer binds to ALK4 (Fig. 3B; Yeo and Whitman 2001). To determine whether these amino acids are also involved in TMEFF1 binding, we coexpressed Cripto mCFC with TMEFF1 and assayed for their interaction by coimmunoprecipitation. We observed no reduction of coprecipitated TMEFF1 using this mutant (Fig. 3D). The result suggests that though both
Figure 3. Cripto and TMEFF1 associate in CHO cells, and this association is attenuated by deletion of the CFC domain of Cripto. (A) TMEFF1 binds to Cripto in CHO cells. (B) Schematic diagrams of wild-type and mutant Cripto. (C) Deletion of the CFC domain in Cripto attenuates the interaction between Cripto and TMEFF1. (D) The Cripto mCFC mutant with two point mutations of the conserved residues in the CFC domain, unlike the CFC deletion mutant, does not affect the binding of Cripto to TMEFF1. CHO cells were transfected with the mutants or the wild-type Flag-tagged Cripto and/or HA-tagged TMEFF1, as indicated. Cell lysates were immunoprecipitated with anti-HA (IP: HA) or anti-Flag (IP:Flag) antibodies. One percent of each sample was run as whole-cell lysate (WCL). Bound protein was detected by anti-HA (IB:HA) or anti-Flag (IB:Flag) antibodies on Western blot. (SP) Signal peptide; (EGF) epidermal growth factor-like domain; (CFC) Cripto-FRL1-Cryptic domain; (FS) follistatin domain; (TM) transmembrane region.
ALK4 and TMEFF1 bind to the CFC motif, they may contact different residues in this domain.

To determine which region in TMEFF1 may be involved in its association with Cripto, we also examined the different domains of TMEFF1 using deletion mutants. There are two highly conserved regions in all TMEFF family members, which contain two follistatin modules and an EGF motif, respectively. When HA-tagged TMEFF1 mutants that lack either the follistatin modules (TMEFF1-ΔFS) or the EGF motif (TMEFF1-ΔEGF; Fig. 3E) were cotransfected with Cripto-F, we found that similar levels of wild-type or mutant forms of TMEFF1 were coprecipitated with Cripto, though we occasionally observed a slight reduction in the level of TMEFF1-ΔFS (Fig. 3F). The data suggest that the sequence outside of the follistatin and the EGF domains may be involved in direct binding of TMEFF1 to Cripto. This biochemical result is also consistent with our previous observation that both TMEFF1-ΔFS and TMEFF1-ΔEGF can still inhibit nodal activity (Chang et al. 2003).

Nodal signal plays essential roles during vertebrate embryogenesis. Strict regulation of nodal activity is therefore important to ensure correct development of vertebrate body plans. Nodal functions can be regulated by multiple factors. Cerberus, for example, blocks nodal signaling through direct binding to the ligand (Piccolo et al. 1999). Lefty, on the other hand, may bind to the nodal receptors to prevent the access of nodal to its receptors (Sakuma et al. 2002). Bone morphogenetic proteins (BMPs) can also inhibit nodal activity by forming heterodimers with nodal (Yeo and Whitman 2001; Eimon and Harland 2002). In this study, we show for the first time that a membrane protein can interact directly with the Cripto coreceptor to block nodal signaling, thus providing a novel
mechanism by which nodal activity can be regulated. Interestingly, we find that the CFC
domain in Cripto, which is critical for its interaction with ALK4, is also important for its
interaction with TMEFF1, but the crucial residues required for association with ALK4
and TMEFF1 may differ. Together with the competition results, our data suggest that
TMEFF1 and ALK4 compete for binding to Cripto, and association of TMEFF1 with
Cripto may physically exclude binding of Cripto to ALK4, thus leading to the inhibition
of the nodal signaling. In addition to its function during early development, Cripto has
also been found to be amplified in several carcinomas (for reviews, see Normanno et al.
2001; Adamson et al. 2002). Cripto may signal in both ALK4-dependent and ALK4-
independent fashion in these carcinoma cells to activate receptor tyrosine kinase and the
downstream mitogen-activated protein (MAP) kinase pathway (Bianco et al. 1999, 2002,
2003). Because TMEFF1 directly associates with Cripto in both Xenopus and in mammal-
ian cell culture, it is possible that TMEFF1 may directly participate in mediating Cripto
signaling or modulate the nodal-independent signal transduction by Cripto in these cell
contexts. Although these issues are currently under investigation, our studies reveal a key
connection between TMEFF1 and Cripto function, and may provide important clues to
our understanding of molecular mechanisms underlying a variety of activities mediated
by Cripto and/or TMEFF family members, including influencing neuronal cell function
and the formation and progression of cancers (Bianco et al. 1999, 2002, 2003; Horie et al.
Materials and Methods

Plasmid Construction for Tagged and Mutant Proteins

PCR-based strategy was used to generate all constructs. For TMEFF1-HA, an N-terminal primer X7365-N(RI) (5'-GGGGAATTCACCATGGATG GATTGCACCCT-3') and two overlapping C-terminal primers, HAC1(XbaI) (5'-GCTCTAGACTACACAGCATAGTCAGGCACGTCGT ACGG-3') and HA-C2(7365) (5'-AGGCACGTCGTACGGGATACACCACCAT CCGGGAAGAAGT-3'), were used for PCR over the pCS2++TMEFF1 template (Chang et al. 2003). The PCR product was digested and inserted into the EcoRI/XbaI sites of pCS2++ vector. TMEFF1ΔFS-HA and TMEFF1ΔEGF-HA were made by replacing the extracellular domain sequence in pCS2++TMEFF1-HA with that from pCS2++ TMEFF1-ΔFS and pCS2++ TMEFF1-ΔEGF, respectively (Chang et al. 2003). TMEFF1-Flag was made by PCR with the N-terminal primer X7365-N(RI) and two C-terminal primers, Flag-C1(XbaI) (5'-GCTCTAGACTACACCTTGT CATCGTCATCCTTGTAGTC-3'), and Flag-C2(7365) (5'-GTCATCCT TGTAGTCGCCCACCATCCGGGAAGAAGT-3'), on the pCS2++TMEFF1 template. The PCR product was digested and inserted into EcoRI/XbaI sites of pCS2++ vector. For CriptoΔCFC-Flag, two PCR reactions were performed with the N-terminal fragment amplified with the primers Cripto-N(SpeI) (5'-GGACTAGTCACCATGGGTACTTCTCA-3') and N3'(BamHI) (5'-GCGGATCCGTGCTTTGCGAACATC-3'), and the C-terminal fragment amplified with the primers C5'(BamHI) (5'-CGG GATTCCGTCACGTGATGGACCAG-3') and C3'(XhoI) (5'-AGGCTC GAGAGGCTTGAATT-3'), on pCS2+Cripto-3Flag template.
The PCR products were digested with SpeI/BamHI and BamHI/XhoI, respectively, and inserted into the SpeI/XhoI sites of pCS2+Cripto-3Flag vector. Sequencing of the construct confirmed that the CFC domain, containing amino acids 99-134, was eliminated. Other Cripto constructs were kindly provided by Drs. T. Keller and M. Whitman (Harvard Medical School, Boston, MA; Yeo and Whitman 2001).

**RT-PCR Assay**

Plasmids were linearized (AscI for pCS2+-based plasmids, NotI for pCS2+-based plasmids) and RNAs were synthesized with mMessage mMachine kit (Ambion) as described (Chang et al. 1997). The RNAs encoding AXnr1 (0.2 ng), TMEFF1/HA/Flag (1 ng), Cripto/ΔEGF/ΔCFC/mEGF1/mEGF2/mCFC (0.5-1 ng), and Cerberus (1 ng) were injected into both animal poles of two-cell-stage embryos. Animal caps were then cut at blastula stages 8.5-9, either dissociated and reaggregated immediately as described (Chang et al. 1997) or left intact, and incubated to early gastrula stages (stages 10-11) before total RNA was extracted for RT-PCR assay for gene expression. The primers used in the RT-PCR experiments were as described (Chang et al. 1997).

**Coimmunoprecipitation Analysis**

Coimmunoprecipitation with *Xenopus* protein embryonic extract was performed as described (Yeo and Whitman 2001), with the exception that no chemical cross-linker was used. Basically, RNAs encoding Cripto-F (2 ng), TMEFF1-HA/F (3 ng), ALK4-HA (3 ng), or AXnr1-HA (4 ng) were injected alone or in combination into two-cell-stage *Xenopus* embryos. The doses of RNA used for the competition experiment were as indi-
cated in the Figure 1 legend. Protein extract was made at early gastrula stages (stage 10+) and split into two halves. One half was immunoprecipitated with an anti-Flag antibody (Sigma), and the other half was precipitated with an anti-HA antibody (Covance). The precipitated samples were separated on 10% PAGE and transferred to Immobilon P membrane (Millipore). Western blot was subsequently performed to detect coprecipitated proteins.

For cell culture assay, CHO cells maintained in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum were transfected at 60%-70% confluence with the plasmids encoding TMEFF1/TMEFF1ΔEGF-HA (4-7 µg), TMEFF1ΔFS-HA (18 µg), and/or Cripto/CriptoΔEGF/CriptoΔCFC-Flag (2-3 µg), using FuGENE 6 reagent (Roche). Two days after transfection, the cells were lysed in the coimmunoprecipitation buffer (Yeo and Whitman 2001) in the presence of protease inhibitor cocktail (Roche), 2 µg/mL aprotinin, 2 µg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride. One percent of cell lysate was set aside for use as whole cell lysate. The remainder was precipitated with the anti-Flag antibody (Sigma) and analyzed by Western blot as described earlier.

Acknowledgments

We thank Drs. Tracy Keller, Malcolm Whitman, Eva Reissman, and Makoto Asashima for kindly providing plasmids used in this study, and Dr. Fannie Lin for technical advice on Western blot. P.W.H. is partly supported by the MSTP program, and C.C. is supported by HHMI Institutional Career Development Award and NIH grant HD43345.
Note Added in Proof

While TMEFF1 has been shown to inhibit nodal signaling in the *Xenopus* embryos, inhibition of nodal signaling by TMEFF1 and the closely related protein TMEFF2 has not been examined in the mammalian system. After this manuscript was accepted, we found evidence that TMEFF1 and TMEFF2 may inhibit nodal signaling in mammalian cells. Transient transfection of 293T cells with the nodal signaling components nodal, Cripto, and FAST2 results in activation of an activin-responsive luciferase reporter (Yan et al. 2002). Using this assay, our preliminary data indicates that human TMEFF1 and TMEFF2 inhibit luciferase activation in 293T cells. This is the first demonstration of TMEFF2 inhibition of Nodal signaling, and the first time that TMEFFs have been shown to inhibit nodal signaling in mammalian cells. In addition, human TMEFF1 and TMEFF2 associate with Cripto in coimmunoprecipitation assays, consistent with a conserved role for TMEFFs in binding Cripto to block nodal signaling in mammalian cells. We thank Drs. Michael Shen, Hiroshi Hamada, and Malcolm Whitman for kindly providing the constructs used for the luciferase assay.

References


cancer that is associated with disease progression and androgen independence. *Int. J. Cancer* **94**: 178-184.


CHAPTER 3

A NOVEL INTERACTION BETWEEN TOMOREGULINS AND ERBB RECEPTORS

by

PAUL HARMS AND CHENBEI CHANG

Prepared according to the guidelines of Cancer Research

Format adapted for dissertation
CHAPTER 3
A NOVEL INTERACTION BETWEEN TOMOREGULINS AND ERBB RECEPTORS

Abstract

Inappropriate activation of ErbB family receptor tyrosine kinase signaling occurs in many cancers and may promote tumor cell proliferation, survival, and migration. Better understanding of how ErbB signaling is regulated may reveal novel therapeutic targets for cancer. Tomoregulins (or TMEFFs, for transmembrane proteins with EGF-like and 2 follistatin domains) are proposed to act as antagonists of tumorigenesis and tumor progression. We have found a novel interaction between ErbB receptors and TMEFFs in coimmunoprecipitation assays. Domain-mapping suggests that TMEFFs bind ErbBs in a manner unlike conventional ErbB ligands. Despite the direct physical association, functional analyses of the consequence of TMEFF/ErbB interaction revealed that TMEFFs do not broadly affect ErbB signaling in tumor cell lines with respect to activation of downstream signaling pathways or stimulation of cell proliferation. TMEFFs may thus have subtle effects on ErbB-regulated processes in a cell type- and/or environment-dependent manner.

Introduction

The accumulation of mutations and changes in gene expression is central to the development and progression of cancer. Specifically, activating mutations or overexpression of oncogenes drives carcinogenesis by promoting inappropriate cell proliferation, survival, motility, and/or dedifferentiation (reviewed in 1). In concert with increased on
cogene activity, inactivating mutation or decreased expression of tumor suppressor genes permits tumor formation by removing checks on cell proliferation, motility, etc. Therefore, identification and characterization of genes with altered expression in tumors is a critical tool in expanding our understanding of the molecular basis of cancer.

In 2000, two labs independently identified a novel gene (hyperplastic polyposis protein 1 [HPP1] or transmembrane protein containing EGF and FS domains [TPEF]) which was silenced by promoter hypermethylation in colon cancers and tumor cell lines (2, 3). Around the same time, an mRNA for a gene product named TENB2 was found to be overexpressed in prostate cancer (4); a novel neuronal survival factor called transmembrane protein with epidermal growth factor-like and two follistatin domains (TMEFF2) was identified (5); and an ErbB4-activating protein named tomoregulin was described (6). It has since become generally accepted that these five molecules are in fact represented by a single gene product called TMEFF2 or tomoregulin-2. TMEFF1, the only known paralog of TMEFF2, has an identical domain structure to TMEFF2. Little progress has been made toward unifying these initial disparate observations into a coherent picture of functional role(s) for TMEFFs in cell homeostasis and tumorigenesis.

The evidence supporting a role for TMEFFs in suppressing tumor formation and/or progression remains largely correlative. TMEFF2 expression is silenced by promoter hypermethylation in a subset of colon neoplasias and polyps (3), as well as in gastric adenocarcinomas (7). The TMEFF2 promoter also undergoes hypermethylation in dysplasias and carcinomas of other sites in the gastrointestinal tract and in other tissues (8-12), although whether promoter hypermethylation corresponds with loss of expression has not been examined in most of these contexts. Likewise, TMEFF1 is downregulated in
primary brain tumors (astrocytomas, glioblastomas, and meningiomas) through an undetermined mechanism (13). However, TMEFF expression may be upregulated in a narrow range of malignancies (4, 14, 15), suggesting that tumor suppressor roles for TMEFFs may be tissue-dependent.

Consistent with roles in suppressing tumorigenesis, evidence suggests that TMEFFs regulate cell proliferation in some cell types. A U118 glioma cell line stably overexpressing TMEFF1 displayed a slower rate of proliferation than a vector control line (13). Stable TMEFF2 overexpression inhibits the proliferation of DU145 and PC3 prostate carcinoma cells (16). Conversely, TMEFF1 expression in canine mammary tumor cells increases cell numbers (17), possibly by stimulating proliferation. This suggests TMEFFs might regulate proliferation in a tissue-specific manner. The mechanism by which TMEFFs modulate cell proliferation remains to be determined.

The predicted domain structure of TMEFFs indicates mechanisms by which TMEFFs may modulate cell signaling to influence tumorigenesis. TMEFFs are cell-surface proteins which possess two follistatin-like (FS) domains, an EGF-like motif, a transmembrane domain, and a short cytoplasmic domain (18).

The presence of an EGF-like motif suggests a role for TMEFFs in signaling through the ErbB family of receptor tyrosine kinases. Four members of the ErbB family are present in mammals: EGFR/ErbB1, ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4 (19, for reviews, see 20). ErbB ligands display specificity for receptor binding; for instance, EGF displays high affinity for EGFR, while neuregulin-1-β1 (also known as heregulin-β1, or HRGβ1) binds ErbB3 and ErbB4 with high affinity. ErbB2 is not thought to bind ligands directly, but rather acts as the preferred heterodimerization
partner for ligand-bound ErbB receptors. EGFR and ErbB2 have oncogenic properties, and are overexpressed in a variety of cancers (19). In contrast, the role of ErbB4 in tumor development is less clear. Activated ErbB4 stimulates intracellular signaling cascades associated with the promotion of cell proliferation and survival, such as the extracellular signal-regulated kinase (ERK) and phosphoinositide-3-kinase (PI3K) pathways (21, for reviews, see 22). However, ErbB4 also promotes differentiation of some breast carcinoma cell lines (23), and ErbB4 expression in breast carcinomas may be associated with a more favorable prognosis (21).

TMEFF2 has been shown to specifically activate ErbB4 but not ErbB1/2/3 (6). It is possible that TMEFF-mediated ErbB4 activation might regulate proliferation and alter differentiation in certain contexts. The mechanism by which TMEFF2 activates ErbB4 has not been determined. The specific activation of ErbB4 but not ErbB1/2/3 by TMEFF2 is unusual, as most ErbB4 ligands also stimulate activation of ErbB2 via receptor heterodimerization (20). To our knowledge, Cripto is the only other factor which specifically activates ErbB4 but not ErbB1/2/3 (24). Cripto does not directly bind ErbB4, but rather binds a distinct cell-surface receptor and transactivates ErbB4 in a c-Src dependent manner (24, 25). This raises the possibility that TMEFF might also activate ErbB4 in an indirect manner.

The presence of FS domains in TMEFFs suggests additional mechanisms by which TMEFFs might regulate proliferation. FS domains are found in many inhibitors of TGF-β family signaling. Consistent with this, TMEFF1 has been shown to inhibit signaling by the TGF-β family factors bone morphogenetic protein-2 (BMP2) and the activin-like factors nodal and Vg1 in the Xenopus embryo (26). In addition to roles in develop-
ment, nodal and BMPs have activities in cancer. Specifically, nodal signaling promotes invasiveness and plasticity of melanoma cells (27), while BMP2 signaling has been shown to suppress proliferation and stimulate motility in breast carcinoma cells (28, 29). It is unknown whether TMEFFs suppress such responses to nodal or BMP in mammalian cells. In addition, an FS domain in the extracellular matrix protein SPARC mediates inhibition of signaling by basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (30-32). Therefore, TMEFF might modulate proliferation via effects on BMP or VEGF/FGF signaling.

The aim of this study was to identify mechanisms by which TMEFF regulates tumor cell proliferation. We produced stable U118 glioma cell lines which inducibly express TMEFF1 or TMEFF2. We found that inducible expression of TMEFFs in these cell lines did not affect cell proliferation, in contrast to a previous report using stable expression (13). We demonstrate a novel interaction between TMEFFs and members of the ErbB family of receptor tyrosine kinases; however, the significance of this interaction remains unclear, since inducible expression of TMEFFs may not modulate ErbB receptor signaling. We also demonstrate an interaction between TMEFF and FGFR1, but find that TMEFF expression does not affect the proliferative response to bFGF.

Materials and Methods

Cell Culture, Growth Factors, and Antibodies

U118, T47D, and COS7 cells were cultured in DMEM with phenol red, 10% FBS, and penicillin/streptomycin. T47D cells were a gift from Dr. Daniel Welch. U118 cells were a gift from Dr. Xinbin Chen. COS7 cells were obtained from the American
Type Culture Collection (ATCC). Recombinant human NRG1-β1 (heregulin-β1) and BMP2 were purchased from R&D Systems. Recombinant human EGF and bFGF were purchased from Sigma. Goat anti-TMEFF1 and goat anti-TMEFF2 were purchased from R&D Systems. Mouse anti-alpha-tubulin (clone B-5-1-2), polyclonal rabbit anti-Flag, and mouse anti-Flag M2 were purchased from Sigma. Polyclonal rabbit anti-HA (HA.11), monoclonal mouse anti-HA, and monoclonal mouse anti-GluGlu were purchased from Covance. Anti-EGFR (sc-03) and anti-ErbB3 (sc-285) were purchased from Santa Cruz Biotechnology. Rabbit anti-phospho-AKT (T308) and rabbit monoclonal anti-phospho-EGFR (Y1173) were purchased from Cell Signaling. Polyclonal rabbit antiphosphorylated (dp) ERK was purchased from Promega. Mouse anti-ERK2 (clone 33) was purchased from BD Transduction Laboratories. Peroxidase-conjugated donkey anti-rabbit and peroxidase-conjugated donkey anti-mouse were purchased from Jackson Immunoresearch.

Plasmid Constructs for Tagged Proteins, Mutant Proteins, and Tetracycline-Inducible Expression

The following constructs were generated previously: EGFR-pCS2++, ErbB3-pCS2++, Flag/ErbB2-pCS2++, and Flag/ErbB4-pCS2++ (Nie and Chang 2006); X7365/HA-ΔC/HA and X7365/HA-ΔTC/HA (Chang et al. 2003); and X7365/HA and X7365ΔFS/HA (33). PCR-based strategy was used to generate all constructs. Final constructs were verified by DNA sequencing. For hTMEFF1/HA-pCS2++, an N-terminal primer hTR1.5’(HindIII): ATTAAGCTTACCATGGCGCGCGCGCATGGATAAAAGC-
CATTCTGGATGAC and hTR1.HA.3’2(XbaI): CCGCTCTAGAT-TATGCGTAGTCAGGGACATCATATGGA were used for PCR over the hTMEFF1/pBlueScriptR template (obtained from the ATCC). The PCR product was digested and inserted into the HindIII/XbaI sites of pCS2++ vector. For hTMEFF2/HA-pCS2++, an N-terminal primer hTR2.5’(HindIII): CCCAAGCTTAC-CATGGTGCTGTGGGAGTC and two C-terminal primers hTR2.HA.3’1: TCAGGGACATCATATTGAGTAGATTACCTCGTGGA and hTR2.HA.3’2(XbaI): GCTCTAGATTATGCGTAGTCAGGGACATCATATGGA were used for PCR over the hTMEFF2-pCMV-Sport6 template (obtained from the ATCC). The PCR product was digested and inserted into the HindIII/XbaI sites of pCS2++ vector. For Glu-Glu/hTMEFF1-pCS2++, two PCR reactions were performed, with the N-terminal fragment that inserted a GluGlu tag N-terminal to proline 43 of hTMEFF1 using the primers hTR1.5’(HindIII), hTR1.EE.N3’1: TGGCATATATTGTTGGAGCGCGCGGCT, and hTR1.EE.N3’2(BamHI): GCGGATCCTCCATTGACGTATATCCTGGGAGTC and a C-terminal fragment amplified using the primers hTR1.EE.C5’: GGATC-CACCGGTGTGGGACGTATATCCTGGGAGTC and hTR1.3’(XbaI): CGCGTCTAGATTAAC-CATTCTGGATGACGTATCGTTGGGAGTC were used for PCR over the hTMEFF1/pBlueScriptR template. Fragments were digested with HindIII/BamHI and BamHI/XbaI, respectively, and inserted into the HindIII/XbaI sites of pCS2++ vector. For GluGlu/hTMEFF1ΔEGF-pCS2++, two PCR reactions were performed, with the N-terminal fragment amplified with the primers hTR1.5’(HindIII) and hTMEFF1ΔEGF.3’(BglII): CTCAGATCTAGG-CATGTGTTTCCAATATAACATC and the C-terminal fragment amplified with the primers hTMEFF1ΔEGF.5’(BglII): CGGAAGATCTGAAAGACGACTTTAG-
TATTCTCTATGTAG and hTR1.3’(XbaI) on GluGlu/hTMEFF1 template. Fragments were digested with HindIII/BglII and BglII/XbaI, respectively and inserted into the HindIII/XbaI sites of pCS2++ vector. The final hTMEFF1ΔEGF construct lacks residues C274-C310, and contains a two-residue insertion (arginine-serine) at the site of the deletion corresponding to the introduced BglII digest site. For Glu-Glu/hTMEFF1ΔEGF+-pCS2++, the primers hTR1.5’(HindIII) and hTR1d236-310.3’(BglII): CTCA-GATCTGCAATGACCAAGATGCCTTATATC were used for PCR over the GluGlu/hTMEFF1ΔEGF-pCS2++ template. The PCR product was digested with HindIII/BglII and inserted into GluGlu/hTMEFF1ΔEGF digested with HindIII/BglII, replacing the N-terminal portion of GluGlu/hTMEFF1ΔEGF. The final hTMEFF1ΔEGF+ construct lacks residues T236-C310, and contains a two-residue insertion (arginine-serine) at the site of the deletion corresponding to the introduced BglII digest site. For Flag/xFGFR1-pCS2++, the primers Flag/FGFR.5’(NotI) CTTGCAGCGCGGCTCCACCTCCCTGAT and CS2(AscI) AGGCCAGCCGCGGATTTAAAAACCT were used for PCR over the xFGFR1/pCS2++ template. The PCR product was digested with NotI/AscI and inserted into Flag/ErbB4-pCS2++ digested with NotI/AscI, replacing ErbB4 with xFGFR1 to produce a construct containing xFGFR1 modified with an N-terminal preprotrypsin signal peptide and Flag tag. For Flag/PDGFRα-pCS2++, the primers Flag/PDGFR.5’(NotI) CTTGCGGCGCGGAAAATCCTTTGCCCACC and CS2(AscI) AGGCCAGCCGCGGATTTAAAAACCT were used for PCR over the PDGFRα/pCS2++ template. The PCR product was digested with NotI/AscI and inserted into Flag/ErbB4-pCS2++ digested with NotI/AscI, replacing ErbB4 with PDGFRα to
produce a construct containing PDGFRα modified with an N-terminal preprotrypsin signal peptide and Flag tag.

The plasmids for the tetracycline inducible system (pcDNA4/TO and pcDNA6/TR) were kindly provided by Dr. Xinbin Chen. TMEFF constructs were subcloned into the pcDNA4/TO vector from the pCS2++ vector by digesting TMEFF/pCS2++ constructs with HindIII/XbaI and inserting the digest product into the HindIII/XbaI sites of the pcDNA4/TO construct.

Establishment of Stable Inducible Cell Lines

All transfections were achieved using Lipofectamine-2000 (Invitrogen) in Opti-MEM (Gibco) as per the manufacturer’s instructions. For T47D cells, cells in 10-cm dishes were co-transfected with three plasmids: (1) 600 ng pBabe-puro (to improve selection efficiency), (2) 6 μg pcDNA4/TO vector (for tetracycline inducible expression), either empty or containing hTMEFF1/HA, and (3) 6.4 μg pcDNA6/TR, which represses transcription from pcDNA4/TO in the absence of tetracycline. Transfectants were selected in 1 μg/ml puromycin and 10 μg/ml blasticidin. Individual clones were screened by immunoblot analysis using anti-HA antibodies. For U118 cells, cells in 6-well plates were co-transfected with three plasmids: (1) 100 ng pBabe-puro (to improve selection efficiency), (2) 1.2 μg pcDNA4/TO vector (for tetracycline inducible expression), either empty or containing hTMEFF1/HA, hTMEFF1, hTMEFF2, or hTMEFF2/HA, and (3) 1.3 μg pcDNA6/TR, which represses transcription from pcDNA4/TO in the absence of tetracycline. Transfectants were selected in 0.5 μg/ml puromycin, 2 μg/ml blasticidin,
and 10 μg/ml Zeocin. Individual clones were screened by immunoblot analysis using anti-TMEFF1, anti-TMEFF2, or rabbit anti-HA antibodies.

**Phosphorylation Studies**

1x10^5 U118 cells were seeded per well of 6-well dish, in the presence or absence of 2 μg/μl tetracycline. After one day of growth, cells were washed twice with PBS and cultured in serum-free DMEM supplemented with 0.1% BSA overnight, with or without tetracycline as indicated. Immediately prior to growth factor treatment, cells were washed once with serum-free DMEM. 100 ng/ml growth factor was added in serum-free DMEM with 0.1% BSA. Cells were treated for five minutes at 37°C. At the completion of treatment, cells were placed on ice, washed twice with chilled PBS, and collected in lysis buffer containing 1% SDS and 60 mM Tris. Lysate was boiled immediately, and then sonicated. The BCA protein assay (Pierce) was used to quantitate protein content of lysates to equalize loading as necessary. 10-20 μg lysate was separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in TTBS with 5% nonfat dry milk and incubated overnight with the indicated phospho-specific antibody in TTBS with 1% BSA. The following dilutions of primary antibody were used: 1:5000 anti-dpERK, 1:3000 anti phospho-EGFR, or 1:3000 anti-phospho-S473-AKT. Bound antibody was detected by peroxidase-conjugated anti-rabbit and Enhanced Chemiluminescence Plus (ECL+) (Amersham Biosciences). For antibodies which were not phospho-specific, antibody was diluted in TTBS with 1% nonfat dry milk. The following dilutions were used: 1:10,000 anti-tubulin, 1:8000 anti-ERK2, 1:1000 anti-TMEFF1, 1:1000 anti-
TMEFF2, and 1:3000 anti-EGFR. Bound antibody was detected by peroxidase-conjugated secondary antibody and ECL+ (Amersham Biosciences).

**Cell Proliferation Assay**

Cells were seeded in triplicate into 6-well plates, in the presence or absence of 2 μg/ml tetracycline. 3x10^4 cells were used, unless otherwise indicated. At the indicated time-points, adherent cells were collected by trypsinization and counted three times using a Coulter cell counter (Coulter, Corporation, Miami, FL, USA). In some cases, remaining cells were collected and lysed for protein expression. For assays of proliferation in serum-free conditions, cells were allowed to attach overnight before washing twice in 1X PBS and addition of serum-free media supplemented with 0.1% BSA ± growth factor.

**Immunoprecipitation and Immunoblotting**

COS7 cells in 10 cm dishes were transiently transfected using Lipofectamine-2000 (Invitrogen) in Opti-MEM (Gibco), as per the manufacturer’s instructions. The following amounts of plasmid were used, as indicated: 8 μg hTMEFF1/HA-pCS2++, 8 μg hTMEFF2/HA-pCS2++, 5 μg EGFR-pCS2++, 5 μg Flag/ErbB2-pCS2++, 5 μg ErbB3-pCS2++, 5 μg Flag/ErbB4-pCS2++, 12 μg Flag/FGFR1-pCS2++, 12 μg Flag/PDGFRα-pCS2++, 4 μg xTMEFF1/HA-pCS2++, 8 μg xTMEFF1ΔFS/HA-pCS2++, 12 μg xTMEFF1ΔC/HA-pCS2++, 12 μg xTMEFF1ΔTC/HA-pCS2++, 8 μg GluGlu/hTMEFF1-pCS2++, 8 μg GluGlu/hTMEFF1ΔEGF-pCS2++, or 8 μg GluGluhTMEFF1ΔEGF+-pCS2++. Two to three days after transfection, cells were washed twice in 1X PBS and lysed in coimmunoprecipitation buffer (34) with 2 μg/ml apro tinin, 2 μg/ml leupeptin, 1
mM phenylmethylsulfonyl fluoride (PMSF), and 1:50 complete EDTA-free protease inhibitor cocktail (Roche). One percent of lysate was set aside for use as whole cell lysate. The remainder of cell lysate was precipitated with 1 μg of the indicated antibody (mouse anti-Flag, mouse anti-HA, rabbit anti-EGFR, or rabbit anti-ErbB3) and protein-G or protein-A agarose beads (Roche) for at least 3 hours at 4°C. Precipitated samples were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Immunoblot was subsequently performed to detect precipitated protein. The following dilutions of antibody in TTBS with 1% milk were used for immunoblotting: 1:1000 anti-Flag rabbit antibody, 1:1000 anti-HA mouse antibody, 1:1000 anti-HA rabbit antibody, 1:1000 anti-GluGlu mouse antibody, 1:1000 rabbit anti-EGFR, 1:4000 anti-ErbBB3, and 1:1000 anti-ErbB4.

Results

TMEFFs and Cell Proliferation

TMEFF1 may inhibit proliferation in U118 glioma cells, since a U118 cell line stably overexpressing TMEFF1 displayed slower growth relative to a vector control cell line (13). To better understand this phenomenon, we produced stable U118 cell lines which inducibly express TMEFF1 or TMEFF2 under control of the tetracycline-on system. Tetracycline-regulated expression of TMEFFs was confirmed by immunoblot (Figure 1A and B). We were unable to demonstrate inhibition of proliferation when expression of TMEFF1 was induced in U118-9J cells cultured in 10% serum (Figure 1C, upper panel). In addition, TMEFF1 failed to affect proliferation in two additional inducible TMEFF1 clonal cell lines examined, or in a TMEFF1/HA inducible cell line (data not
Figure 1. TMEFFs do not modulate glioma proliferation. (A) Inducible TMEFF1 expression in U118-9J cells. (B) Inducible TMEFF2 expression in U118-11A cells. Cells were cultured for 3 days in the presence or absence of tetracycline, then lysate was collected and TMEFF expression determined by immunoblotting (IB). (C) TMEFF1 induction does not affect U118-9J proliferation in 10% or 0% serum. 3x10^4 cells were seeded per well in the presence (+) or absence (-) of tetracycline to regulate TMEFF1 expression. For assays in 0% serum, cells were allowed to attach overnight in 10% serum before serum starvation. Adherent cells were collected and counted by Coulter counter at the indicated timepoints. Media and tetracycline were renewed every 3 days. (D) TMEFF2 induction does not affect U118-11A proliferation in 10% or 0% serum. Proliferation was measured as described in (C).
shown). TMEFF2 or TMEFF2/HA did not modulate glioma proliferation in inducible lines grown in 10% serum (Figure 1D, upper panel, and data not shown). While TMEFFs did not inhibit glioma proliferation, they also did not promote proliferation in serum-free conditions (Figure 1C and D, lower panels). A slight increase in cell numbers of T-MEFF1-induced cells relative to uninduced cells in serum-free conditions was sometimes observed, but this was not a strong or consistent effect. We also addressed the effect of TMEFF1 overexpression on T47D breast carcinoma proliferation. A T47D cell line was produced which inducibly expresses TMEFF1/HA under the tetracycline-on system (Figure 2A). We found that TMEFF1/HA overexpression had no significant effect on breast carcinoma proliferation when cells were cultured in 10% fetal bovine serum (Figure 2B). Furthermore, TMEFF1/HA did not promote proliferation in serum-free conditions (Figure 2C; see also Figures 6 and 7). Over many experiments we observed a weak trend toward increased cell number of TMEFF1-expressing cells relative to uninduced cells in serum-free conditions, but this increase did not typically reach statistical significance.

A Novel Interaction Between TMEFFs and ErbB Receptors

As TMEFFs did not have a direct effect on cell proliferation in our hands, we addressed the possibility that TMEFFs might regulate proliferation in a more specific manner by activating or modulating ErbB4. To determine whether TMEFF activates ErbB4 via direct binding, or mediates transactivation via a distinct receptor, we performed a series of coimmunoprecipitations to determine whether TMEFF directly associates with ErbB4. We found that TMEFF1 and TMEFF2 coimmunoprecipitate with ErbB4, consistent with a role for TMEFFs as direct ligands, coreceptors, or modulators for ErbB4.
Figure 2. TMEFF1/HA does not modulate breast carcinoma proliferation. (A) Inducible TMEFF1/HA expression in T47D cell lines. Cells were cultured for 3 days in the presence or absence of tetracycline, then lysate was collected and TMEFF expression determined by immunoblotting (IB). (B) TMEFF1/HA induction does not affect T47D-2T proliferation in 10% serum. 3x10⁴ cells were seeded per well in the presence (+) or absence (-) of tetracycline to regulate TMEFF1 expression. (C) TMEFF1/HA induction does not stimulate proliferation in serum-deprived T47D-2T cells. 6x10⁴ cells were seeded per well in the presence (+) or absence (-) of tetracycline and allowed to attach overnight prior to serum starvation in the presence or absence of tetracycline. For (B) and (C), adherent cells were collected at the indicated timepoints and counted by Coulter counter. Media and tetracycline were renewed every three days.
(Figure 3A, lanes 7 and 8). However, TMEFF1 and -2 also associated with ErbB2, which is not thought to directly bind ligands (Figure 3A, lanes 4 and 5). In addition, we observed that TMEFFs associate with EGFR and ErbB3 (Figure 3B, C), and that TMEFF1 associates with FGFR1 (Figure 3D, lane 3). Some experiments suggested a weak association between TMEFF1 and PDGFRα, although this interaction was not consistently observed (Figure 3D [lane 4] and data not shown). Therefore, we were unable to demonstrate specificity with regard to the receptor tyrosine kinase associating with TMEFFs.

*Xenopus* TMEFF1 (xTMEFF1) was also capable of associating with ErbB4, suggesting that the TMEFF/ErbB association may be conserved through evolution (Figure 4B, lane 2).

We reasoned that if TMEFF were binding ErbBs in the manner of a conventional ligand, the EGF domain would be critical for this interaction. Therefore, we performed coimmunoprecipitations with TMEFF deletion mutants to determine the domain of TMEFF involved in the TMEFF/ErbB interaction. Figure 4A shows a diagrammatic representation of deletion mutants used in this study. We found that the transmembrane and cytoplasmic domains (Figure 4B), the FS domain (Figure 4C), and the EGF domain (residues C274-C310) (Figure 4D, lane 4) of TMEFF1 were all dispensable for TMEFF association with ErbB4. However, the TMEFF1ΔEGF+ mutant, which lacks the EGF domain and the adjacent region between the EGF and FS domains (residues T236-C310), was severely impaired in its ability to associate with ErbB4 (Figure 4D, lane 6). The lack of requirement for the EGF domain in TMEFF/ErbB interaction suggests that TMEFFs may bind ErbB receptors in a manner unlike orthodox ErbB ligands. Thus, TMEFFs on
Figure 3. TMEFFs associate with ErbB receptors. (A) TMEFF1 and TMEFF2 coimmunoprecipitate with ErbB2 and ErbB4. (B) TMEFF1 and TMEFF2 coimmunoprecipitate with EGFR. (C) TMEFF1 and TMEFF2 coimmunoprecipitate with ErbB3. (D) TMEFF1 coimmunoprecipitates with FGFR1 and PDGFRα. Immunoprecipitation (IP) and immunoblotting (IB) was performed on lysate from COS7 cells transiently transfected with the indicated constructs.
Figure 4. TMEFF residues 236-310 influence TMEFF/ErbB association. (A) Deletion mutants used for domain mapping. SP = signal peptide, GG = GluGlu tag, FS = follistatin domains, EGF = epidermal growth factor-like motif, EGF+ = EGF and flanking region (residues 236-310), TM = transmembrane domain, C = C-terminal cytoplasmic domain, HA = HA tag. (B) TMEFF transmembrane and cytoplasmic domains are dispensable for TMEFF/ErbB4 association. (C) TMEFF FS domains are dispensable for TMEFF/ErbB4 interaction. (D) The TMEFF EGF domain is dispensable for TMEFF/ErbB interaction, but deletion of EGF and flanking region (EGF+, residues 236-310) is strongly attenuates association. For all experiments, immunoprecipitation (IP) and immunoblotting (IB) was performed on lysate from COS7 cells transiently transfected with the indicated constructs.
the cell surface might be positioned to directly modulate ErbB signaling in a novel manner.

*TMEFFs Do Not Clearly Modulate ErbB Signaling*

To examine the possibility that TMEFFs modulate ErbB signaling, we utilized U118 glioma cells inducibly expressing TMEFF1 or TMEFF2. We found that expression of TMEFF1 in U118-9J glioma cells did not modulate response to EGF or the ErbB3/4 ligand HRG with respect to ERK phosphorylation (Figure 5A). The same was true for TMEFF2 expression in U118-11A cells (Figure 5B). However, U118 cells were of limited use in this study, as in our hands they did not show a consistent response to HRG, nor did these cells display a proliferative or survival response to EGF or HRG (data not shown). Our results suggested that basal levels of ERK phosphorylation might be affected by induction of TMEFF expression; we are currently investigating this possibility.

T47D breast carcinoma cells express all four ErbB receptors and respond to both to EGF and HRG (35-37), and therefore are more suitable to a broad study of ErbB signaling. We found that TMEFF1/HA expression did not significantly affect the proliferative response to EGF or HRG in T47D-2T cells (Figure 6A and B).

*TMEFF1 May Not Modulate the Proliferative Response to bFGF*

TMEFF association with FGFR1 (see Figure 3D) suggested another possible mechanism by which TMEFF might affect tumor growth, as FGF signaling enhances proliferation of tumor cells, including breast carcinoma and glioma cells (36, 38-40). Expression of TMEFF1/HA was associated with a transient decrease in proliferation in only
Figure 5. TMEFFs do not modulate ErbB signaling in glioma cells. 

(A) TMEFF1 induction does not modify EGF signaling in U118-9J cells. Cells were cultured in the presence or absence of tetracycline for 24 hours to regulate TMEFF1 expression. Cells were serum-starved overnight in the presence or absence of tetracycline, then treated at 37°C for 5' with 100 ng/ml EGF or HRG, or left untreated. 

(B) TMEFF2 induction does not alter EGF signaling in U118-11A cells. U118-11A cells inducibly expressing TMEFF2 were treated as in (A). IB = immunoblot, dpERK = diphosphorylated ERK, pEGFR = phospho-EGFR (Y1173).
Figure 6. TMEFF1 does not alter the proliferative response to EGF or HRG in T47D-2T breast carcinoma cells. $6 \times 10^4$ T47D-2T cells were allowed to attach overnight in the presence (+) or absence (-) of tetracycline to regulate TMEFF1 expression, then serum-starved in the presence or absence of growth factor and the presence or absence of tetracycline. Adherent cells were collected at the indicated timepoints and counted by Coulter counter. Growth factors, tetracycline, and media were renewed every 3 days.
one out of three experiments (Figure 7), thus TMEFF1 is not likely to affect the proliferative response to bFGF in T47D cells. In our hands U118 cells did not display a proliferative response to bFGF (data not shown), so the effect of TMEFF on bFGF signaling was not examined in these cells.

Discussion

Despite evidence that TMEFF expression is altered in a number of malignancies, little is understood regarding how TMEFFs might contribute to or inhibit tumorigenesis. The goal of these studies was to examine ways in which TMEFF expression might alter tumor cell characteristics such as growth factor signaling and proliferation.

To directly examine the effect of TMEFF on cell proliferation, we utilized U118 glioma cells, in which stable TMEFF1 overexpression is reported to inhibit proliferation (13). RT-PCR and Western blot analyses of TMEFF expression suggest we achieved robust inducible expression relative to endogenous levels of TMEFFs (data not shown). We found that stable inducible U118 cell lines did not display decreased growth upon expression of TMEFF1. There are several possible explanations for the discrepancy between our findings and published results. TMEFF1 inhibition of proliferation was observed using a stable cell line which constitutively overexpressed TMEFF1, whereas we utilized stable inducible cell lines. When comparing stable cell lines with constitutive expression to empty vector or parental control lines, differences in proliferation may be due to clonal differences between the cell lines (e.g. in the genomic insertion site of the expression construct) rather than overexpression of the protein of interest. This concern is mostly eliminated when using inducible lines. In addition, the previous study reported results
Figure 7. TMEFF1 may not alter the proliferative response to FGF signaling in T47D-2T breast carcinoma cells. 3x10⁴ T47D-2T cells were allowed to attach overnight in the presence (+) or absence (-) of tetracycline to regulate TMEFF1 expression, then serum-starved 24 hours prior to addition of growth factor in the presence or absence of tetracycline. Adherent cells were collected after 3 days (A, B) or 2 days (C) of treatment and counted by Coulter counter. Each graph represents results from one experiment done in triplicate.
from a single clone, whereas we examined several separate lines to rule out idiosyncrasies of any particular clone. Along the same lines, it is plausible that the disparity between our results is due to clonal variations or differences which have arisen during passaging, such that one group of U118 cells has become refractory or sensitized to the effects of TMEFF1 overexpression. Another possible explanation for the discrepancy is that our inducible lines may express lower levels of TMEFF1 than the line in which constitutive TMEFF1 expression was found to inhibit proliferation. Finally, the previous study assessed cell proliferation by the MTT assay, which estimates cell number by measuring cellular conversion of a metabolic substrate introduced into the culture media. In contrast, we counted cells directly by Coulter counter, which does not account for floating or loosely adherent cells (which are removed by washes prior to counting), nor does it measure cell viability (although typically most dead cells are removed by washing). The possibility that TMEFF1 expression alters U118 survival and/or adhesion requires further investigation.

While we did not find a general role for TMEFFs in regulating cell proliferation, the possibility remains that TMEFFs exert more restricted effects on cell cycle progression by modulating growth factor/receptor tyrosine kinase signaling. We demonstrate here for the first time an interaction between TMEFFs and members of the ErbB family of receptor tyrosine kinases. As TMEFF2 is reported to activate ErbB4, the association of TMEFFs with ErbB4 in coimmunoprecipitation assays suggested TMEFFs might bind ErbB4 as ligands. However, we also find that TMEFFs associate with ErbB1/2/3, receptors that TMEFFs are not reported to activate. This suggests that TMEFF is not binding ErbBs in the manner of a conventional ligand. Consistent with this, we found that dele-
tion of the EGF domain of TMEFF1 did not abrogate association with ErbB4, but deletion of both the EGF domain and a flanking region severely attenuated TMEFF1/ErbB4 association. In addition, TMEFFs associated with the receptor tyrosine kinase FGFR1. The lack of specificity in TMEFF binding to receptor tyrosine kinases suggests TMEFFs might have a broad role in modulating receptor tyrosine kinase activity. However, inducible expression of TMEFFs in U118 glioma cells did not alter ERK phosphorylation in response to EGF. In addition, inducible expression of TMEFF1/HA did not modulate the proliferative response of T47D breast carcinoma cells to EGF or the ErbB3/4 ligand HRG. TMEFFs might modulate ErbB function more subtly, by altering the kinetics or dose-response of ErbB signaling. It is also possible that TMEFFs specifically modulate ErbB signaling via pathways not examined in this study, or that TMEFFs regulate the interaction of ErbBs with other modulators of ErbB signaling (e.g. proteins which associate with ErbBs to mediate ligand-independent ErbB transactivation). Also, ErbBs might modulate TMEFF function without significant effect on ErbB signaling. An additional consideration is that we examined only full-length TMEFFs in this study. Since TMEFF2 ectodomain can be cleaved and released from the plasma membrane (41), TMEFFs might require shedding from the membrane to become activated for modulation of ErbB signaling; overexpressed membrane-bound TMEFFs would be nonfunctional in such a setting unless shedding was stimulated.

Previous studies have shown that TMEFF1 and -2 localize to the plasma membrane (13, 42). One concern is whether inducibly expressed TMEFFs are localized to the cell membrane in the cell lines used in this study. Preliminary data suggests TMEFF2/HA may not localize to the plasma membrane in our cell lines. Glycoproteins with impaired
maturation and retention in the ER may be sensitive to digestion with Endoglycosidase H (Endo H). Our preliminary data indicates that TMEFF2/HA expressed in U118 inducible cell lines are sensitive to Endo H digestion (data not shown). In line with retention of TMEFFs in the ER, TMEFF2/HA expressed in a U118 inducible line displayed punctate cytoplasmic staining consistent with localization to the secretory pathway in a preliminary immunofluorescence study (data not shown). We have not examined the Endo H sensitivity and cellular localization of untagged TMEFFs. If TMEFFs are indeed retained in the ER in our cell lines, this would prevent them from interacting with ErbBs at the cell surface. Therefore, our inability to detect modulation of ErbB signaling by TMEFFs may be due to protein mislocalization.

The association of TMEFF with FGFR1 is intriguing because another FS domain-containing protein, the extracellular matrix protein SPARC, inhibits bFGF promotion of angiogenesis via its FS domain (32). However, TMEFF1 did not modulate cell proliferation in response to bFGF.

Overall, the demonstration of an interaction between TMEFF and ErbB receptors suggests new functions for TMEFFs in modulation of growth factor signaling. While TMEFF1 does not appear to alter the proliferative response to EGF or HRG, TMEFFs may regulate other aspects of ErbB signaling, such as stimulation of cell motility or survival. Further investigation of the TMEFF/ErbB interaction promises to reveal novel molecular roles for TMEFFs in tissue homeostasis and cancer.
Acknowledgments

We are grateful to Yun-Ju Lai, Gordon Meares, and Dr. Fang-Tsyrr Lin for technical advice on Western blot studies of signaling protein phosphorylation. We thank Drs. Monica Richert and Daniel Welch for kindly providing the T47D cell line used in this study. We also thank Drs. Mark Stonecypher and Steven Carroll for technical advice on immunoprecipitation of ErbB receptors. We are also grateful to Kelly Harms and Dr. Xinbin Chen for generously providing resources, plasmids, and technical advice related to the establishment of stable inducible lines and cell proliferation assays, as well as the U118 cell line.

References


CHAPTER 4
SUMMARY AND DISCUSSION

The similarity between tumors and embryonic tissues has been recognized for over a century. Although the pathogenesis of cancer is rarely so straightforward as a simple reversion to embryonic form, it has become increasingly evident that many of the same genes and signaling pathways which play strictly regulated roles in embryogenesis may also become dysregulated in adult tissues to promote tumorigenesis. Each of the major signaling axes critical for embryogenesis (including fibroblast growth factor [FGF], Hedgehog, Wnt, and TGF-β) has been shown to become imbalanced in human cancers (for reviews, see Calvo and Drabkin 2000; Grose and Dickson 2005). Therefore, further understanding of the control of signaling pathways in embryogenesis will yield insight into how these same pathways may become dysregulated in tumorigenesis, potentially revealing novel molecular targets for cancer therapy.

TMEFFs act to inhibit TGF-β family signaling in developing embryos (Chang et al. 2003). In addition, expression of TMEFFs is altered in cancers, suggesting TMEFFs might act as tumor suppressors in certain tissues (Young et al. 2001; Shibata et al. 2002; Gery et al. 2003; Geddert et al. 2004; Takahashi et al. 2004; Suzuki et al. 2005; Brucher et al. 2006b; Suzuki et al. 2006). In these studies, we have addressed mechanistic roles for TMEFFs in regulating cell signaling in embryogenesis and tumor cells. We demonstrate that TMEFFs associate with the nodal coreceptor Cripto to inhibit nodal signaling in *Xenopus* embryos and mammalian cells. We also examine mechanisms by which
TMEFFs might regulate cell proliferation. We demonstrate a novel interaction between TMEFFs and ErbB receptor tyrosine kinases. However, the significance of this association is unclear, as overexpression of TMEFFs may not modulate ErbB activation in response to ligands. In addition, we demonstrate that TMEFF does not play clear roles in modulating control of breast carcinoma cell proliferation by FGF signaling.

TMEFF1 Inhibits Nodal Signaling Through Direct Binding the Nodal Coreceptor Cripto

Nodal is a TGF-β family ligand which plays critical and highly conserved roles in mesendoderm induction, left/right asymmetry, and other patterning events during embryogenesis (for reviews, see Whitman 2001; Schier 2003). Six nodal-related genes (Xnr1-6) are present in Xenopus, whereas only one nodal gene is present in mammals. Nodals signal through the type I receptor activin receptor-like kinase (ALK)-4 or -7 and the type II receptor ActRIIA/B. In addition, the presence of an EGF-CFC coreceptor such as Cripto is required for assembly of a functional nodal/ALK4/ActRII signaling complex (Gritsman et al. 1999; Reissmann et al. 2001; Yeo and Whitman 2001; Bianco et al. 2002; Yan et al. 2002).

Due to its central role in embryogenesis, nodal signaling is subject to stringent spatiotemporal regulation by many factors. Expression of nodal ligand can be induced by transcription factors such as VegT or in response to Notch signals, signaling components of the Wnt pathway, or nodal itself (Schier 2003). The stability and signaling activity of newly synthesized nodal proteins are modified by prodomain cleavage and homo- or heterodimerization with other TGF-β family ligands (Chang 2006 [in press]). Secreted nodal factors are inhibited by soluble antagonists such as Cerberus. Additional regulation
occurs at the levels of receptor stability and activation, signal transduction by Smads, and target gene activation/repression (Schier 2003; Chang 2006 [in press]).

Here, for the first time, we show that TMEFF1 inhibits nodal signaling at the receptor level by binding the nodal coreceptor Cripto, an EGF-CFC family protein. TMEFF1 association blocks the CFC domain of Cripto from associating with the nodal receptor ALK4. Thus, TMEFF1 prevents the formation of a functional nodal/Cripto/ALK4/ActRII signaling complex.

The manner in which TMEFF inhibition of nodal signaling contributes to embryonic patterning is still unknown, but the expression pattern of *Xenopus* TMEFF1 suggests several possibilities. (There is no apparent TMEFF2 homolog in *Xenopus.*) Expression of TMEFF1 in the *Xenopus* embryo does not markedly correlate with patterns of nodal expression, suggesting that TMEFF restricts nodal signaling in a spatiotemporal rather than general manner. In *Xenopus*, TMEFF1 expression begins during gastrulation, where TMEFF1 is strongly expressed in the animal region (future ectoderm) (Chang et al. 2003). Since nodal is a potent inducer of mesendoderm, perhaps TMEFF1 plays a role in preventing the mesoderm-inducing activity of nodal from influencing the presumptive ectoderm in the animal region. In line with this, TMEFF1 is only weakly expressed in the marginal zone, which is destined to become mesoderm and endoderm (Chang et al. 2003). During late neurulation, TMEFF1 expression is restricted to neural folds and the dorsal neural tube in the trunk region. At this stage, TMEFF1 might act to limit the signaling range of the nodal homolog Xnr4, which is expressed in the notochord and ventral neural tube (Jones et al. 1995). In mice, TMEFF1 is expressed throughout embryogenesis, suggesting a broader role for TMEFF1 in the patterning of mammalian embryos (De
Groot et al. 2000). Additional studies are needed to determine whether TMEFF1 indeed modulates nodal signaling in these settings.

In addition to nodal, TMEFF1 also inhibits signaling by Vg1, a TGF-β family factor which requires Cripto for signaling (Chang et al. 2003; Cheng et al. 2003). Future investigations might also address whether TMEFF inhibits signaling by other TGF-β family members that utilize the Cripto coreceptor, such as derriere and GDF3 (Chen et al. 2006; Dorey and Hill 2006; Onuma et al. 2006). In *Xenopus*, three EGF-CFC family members (FRL1/XCR1, XCR2, and XCR3) are present, and display differential affinities for different nodal ligands (Dorey and Hill 2006; Onuma et al. 2006). Although we demonstrated TMEFF1 association with the *Xenopus* EGF-CFC family member FRL1/XCR1, TMEFF1 association with XCR2 and XCR3 remains to be examined. In addition, EGF-CFC family proteins have been shown to participate in signaling pathways independent of nodal/ALK4, including activation of the ERK pathway (Kannan et al. 1997), inhibition of activin signaling (Adkins et al. 2003; Gray et al. 2003), and participation in Wnt signaling (Tao et al. 2005). Further studies are needed to determine whether TMEFF association with EGF-CFC family members inhibits these nodal/ALK4-independent activities.

Understanding the mechanism by which TMEFFs regulate nodal signaling may also yield insight into the differential roles played by TGF-β family members in development. There is a dichotomy between Cripto and TMEFF with respect to signaling by the TGF-β family members nodal and activin. Cripto facilitates nodal signaling and inhibits activin signaling (Reissmann et al. 2001; Yeo and Whitman 2001; Bianco et al. 2002; Yan et al. 2002; Adkins et al. 2003; Gray et al. 2003), whereas TMEFF inhibits
nodal signaling but is permissive toward activin signaling (Chang et al. 2003). Perhaps Cripto and TMEFF are expressed as molecular switches to regulate signaling by activin or nodal. Interestingly, another level of control may be exerted by regulation of the TMEFF/Cripto association. In line with this, TMEFF2 may be proteolytically shed from the cell surface (Lin et al. 2003) and soluble TMEFF1 does not inhibit nodal signaling (Chang et al. 2003). Thus, stimulation of TMEFF shedding from the cell surface might abolish the inhibition of nodal signaling by TMEFFs.

TMEFFs are also expressed postnatally, raising the question of whether TMEFF inhibition of nodal signaling might also play roles in postnatal physiology and tumorigenesis. However, to date, postnatal roles for nodal have not yet been determined, due to the embryonic lethality of loss of nodal function. Nodal might have roles in the mammary gland, since nodal and its receptors are expressed in mammary tissue (Bianco et al. 2002) and several other members of the TGF-β family are known to play central roles in mammary gland development (reviewed in Serra and Crowley 2005)).

Inhibition of nodal signaling by TMEFFs also suggests novel roles for TMEFFs in tumorigenesis. Depending on context, TGF-β family factors display properties of either tumor suppressors or promoters of malignancy (for reviews, see Massague et al. 2000; Derynck et al. 2001). TGF-β signaling antagonizes tumorigenesis by promoting growth arrest and/or apoptosis in many epithelial cell types. However, in tumors resistant to these antiproliferative/proapoptotic effects, TGF-β signaling promotes angiogenesis, suppresses immune surveillance, and may stimulate epithelial-mesenchymal transition of tumor cells (which is associated with decreased cell-cell adhesion and enhanced invasiveness). In line with a pro-malignant role for nodal in malignancy, melanomas secrete
nodal to promote invasiveness, inhibit differentiation, and stimulate transformed growth of melanoma cells (Topczewska et al. 2006). Further studies are needed to determine whether TMEFFs are competent to inhibit these responses to nodal in melanomas. The role of nodal signaling in other cancer types has not been examined.

**TMEFFs and Cell Proliferation**

In addition to examining TMEFF1 inhibition of nodal signaling during embryogenesis, we also examined roles that TMEFFs might play in tumorigenesis. The observation that TMEFF1 or TMEFF2 expression is downregulated in certain tumors (Liang et al. 2000; Young et al. 2001; Gery et al. 2003) suggested that TMEFFs may inhibit tumor formation or progression. Consistent with this hypothesis, it has been shown that overexpression of TMEFF1 and TMEFF2 leads to slower growth rates in glioma and prostate cancer cells, respectively (Gery et al. 2002; Gery et al. 2003). We failed to find evidence of a role for TMEFFs in controlling proliferation of glioma or breast cancer cells. Therefore, we investigated the possibility that TMEFFs might exert more specific effects on proliferation by modulation of pro-growth signals, with special focus on the EGF/ErbB axis of growth factor signaling.

**TMEFFs and ErbBs**

TMEFFs possess an EGF domain in their extracellular region. EGF domains are motifs of 6 cysteines found in a range of secreted proteins, including extracellular matrix proteins, blood clotting factors, and the EGF family of growth factors (which signal via the ErbB family of receptor tyrosine kinases). EGF motifs may serve as protein-protein
interaction domains, recognition sites for posttranslational modifications such as O-fucosylation, or growth factor signaling domains. EGF domains with ErbB signaling activity possess the consensus sequence \( CX_7CX_{10-13}CX_3YXGXRCX_4L \) (Carpenter and Cohen 1990). The EGF domains of TMEFFs conform to this consensus sequence with the exception of the sole arginine. In neuregulin (NRG) \( \beta \), replacement of this arginine with an alanine results in a mutant with dramatically diminished ErbB binding and a reduced ability to stimulate ErbB2 and ErbB4 phosphorylation (Jones et al. 1998). However, TMEFF2 may be an active ErbB ligand, as treatment of MKN28 gastric cancer cells, which express all four ErbB receptors, with soluble TMEFF2 stimulates phosphorylation of ErbB4 but not ErbB1/2/3 (Uchida et al. 1999). The specific activation of ErbB4 but not ErbB2 by TMEFF2 is highly unusual, as ligand-bound ErbB receptors preferentially dimerize with and activate ErbB2 (Yarden and Sliwkowski 2001). According to crystallographic evidence, the ligand does not directly recruit or interact with the dimerization partner, as the ligand-binding site is positioned on the outside surface of the receptor dimer (Burgess et al. 2003). To our knowledge, the only factor besides TMEFF2 reported to specifically stimulate ErbB4 without concomitant ErbB2 activation is Cripto (Bianco et al. 1999). While Cripto does not bind ErbB4 as a ligand, Cripto is thought to indirectly activate ErbB4 via transactivation after binding to distinct cell-surface receptor(s) including glypican-1 (Bianco et al. 1999; Bianco et al. 2003). This raises the question of whether TMEFF2 activates ErbB4 via direct binding, or whether an unidentified cell-surface receptor mediates ErbB4 transactivation by TMEFF2.

Importantly, we found that TMEFF1 and TMEFF2 associate with ErbB4 in coimmunoprecipitations assays, supporting a model wherein TMEFFs activate ErbB4 (or oth-
erwise affect ErbB function) by direct binding. We also demonstrated that TMEFFs associate with ErbB1/2/3. This was surprising, since TMEFF2 is not reported to activate these receptors (Uchida et al. 1999). We are currently investigating whether TMEFFs activate ErbB1/2/3. Interestingly, we found that the EGF domain of TMEFF1 is dispensable for its interaction with ErbB4. Thus, TMEFFs bind ErbB receptors in a manner unlike ortho-
dox ligands, and may be instead positioned to modulate ErbB signaling in a novel manner.

We found that overexpression of TMEFF1 or TMEFF2 did not broadly inhibit or enhance signaling by the EGFR/ErbB1 ligand EGF in glioma cells. Furthermore, the pro-
iferative response of breast carcinoma cells to EGF and HRG signaling was not modified in the presence of TMEFFs. Although we have not identified the functional significance for the TMEFF/ErbB interaction, several possibilities remain. Importantly, it has been found that a number of cell-surface proteins complex with ErbBs in order to mediate ac-
tivation or modulation of ErbB signaling, such as the adhesion protein E-cadherin (Pece and Gutkind 2000), integrins (Moro et al. 1998), the mucins MUC1 and MUC4 (Carraway et al. 1999; Schroeder et al. 2001; Funes et al. 2006), the proteoglycan CD44 (Yu et al. 2002), and the leucine-rich protein LRIG1 (Laederich et al. 2004). Such regula-
tors may interact with all four ErbB receptors, or specifically bind one or several family members. In some cases, association with regulators leads to ligand-independent ErbB phosphorylation. Cell-surface regulators may also modulate ErbB localization and/or ligand responsiveness. Regulators may modify downstream signaling by ErbB receptors in a general manner, or may specifically modulate one or two cytoplasmic signaling cas-
cades. For example, MUC4 has been shown to increase p38 but not c-Jun N-terminal
kinase or extracellular signal-regulated kinase (ERK) activation by ErbB2 (Ramsauer et al. 2006). In addition, some proteins may differentially modulate ErbB signaling in a context-dependent manner; for example, E-cadherins transiently stimulate ligand-independent EGFR activity upon adherens junction formation, but inhibit EGF activation of EGFR in other contexts (Pece and Gutkind 2000; Qian et al. 2004).

Of immediate concern is whether the TMEFF/ErbB association is an artifact of the overexpression/coimmunoprecipitation assay. To rule out the possibility that TMEFFs are incapable of binding ErbB receptors at physiological concentrations, the association of TMEFFs with endogenously expressed ErbBs must be demonstrated. Unfortunately, antibodies capable of detecting TMEFFs at endogenous levels are unavailable, thus overexpression of TMEFFs must be used in this assay. Another possibility is that the TMEFF/ErbB association is occurring in cell lysate but not in living cells. To exclude this possibility, colocalization of TMEFFs and ErbBs by immunofluorescence may be examined. Although we found that the TMEFF/ErbB association is abrogated upon deletion of the EGF and flanking regions of TMEFF1, the TMEFF/ErbB association might be further confirmed and characterized by demonstration of the ErbB domain involved in the interaction.

TMEFFs may modulate ErbB signaling at any step of the pathway, including ligand binding, receptor dimerization and activation, recruitment of downstream signaling effectors, and signal termination by receptor internalization, dephosphorylation, and/or proteolysis. The importance of a region in the TMEFF1 extracellular domain for TMEFF1/ErbB4 interaction suggests TMEFFs may modulate ErbB signaling at the level of ligand/receptor interaction or receptor dimerization. The failure of TMEFFs to broadly
modulate ErbB signaling suggests TMEFFs do not strongly inhibit or promote ligand binding or receptor dimerization/activation. In addition, TMEFFs probably do not affect recruitment of effectors responsible for activation of the ERK pathway. However, TMEFFs might modulate ErbB signaling in a more subtle manner. For example, the same ligand may signal through different ErbB receptor heterodimers to elicit distinct signaling responses (Olayioye et al. 2000). Therefore, TMEFF might modulate ErbB signaling with respect to the receptor dimerization partner selected.

Due to the diversity of ErbB signaling input and output and to the subtlety with which ErbB signaling may be modulated, determining how TMEFFs might regulate ErbB signaling may be challenging. It is possible that the consequences of TMEFF/ErbB interaction may be more readily apparent at the level of cellular or tissue functions. For example, soluble TMEFF2 ectodomain has been shown to stimulate survival of both hippocampal and mesencephalic neurons (Horie et al. 2000). Since NRG enhances survival of mesencephalic neurons (Gerecke et al. 2004; Zhang et al. 2004a) and EGF promotes hippocampal neuron survival (Chen et al. 2005), perhaps TMEFF2 activates ErbB signaling to promote neuronal survival in these contexts.

**TMEFFs and FGF Signaling**

Since we found that TMEFF1 associates with FGFR1, we examined whether TMEFFs might modulate FGF signaling. Since the follistatin (FS) module of the extracellular matrix protein SPARC antagonizes basic FGF (bFGF)-stimulated migration and angiogenesis (Chlenski et al. 2004), the FS modules of TMEFF might also have FGF-inhibitory activity. However, we found that TMEFF1 expression had no discernable
effect on bFGF stimulation of cell proliferation in breast carcinoma cells. Signaling by bFGF may proceed through binding to several isoforms of FGFR family members, including FGFR1b, FGFR1c, FGFR2c, FGFR3c, and FGFR4 (for reviews, see Powers et al. 2000; Eswarakumar et al. 2005). Of these, T47D cells are reported to express FGFR2c, FGFR3c, and FGFR4, but not FGFR1 (Johnson et al. 1998). It is possible that TMEFF1 may specifically associate with FGFR1 but not FGFR2-4, thus permitting bFGF signaling through FGF receptors other than FGFR1. Since there is great variability in the functional activities of FS modules, an additional possibility is that the FS modules of TMEFFs may lack the capacity to inhibit bFGF signaling. Alternatively, inhibition of FGF signaling by FS modules may be limited to the context of angiogenesis and/or other functional outputs of FGF signaling.

Taken together, our findings do not support a broad role for TMEFFs in the regulation of cell proliferation. TMEFFs might oppose tumor progression by other mechanisms. As ErbB4 signaling promotes differentiation in certain cell types (reviewed in Carpenter 2003), TMEFFs might signal through ErbB4 to induce or maintain a differentiated phenotype. In addition, TMEFFs have been hypothesized to regulate cell adhesion (Young et al. 2001); therefore, TMEFFs may modulate cell adhesion in certain physiological contexts, with consequences for proliferation and/or motility that are not apparent in standard tissue culture conditions. In addition, several studies suggest roles for TMEFFs in regulating cell survival (Horie et al. 2000; Penning et al. 2006). It is also possible that, while silencing or downregulation of TMEFFs may be useful biomarkers for malignancy or other conditions, decreased TMEFF expression is only incidental to tumor progression.
Concluding Remarks

Strict regulation of extracellular signaling pathways is critical for proper embryonic patterning and the prevention of malignancy in differentiated tissues. Here we have examined mechanistic roles for the putative antitumor factors TMEFF1 and TMEFF2 in regulation of signaling and cell proliferation. We show that TMEFF1 inhibits nodal signaling by binding the nodal coreceptor Cripto. The TMEFF1/Cripto interaction suggests that TMEFFs may play roles in embryonic patterning via modulation of numerous signaling pathways, including nodal/Vg1/GDF3/derriere, Cripto-dependent Wnt signaling, and Cripto activation of ERK (Kinoshita et al. 1995; Kannan et al. 1997; Chang et al. 2003; Tao et al. 2005; Dorey and Hill 2006; Onuma et al. 2006). We also demonstrate a novel interaction between TMEFFs and ErbB receptors, suggesting that TMEFFs are positioned to modulate one of the major growth factor signaling pathways implicated in tumorigenesis. Further investigation is needed to characterize the functional consequences of the TMEFF/ErbB interaction.
GENERAL REFERENCES


-. 2006b. Hypermethylation of hMLH1, HPP1, p14(ARF), p16(INK4A) and APC in primary adenocarcinomas of the small bowel. Int J Cancer 119(6): 1298-1302.


Feldman, B., Gates, M.A., Egan, E.S., Dougan, S.T., Rennebeck, G., Sirotkin, H.I.,
Schier, A.F., and Talbot, W.S. 1998. Zebrafish organizer development and germ-

Muc4 potentiates neuregulin signaling by increasing the cell-surface populations

Gedert, H., Kiel, S., Iskender, E., Florl, A.R., Krieg, T., Vossen, S., Gabbert, H.E., and
Sarbia, M. 2004. Correlation of hMLH1 and HPP1 hypermethylation in gastric,
but not in esophageal and cardiac adenocarcinoma. *Int J Cancer* **110**(2): 208-211.


androgen-regulated gene exhibiting antiproliferative effects in prostate cancer

Gery, S., Yin, D., Xie, D., Black, K.L., and Koeffler, H.P. 2003. TMEFF1 and brain tu-

echusetts.

Glynne-Jones, E., Harper, M.E., Seery, L.T., James, R., Anglin, I., Morgan, H.E., Taylor,
K.M., Gee, J.M., and Nicholson, R.I. 2001. TENB2, a proteoglycan identified in
prostate cancer that is associated with disease progression and androgen inde-


Grose, R. and Dickson, C. 2005. Fibroblast growth factor signaling in tumorigenesis. *Cyto-


Hackel, P.O., Zwick, E., Prenzel, N., and Ullrich, A. 1999. Epidermal growth factor re-


APPENDIX

IACUC APPROVAL FORM
NOTICE OF APPROVAL

DATE: January 27, 2006

TO: Chenbei Chang, Ph.D.
MCLM-360 0005
FAX: 975-5848

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Twisted Gastrulation Gene in Vertebrate Development
Sponsor: NIH
Animal Project Number: 060106640

On January 26, 2006, the University of Alabama at Birmingham Institutional Animal Care and
Use Committee (IACUC) reviewed the animal use proposed in the above referenced application.
It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frogs</td>
<td>B</td>
<td>30</td>
</tr>
<tr>
<td>Frogs</td>
<td>A</td>
<td>100</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from January 2006. Approval from the IACUC must
be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate
granting agency.

Refer to Animal Protocol Number (APN) 060106640 when ordering animals or in any
correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this
study. If you have concerns or questions regarding this notice, please call the IACUC office at
934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
1717 7th Avenue South
205.934.7692 • Fax 205.934.1188
iacuc@uab.edu
www.uab.edu/iacuc

The University of
Alabama at Birmingham
Mailing Address:
VH B10
1530 3RD AVE S
BIRMINGHAM AL 35294-0019