REGULATION OF NAKED2 BY TRANSFORMING GROWTH FACTOR-ALPHA AND WNT SIGNALING

By

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To my parents,

my wife and our baby boy

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William Hutchinson Murray once said: “Until one is committed, there is hesitance, the chance to draw back, always ineffectiveness. Concerning all acts of initiative (and creation), there is one elementary truth, the ignorance of which kills countless ideas and splendid plans: that the moment one definitely commits oneself, then providence moves, too. A whole stream of events issues from the decision, raising in one’s favor all manner of unforeseen incidents and meetings and material assistance, which no man could have dreamt would come his way.” It has not been an easy journey for my Ph.D study; therefore, I would not have accomplished it without the commitment in the first place.

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LIST OF ABBREVIATIONS

ADAM	a disintegrin and metalloproteinase
AEL	after egg laying
AOM	azoxymethane
AP	alkaline phosphatase
AP-1(2)	adaptor protein-1 (2)
APC	adenomatous polyposis coli
AR	amphiregulin
arm	armadillo
Bad	Bcl-2/Bcl-X(L)-associated death promoter
Bcl	B cell lymphoma/leukemia
bp	base pair
BSA	bovine serum albumin
BTC	betacellulin
CaRT	cargo recognition and targeting
cDNA	complementary DNA
CDK	cyclin dependent kinase
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHX	cycloheximide

CKI	casine kinase I
CMV	cytomegalovirus
CNS	central nervous system
CRC	colorectal cancer
CYLD	cylindromatosis
DEPC	diethyl pyrocarbonate
DIG	digoxygenin
Dkk	Dickkopf
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dsh	Dishevelled
DTT	dithiothreitol
DUB	deubiquitinase
Dvl-1	Dishevelled-1
EDTA	disodium ethylenediamine tetra-acetate
EGF	Epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
eIF-4E	eukaryotic translation initiation factor 4E
EKI	EGFR tyrosine kinase inhibitor

EMT	epithelial-mesenchymal transition
en	engrailed
EPR	epiregulin
ErbB	avian erythroblastosis virus
Erk	extracellular response kinase
FAP	Familial Adenomatous Polyposis
FAVS	fluorescence-activated vesicle sorting
FBS	fetal bovine serum
FKHR	Forkhead in Rhabdomyosarcoma
fz	Frizzled
FYVE domain	Fab1p, YOTB, Vac1p, EEA1 domain
GPCR	G protein-coupled receptor
GRB	growth factor receptor bound protein
GSK-3	glycogen synthase kinase-3
GST	Glutathionine-S-Transferase
HBD	heparin-binding domain
HB-EGF	heparin-binding EGF-like growth factor
HCl	hydrochloric acid
HDAC	histone deacetylase
HEK	human embryonic kidney
HER	human EGF receptor

hh	hedgehog
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
HSPG	heparan sulfate proteoglycan
IP_3	inositol triphosphate
IPTG	Isopropyl-b-D-thiogalactoside
IRES	internal ribosome entry site
IUP	intrinsically unstructured protein
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LiCl	lithium chloride
LRP	LDL receptor-related protein
MAPK	Mitogen-Activated Protein Kinase
MDCK	Madin Darby Canine kidney
MEK	mitogen-activated or extracellular signal-regulated protein kinase kinase (=MAPKK)
µg	microgram
µl	microliter
ml	milliliter
µm	micrometer
mM	millimolar
$MgCl_2$	magnesium chloride

MTGR-1	myeloid translocation gene-related protein 1
mTOR	mammalian Target Of Rapamycin
MT-TGFα	metallo-thionein-TGFα
Myc	avian myelocytomatosis
NaCl	sodium chloride
Nemo	NF-κB essential modulator
NH_4Cl	ammonium chloride
nkd	naked cuticle
NF-κB	nuclear factor κB
NHERF	Na^+/H^+ exchanger regulatory factor
NLK	NEMO-like kinase
Ras	red sarcoma virus
RING	really interesting new gene
PAGE	Polyacrylamide Gel Electrophoresis
PBS	phosphate buffered saline
PCP	planar cell polarity
PCR	Polymerase Chain Reaction
PDK1	3-phosphoinositide-dependent protein kinase 1
PFA	paraformaldehyde
PH domain	pleckstrin homology domain
PI3K	phosphatidylinositol 3' kinase

PKC	protein kinase C
PLC	phospholipase C
PtdIns(3,4,5)P_3	phosphatidylinositol-3,4,5-trisphosphate
PtdIns(4,5)P_2	phosphatidylinositol-4,5-bisphosphate
PTEN	phosphatase and tensin homolog
RNA	ribonucleic acid
RNasin	RNase inhibitor
rhTGFα	recombinant human TGFα
RT	room temperature
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
sFRP	secreted frizzled-related protein
SH2	Src-homology 2
siRNA	short interfering RNA
SSC	standard saline citrate
SSDNA	salmon sperm DNA
SWI/SNF	switching-defective and sucrose nonfermenting
TACE	TNFα-converting enzyme
TGFα	Transforming growth factor α
TGFβ	Transforming growth factor β
TK	thymidine kinase

TNF	tumor necrosis factor
Tris	tris(hydroxymethyl)aminomethane
TTB	TGFα cytoplasmic tail-binding domain
VSV-G	vesicular stomatitis virus-G protein
wg	wingless
WIF1	Wnt inhibitory factor-1

CHAPTER I

GENERAL INTRODUCTION

Naked family members (*Drosophila Naked Cuticle* and mammalian Naked1 and Naked2) have been identified as inducible antagonists of canonical Wnt signaling. We previously reported that Naked2, but not Naked1, interacts with the cytoplasmic tail of transforming growth factor α (TGFα), thereby coating TGFα-containing exocytic vesicles and directing these vesicles to the basolateral corner of polarized epithelial cells. We have designated Naked2 a cargo recognition and targeting (CaRT) protein required for TGFα basolateral cell surface delivery. Despite the importance of its role in both Wnt signaling and TGFα trafficking, the regulation of Naked2 has not been characterized. Moreover, *in vivo* roles for Naked2 in these biological processes are largely unknown. To address these deficiencies, I have examined the regulation of Naked2 expression in the context of canonical Wnt signaling and epidermal growth factor receptor (EGFR) signaling, and I have generated a targeted conditional disruption of Naked2 in the mouse.

Part I: Human colorectal cancer

At least 50% of individuals within Western society will develop a colorectal tumor by age 70. Although only one in 10 benign adenomas progress to malignancy, colorectal cancer remains the second leading cause of cancer deaths in the United States (Parker, Tong et al. 1996) (American Cancer Society Cancer Statistics, 2006). Colorectal cancer results from an accumulation of genetic and epigenetic events that develop over decades (Vogelstein, Fearon et al. 1988). As many as 15% of colorectal cancers may be hereditary; these are best exemplified by Familial Adenomatous Polyposis (FAP) and Hereditary Nonpolyposis Colorectal Cancer (HNPCC) (Kinzler and Vogelstein 1996). Individuals with FAP bear an inherited mutation in the gatekeeper *adenomatous polyposis coli* (APC) gene and develop hundreds of benign adenomatous polyps in early adulthood with somatic loss of the second allele in the involved tissue. Over time, a fraction of these polyps progress to carcinomas, and colon cancer develops in almost all patients by age 40 if prophylactic colectomy is not performed. Patients with FAP are also at increased risk for duodenal ampullary carcinoma, gastric carcinoma, thyroid cancer and CNS tumors. In contrast, HNPCC patients carry a germline mutation in one of the DNA mismatch repair genes, and with somatic inactivation of the second allele of the affected gene, there is accelerated progression to neoplasia in the involved tissue. Patients with these germline defects in DNA mismatch repair are also at increased risk for other extracolonic tumors as well, including endometrial cancer, ovarian cancer, gastric cancer and cancers of the renal pelvis or ureter. Although the majority of colorectal cancers arise from somatic

events, many of the genes affected in hereditary colorectal cancer are also altered in sporadic colorectal cancer. For example, approximately 80% of sporadic colorectal cancers exhibit loss of functional APC.

Colorectal cancer refers to cancers of the colon and rectum, the terminal 6 to 7 feet of the digestive tract. The colonic mucosa consists of large numbers (~10^7) of crypts that are invaginations of the flat surface epithelium (Fig. 1.1). The colon is a dynamic, self-renewing epithelium. It is thought that a small number of stem cells occupy the bottom of the crypt with nearby, rapidly dividing transient amplifying cells that cease to proliferate and then differentiate as they migrate up the crypt. As the differentiated cells reach the luminal surface, they may undergo apoptosis before being sloughed into the lumen. In humans, the life span of an individual colonic epithelial cell is less than a week. Each day, about 10^{10} dead cells are shed by the colon. This loss is compensated for by the ordered migration of the rapidly dividing transient amplifying cells. Therefore, homeostasis of the colonic epithelium is maintained by the perpetual upward movement of a contiguous two-dimensional sheet of epithelial cells. This large-scale self-renewal of epithelial cells not only ensures the integrity of colon mucosa, but it also presents ample opportunities for the acquisition of somatic mutations that can occur during normal DNA replication and chromosome segregation. In addition to mutations that affect cellular proliferation and viability, other events impair migration so that initiated cells are retained, enabling accumulation of additional events that are required for a fully developed carcinoma.

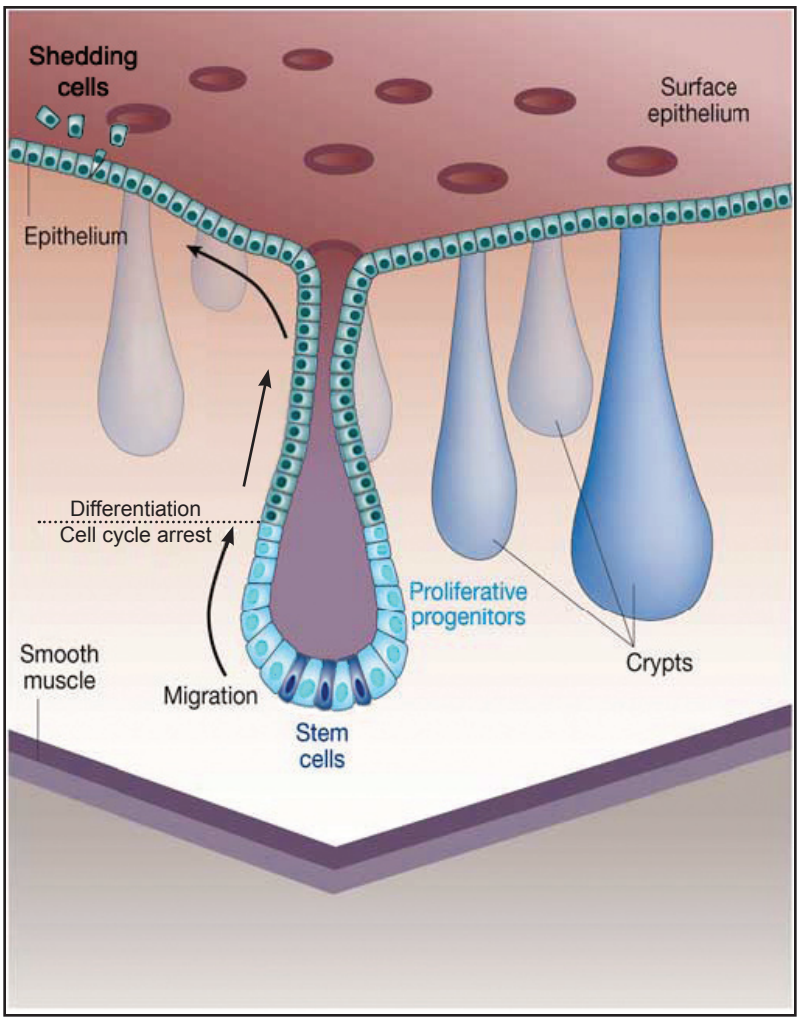


Figure 1.1 Tissue anatomy of the colonic epithelium. Recent data suggests that Lgr5 marks colonic stem cells, and 2-4 of these non-quiescent cells reside at the base of each crypt (Barker, 2007). Progenitor/transient amplifying cells (light blue) occupy the bottom one-third of the crypt. Differentiated cells (green) populate the remainder of the crypt and the flat surface epithelium. As the differentiated cells reach the luminal surface, they may undergo apoptosis before being sloughed into the lumen (Adapted from Reya & Clevers, 2005).

Epithelial cells do not exist in a vacuum. Each crypt is encased in a single layer of pericryptal fibroblasts. These cells also migrate from the crypt base to the cell surface, albeit at a much slower rate than their epithelial counterparts. The stroma also contains additional cellular elements such as endothelial cells and a number of different types of inflammatory cells. It is increasingly recognized that these non-epithelial elements may contribute to the neoplastic process (Bosman, de Bruine et al. 1993; Martin, Pujuguet et al. 1996; Cutler, Graves-Deal et al. 2003).

The etiology of colorectal cancer has been extensively studied over the past twenty years (Fig. 1.2). The initiating event in most colorectal cancers is an activating mutation in the canonical Wnt signaling pathway. Some 85% of all sporadic and hereditary colorectal tumors display loss-of-function mutation in the APC gene (Kinzler and Vogelstein 1996), a key negative regulator of Wnt signaling. Activating mutations in a Wnt agonist, β-catenin, are found in half of the remaining cases. In the small number of cases in which mutations in APC and β-catenin are not detected, it is likely that other components of canonical Wnt signaling are dysfunctional. In any case, dysregulated canonical Wnt signaling results in an expansion of the proliferating crypt compartment that eventually leads to an abnormal tissue architecture, known as an adenoma or adenomatous polyp. This step is thought to be the rate-limiting event in colorectal tumorigenesis because of the low somatic mutation rate in normal epithelial cells that is required for the loss of the wild-type APC allele in FAP patients. In sporadic colon cancer, it takes an even longer period of time to accumulate mutations in both APC

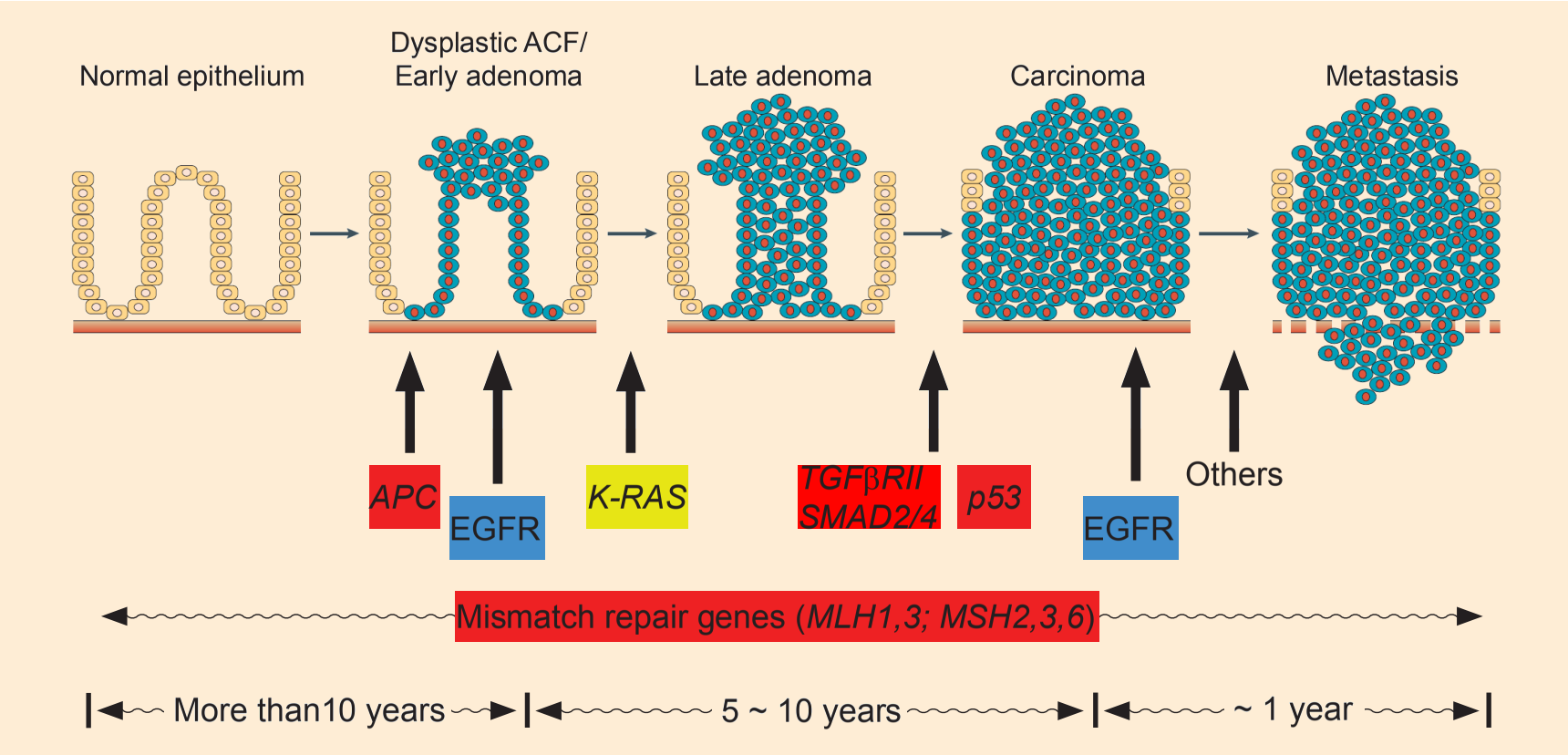


Figure 1.2 A step-wise model of colorectal tumorigenesis. Colorectal cancers are believed to develop over the course of 20–40 years as a consequence of accumulated mutations in oncogenes (yellow box) such as K-RAS and tumour suppressors (red boxes) such as APC, TGFβRII and p53. These mutations may arise within the tumor in a characteristic sequence as shown. A single cell within a heterogeneous population acquires a mutation in one such gene, and this mutation confers a selective advantage to the growth of the cells. Genetic instability caused by CIN and MSI (Rajagopalan, 2003) and epigenetic change by CIMP (Issa, 2000) are thought to occur somewhere during the process of colorectal tumorigenesis to accelerate the rate of mutation in dividing cancer cells. In addition, enhanced EGFR signaling (blue boxes) is used iteratively both at the establishment stage and later during tumor progression (Adapted from Rajagopalan et al., 2003).

alleles or in other Wnt component genes to initiate tumors. This coincides with the classic "two-hit" model proposed by Knudson (Knudson 1993).

Adenomas are benign tumors, and only a small fraction of them progress to larger adenomas and carcinomas, which require acquisition of further genetic alterations (Fig. 1.2). Oncogenic activation of one K-Ras allele is detected in approximately 50% of human colorectal cancers. Although it is not generally thought to initiate tumors by itself (Haigis, in press) (Janssen, el-Marjou et al. 2002; Janssen, Alberici et al. 2006), mutant K-Ras appears to contribute to the transition from moderate to late adenomas (Lamlum, Papadopoulou et al. 2000). In late adenomas, mutations in the type 2 TGF-β receptor are detected (Grady, Rajput et al. 1998). TGF-β ligands and receptors are normally expressed in differentiated cells to inhibit proliferation (Polyak 1996; Oshima, Oshima et al. 1997). In addition to mutations in type 2 TGF-β receptors, loss of function mutations in Smad4 and Smad2, key transducers of TGF-β signaling, have also been found in a small but significant fraction of colorectal cancers (Moskaluk and Kern 1996; Riggins, Thiagalingam et al. 1996). Compound heterozygous mice, carrying inactivating mutations in both APC and Smad4 alleles, develop histologically advanced, invasive malignant intestinal tumors (Takaku, Oshima et al. 1998). These studies suggest that loss of responsiveness to TGF-β signaling confers growth advantages and may contribute to the progression from adenomas to malignant tumors. However, during the late stages of colorectal carcinogenesis, TGF-β promotes tumor progression (Xu and Pasche 2007). Moreover, loss of function mutations in the p53 tumor suppressor gene are also a

common late event in colorectal carcinogenesis (Kinzler and Vogelstein 1996). In APC mutant mice, p53 deficiency enhances the multiplicity and invasiveness of intestinal tumors (Halberg, Katzung et al. 2000), identifying an important role for wild-type p53 in suppressing colorectal tumor progression.

EGFR signaling also contributes to the pathogenesis of colorectal neoplasia (Fig. 1.2). EGFR is a transmembrane receptor tyrosine kinase member of the ErbB family that transduces intracellular signals through various pathways, such as Ras-MAPK and PI3K-AKT cascades. Activation of MAPK leads to increased activity of Elk1 and c-fos transcription factors that may induce expression of target genes, such as cyclin D, and prime the cell for cell cycle progression and enhanced proliferation (Prenzel, Fischer et al. 2001). On the other hand, EGFR activation promotes tumor cell survival by inhibiting apoptosis, and this effect has been shown to be mediated by PI3K-AKT signaling cascade and may involve induction of NF-κB signaling. Moreover, EGFR signaling also contributes to tumor invasiveness and metastasis. EGFR ligands, TGFα and EGF, upregulate the expression of vascular endothelial growth factor (VEGF) and induce angiogenesis in tumor cells (Raymond, Faivre et al. 2000). Activated EGFR also influences cell-cell adhesion by modulating the interaction of E-cadherin and the actin cytoskeleton (Hazan and Norton 1998; Prenzel, Fischer et al. 2001). Cell motility is also promoted by EGFR tyrosine kinase through activation or upregulation of matrix metalloproteinases (Khazaie, Schirrmacher et al. 1993; Woodburn 1999; Ellerbroek, Halbleib et al. 2001).

The role of EGFR signaling in the early events in intestinal neoplasia has been examined genetically by introducing the hypomorphic *Egfr*^{wa2} allele into the *Apc*^{Min} mouse model of FAP (Roberts, Min et al. 2002). Homozygous *Egfr*^{wa2}; *Apc*^{Min} mice exhibited a 90% reduction in the number of macroscopic intestinal tumors (macroadenomas) at 3 months of age compared to Egfr wild-type *Apc*^{Min} mice. However, there were an equal number of microadenomas (defined microscopically as 5 or fewer dysplastic crypts or crypts with nuclear β–catenin) in the two groups at one month of age. Thus, there was no difference in the number of initiated events. However, EGFR signaling was needed for the initiated tumors to "establish" themselves. The lack of any differences in proliferative indices in the microadenomas between the two groups suggests that EGFR signaling may confer an anti-apoptotic effect in the establishment phase of intestinal tumorigenesis. Macroadenomas that arose in homozygous *Egfr*^{wa2}; *Apc*^{Min} mice did not differ in size and histology from those arising in Egfr wild-type *Apc*^{Min} mice. In a small cohort followed over an additional 9 months, polyp progression in the two groups was indistinguishable by these two parameters. In separate experiments using conventional nude mouse xenografts, an irreversible EGFR tyrosine kinase inhibitor reduced the growth of two human colorectal cancer cell lines, HCA-7 and HCT-116 (Roberts, Min et al. 2002). Thus, as in Drosophila eye development (Freeman and Bienz 2001; Freeman 2002; Brown, Kerr et al. 2007), there appears to be an iterative use of EGFR signaling in intestinal neoplasia.

EGFR has emerged as a promising therapeutic target in colorectal cancer. The

recombinant, chimeric, IgG1 antibody cetuximab has been shown to have consistent clinical activity as a monotherapy in 10% of patients with chemotherapy-resistant metastatic colorectal cancer (Costa, Sander et al. 2004; Cunningham, Humblet et al. 2004). However, no responses were observed in 115 metastatic colorectal cancer patients treated in a clinical trial with two doses of gefitinib, a reversible tyrosine kinase inhibitor (Rothenberg, LaFleur et al. 2005). Nevertheless, a 46% response rate has been observed in a small group of patients with advanced colorectal cancer treated with combined cetuximab and gefitnib (Merchant, in press). The Coffey lab championed the notion that EGFR axis may be a tractable therapeutic target in colorectal cancer. The EGFR axis is defined as the proximal events associated with activation of the EGFR and include cell surface EGFR ligand cleavage, ligand uptake by receptor and EGFR tyrosine kinase phosphorylation and activation. The Coffey lab has shown recently *in vitro* that combined pharmacological blockade at each of these steps results in cooperative growth inhibition (Merchant, Voskresensky et al. 2008).

Taken together, human colorectal cancer is a disease caused by multiple genetic alterations in colonic epithelial cells (Fig. 1.2). It involves extremely complicated, often convergent, signaling networks that remain poorly understood. However, the prevailing model can be simply stated: deregulation of canonical Wnt signaling initiates formation of benign tumors, followed by a further acquisition of mutations in key components of EGFR, TGF-β and p53 signaling pathways that promote malignant tumor progression and metastasis. It appears that c-MYC is a central node in the pathogenesis of colorectal

cancer (Sansom, Meniel et al. 2007).

To advance a better understanding of human colorectal tumorigenesis, my thesis focuses on the transcriptional and post-transcriptional regulations of Naked2, a protein that is involved in both Wnt signaling (Wharton, Zimmermann et al. 2001; Van Raay, Coffey et al. 2007) and EGFR-related events, i.e. the basolateral sorting of TGFα (Li, Franklin et al. 2004; Li, Hao et al. 2007).

Based upon its widespread pattern of expression, rapid cell surface cleavage and avid capture by the EGFR, I postulate that TGFα is the ligand that most often binds the EGFR *in vivo*. It appears that delivery of TGFα to the cell surface is a critical, and possibly rate-limiting, step in regulating endogenous TGFα activity. In polarized epithelial cells, TGFα assures its efficient delivery to the EGFR-restricted basolateral surface by stabilizing Naked2 protein.

In *Drosophila* development, EGFR signaling antagonizes Wg signaling, both in segmental patterning of the larva epidermis (Szuts, Freeman et al. 1997; Sanson 2001) and eye development (Freeman and Bienz 2001). However, both EGFR and Wnt signaling are upregulated in many malignant tumors, and, in some instances, the two pathways cooperate (Schroeder, Troyer et al. 2000; Civenni, Holbro et al. 2003; Schlange, Matsuda et al. 2007). Naked2 may provide a convergent point of TGFα trafficking and canonical Wnt signaling through its critical functions in both pathways.

In Chapter V (General discussion), I will present a two step model by which Naked2 participates in normal epithelial homeostasis (Fig. 5.2). First, it delivers TGFα to the

basolateral surface. Upon discharging that function, it then binds and degrades any excess Dvl-1 so as to maintain tight control of canonical Wnt signaling. Thus, Naked2 may provide a convergence point between EGFR-related events (i.e., cell surface delivery of TGFα) and canonical Wnt signaling.

In colorectal neoplasia, Naked2 is downregulated (Chapter III); this downregulation appears to be a late event in that I observed decreased expression of Naked2 in 10/16 (60%) carcinomas but in 0/6 adenomas. A consequence of this loss would be impaired cell surface delivery of TGFα, resulting in a relatively unoccupied EGFR to which additional ligands might bind. Amphiregulin is an attractive candidate. Like TGFα, amphiregulin is delivered to the basolateral surface of polarized epithelial cells where it is cleaved by TACE. In contrast to TGFα, amphiregulin, through its heparin-binding domain, binds to heparin sulfate proteoglycans more efficiently that to the EGFR. Thus, normally amphiregulin may exist in a depot form in the extracellular matrix. With the increased proteolytic activity associated with cancer, amphiregulin may be released from the extracellular matrix to bind the EGFR. Of note, the Coffey lab has shown recently that amphiregulin, but not TGFα, confers an EMT-like transition when added to epithelial cells (Chung, Cook et al. 2005; Chung, Graves-Deal et al. 2005). Simply stated, I propose that the loss of Naked2 impairs cell surface delivery of TGFα, resulting in an unoccupied EGFR to which amphiregulin binds and drives tumor progression.

To test this model *in vivo*, I have created a conditional targeted disruption of *Naked2* in the mouse (see Chapter IV). Experiments are underway to selectively eliminate

Naked2 in the small and large intestine. To elicit a neoplastic phenotype, I anticipate that these mice may need to be crossed to *MT-TGFα* mice and/or Apc^{Min} mice. Alternatively, the colon carcinogen azoxymethane may need to be administered to these mice.

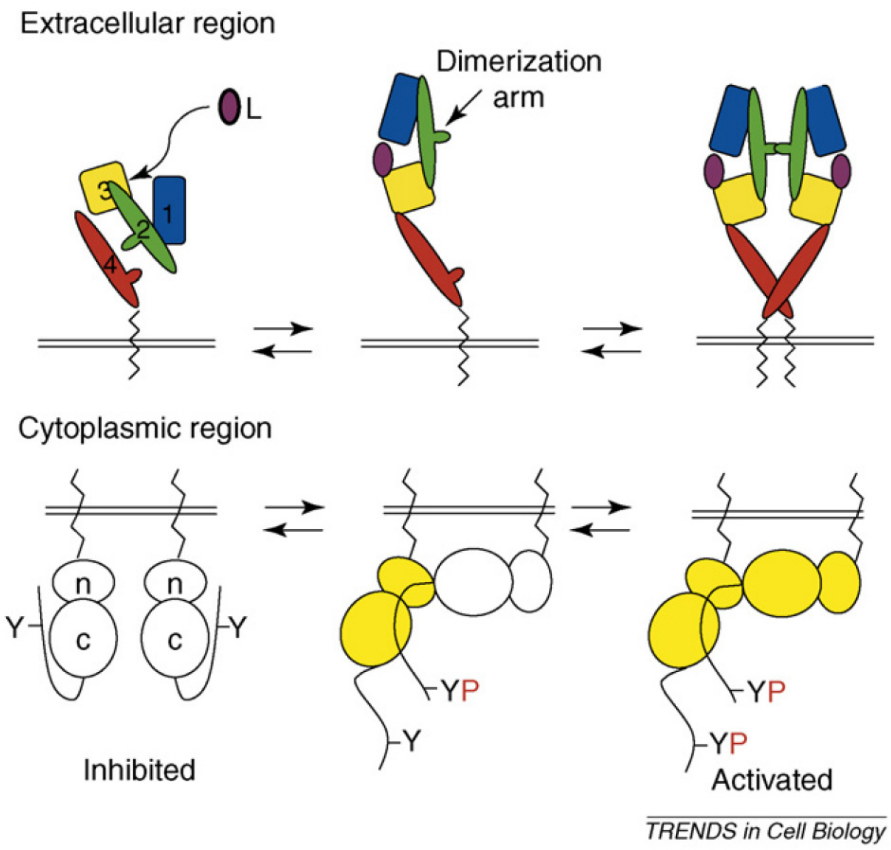
Part II: EGFR signaling and EGFR ligands

EGFR and EGFR signaling

A major focus of the Coffey lab has been EGFR signaling and its role in gastrointestinal neoplasia with an emphasis on trafficking of the EGFR ligands in the context of a battery of human colorectal cancer cell lines that retain the capacity to form a uniform polarizing monolayer when cultured on Transwell filters. EGFR belongs to the family of ErbB receptor tyrosine kinases comprised of four closely related members (Fig. 1.3B) — epidermal growth factor receptor (EGFR, also known as ErbB-1 or HER1), ErbB-2 (HER2), ErbB-3 (HER3) and ErbB-4 (HER4). Upon binding of extracellular growth factor ligands to the ectodomain, EGFR homo- or heterodimerizes with other ErbB receptors and this results in activation of intrinsic tyrosine kinase acitivities that reside in the cytoplasmic tail of the receptors (Fig. 1.3A, with the exception of ErbB-3). Subsequent auto-phosphorylation at multiple tyrosine residues within the cytoplasmic tail of the receptor creates docking sites for appropriate binding of adaptor proteins or signaling molecules (such as GRB-2, Shc, PLC-γ and Src) to transduce a myriad of intracellular signals. Heterodimerization leads to an expansion in the number of possible signaling pathways stimulated even by a single ligand. The specificity of downstream

Figure 1.3 Activation of ErbB receptors and their downstream signalings. (A) Receptor dimerization and auto-phosphorylation. Before ligand binding, the arm (domain II) is sequestered by subdomain IV within the monomer. Ligand binding leads to a conformational change that exposes the arm to facilitate intermonomer associations between dimerization arms. The consequence of ectodomain dimerization is the asymmetric interaction of kinase domains and auto-phosphorylation in tyrosine residues (Reprint Linggi & Carpenter, 2006). P, phosphorylation; Y, tyrosine. (B) Differential ligand engagement of the receptor and different dimeric receptor combinations activate receptor tyrosine kinase activities that signal through a variety of downstream cascades. Each of these receptor combinations translates into diversity as to different cell responses, including apoptosis, migration, proliferation, differentiation and cell adhesion. (Adapted from Yarden and Sliwkowski, 2001)

A


Extracellular region
Dimerization arm
L
1
2
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Cytoplasmic region
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YP
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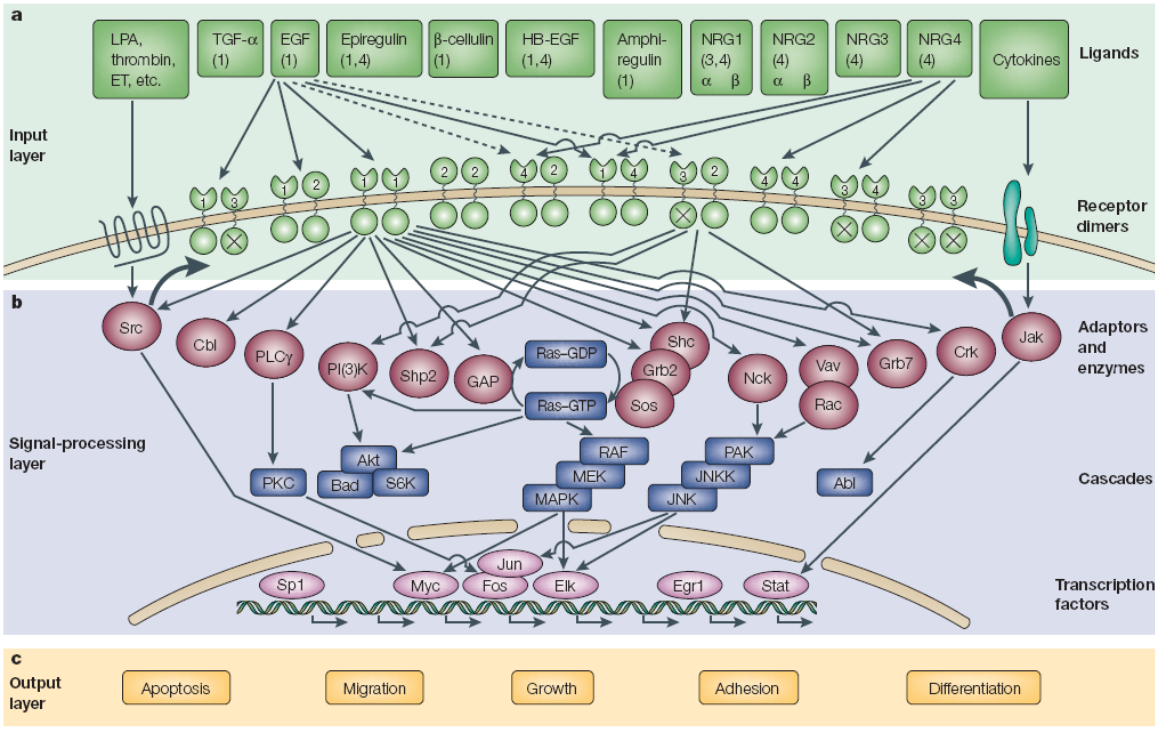
Activated
TRENDS in Cell Biology

B


a
LPA, thrombin, ET, etc.
TGF-α (1)
EGF (1)
Epiregulin (1,4)
β-cellulin (1)
HB-EGF (1,4)
Amphi-regulin (1)
NRG1 (3,4) α β
NRG2 (4) α β
NRG3 (4)
NRG4 (4)
Cytokines
Ligands
Input layer
Receptor dimers
b
Src
Cbl
PLCγ
PI(3)K
Shp2
GAP
Ras–GDP
Ras–GTP
Shc
Grb2
Sos
Nck
Vav
Rac
Grb7
Crk
Jak
Adaptors and enzymes
Signal-processing layer
Akt
PKC
Bad
S6K
RAF
MEK
MAPK
PAK
JNKK
JNK
Abl
Cascades
Jun
Sp1
Myc
Fos
Elk
Egr1
Stat
Transcription factors
c
Output layer
Apoptosis
Migration
Growth
Adhesion
Differentiation

signaling pathways is determined by which ligand binds to the receptor, the composition of the receptor pairs and the particular tyrosine residues that are auto-phosphorylated. ErbB-2 and ErbB-3 are the two special members of the family (Citri, Skaria et al. 2003). ErbB-2 does not bind to any of known ligands with high affinity (King, Borrello et al. 1989; Lonardo, Di Marco et al. 1990), but it is the preferred heterodimeirc partner for other ErbB receptors including EGFR. Furthermore, homodimerization of ErbB-2 receptors causes robust ligand-independent receptor tyrosine kinase activity that is thought to contribute to tumor progression in mammary tissues (Bacus, Zelnick et al. 1994; Karunagaran, Tzahar et al. 1996; DiGiovanna, Stern et al. 2005). On the other hand, ErbB-3 is deficient in its intrinsic receptor tyrosine kinase activity, but it couples with other receptors to start a unique repertoire of downstream cascades. Interestingly, herterodimerization of ligand-less ErbB-2 and kinase-inactive ErbB-3 seems to provide the most potent mitogenic and angiogenic signal (Alimandi, Romano et al. 1995; Yen, Benlimame et al. 2002).

By recruiting different adaptor and signaling proteins, activated EGFR initiates signalings that regulate diverse biological responses including proliferation, differentiation, cell motility and survival (Marmor, Skaria et al. 2004). The three best characterized cascades are Ras-mitogen-activated protein kinase (Ras-MAPK), phosphatidylinositol 3' kinase-protein kinase B (PI3K-Akt) and phospholipase C-protein kinase C (PLC-PKC) pathways (Fig. 1.3B).

Ras-MAPK pathway is a major cascade that can be activated by EGFR ligands and

ErbB receptors. Following activation of EGFR tyrosine kinase and auto-phosphorylation, Grb2 is recruited to the EGFR directly through its SH2 domain to the phosphotyrosine Y^{1068} and Y^{1086}, or indirectly through PTB domain-mediated association of Shc to EGFR. In either case, the cytosolic Grb2/Sos (the Ras exhange factor) protein complex relocates to the activated receptor at the plasma membrane to facilitate the interaction of membrane-associated Ras and Sos. Sos catalyzes the exhange of Ras-bound GDP for GTP thereby activating Ras, which in turn activates the serine/threonine kinase Raf-1. Activation of Raf-1 initiates a kinase cascade involving serine phosphorylation of MEK1/2 (MAPKK) and tyrosine and threonine phosphorylation of Erk1/2 (MAPK). Erk phosphorylates multiple cytoplasmic proteins, such as MAPK-activated protein kinase and the ribosomal p70-S6 kinase. Activated Erk can also translocate into the nucleus, where it phosphorylates and activates a number of transcription factors including Sp1, PEA3, E2F, Elk1and AP1.

Activation of PI3K occurs through SH2-mediated recruitment of the p85 regulatory subunit to a consensus phosphotyrosine site on activated receptors, resulting in allosteric activation of the p110 catalytic subunit that may be involved in GTP-Ras binding. This process also requires additional adaptor proteins since p85 binding to EGFR is indirect. Active PI3K produces phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P_3] from PtdIns(4,5)P_2, resulting in the recruitment of Akt kinase and other signaling effectors that contain the lipid-binding FYVE or pleckstrin homology (PH) domains. Akt is then phosphorylated and activated by PDK1, another PH domain-containing kinase. Akt is a

key effecter of PI3K and has many cytosolic and nuclear targets. In cytoplasm, phosphorylation of Bad by Akt promotes cell survival by blocking binding of this pro-apoptotic molecule to Bcl-2 and Bcl-X(L) proteins. Akt also inhibits Raf and glycogen synthase kinase-3 （GSK-3) kinase functions, while promoting protein translation through mTOR, eIF-4E and ribosomal p70-S6. Once translocated to the nucleus, Akt promotes cell cycle progression by regulating the cell cycle regulatory transcription factor FKHR and downregulating the cyclin dependent kinase inhibitor $p27^{KIP1}$. Taken together, PI3K signaling pathway plays versatile roles in the regulation of cell proliferation and survival. Thus, PTEN has been shown a critical tumor suppressor gene by dephosphorylating the 3' position of PI3K products.

Phospholipase Cγ (PLCγ) is recruited to the activated EGFR through SH2-mediated docking or indirect binding to PI3K products through their PH domains. Subsequent phosphorylation by EGFR tyrosine kinase activates PLCγ, resulting in hydrolysis of $PtdIns(4,5)P_2$, to generate the second messengers diacylglycerol and inositol triphosphate [$Ins(1,4,5)P_3$ or IP_3]. IP_3 releases intracellular calcium from endoplasmic reticulum and thereby activates calcium/calmodulin-dependent kinases. In addition, calcium collaborates with diacylglycerol to simulate protein kinase C that phosphorylates a large variety of substrates.

Thus EGFR signaling is intrinsically complex and context-dependent. It remains challenging to dissect each individual cascade under particular circumstances. As discussed above, ligand engagement of the EGFR appears to be the first step in the

regulation and specification of downstream signaling.

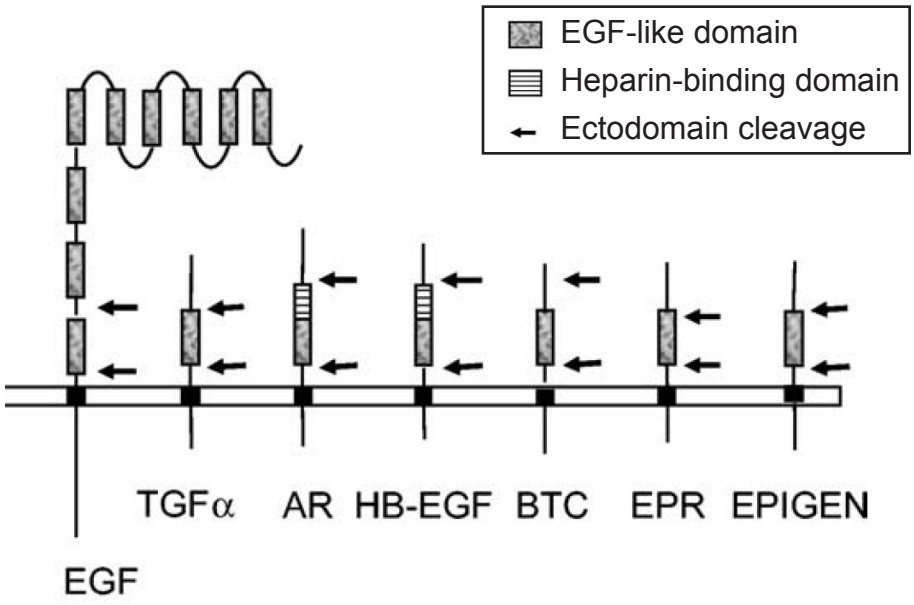
EGFR ligands

Seven mammalian ligands bind the ErbB family receptors (Fig. 1.4A); these include EGF, TGFα, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epiregulin (EPR) and epigen (Shoyab, McDonald et al. 1988; Massague 1990; Higashiyama, Lau et al. 1992; Toyoda, Komurasaki et al. 1995; Strachan, Murison et al. 2001). All of the ligands are made as type I transmembrane proteins that are inserted into the plasma membrane where they undergo proteolytic cleavage to release mature soluble growth factors. All of the actions of these growth factors are thought to be mediated by binding to the EGFR. TGFα, EGF, AR and epigen bind solely to EGFR, whereas HB-EGF, betacellulin and epiregulin can also bind ErbB4.

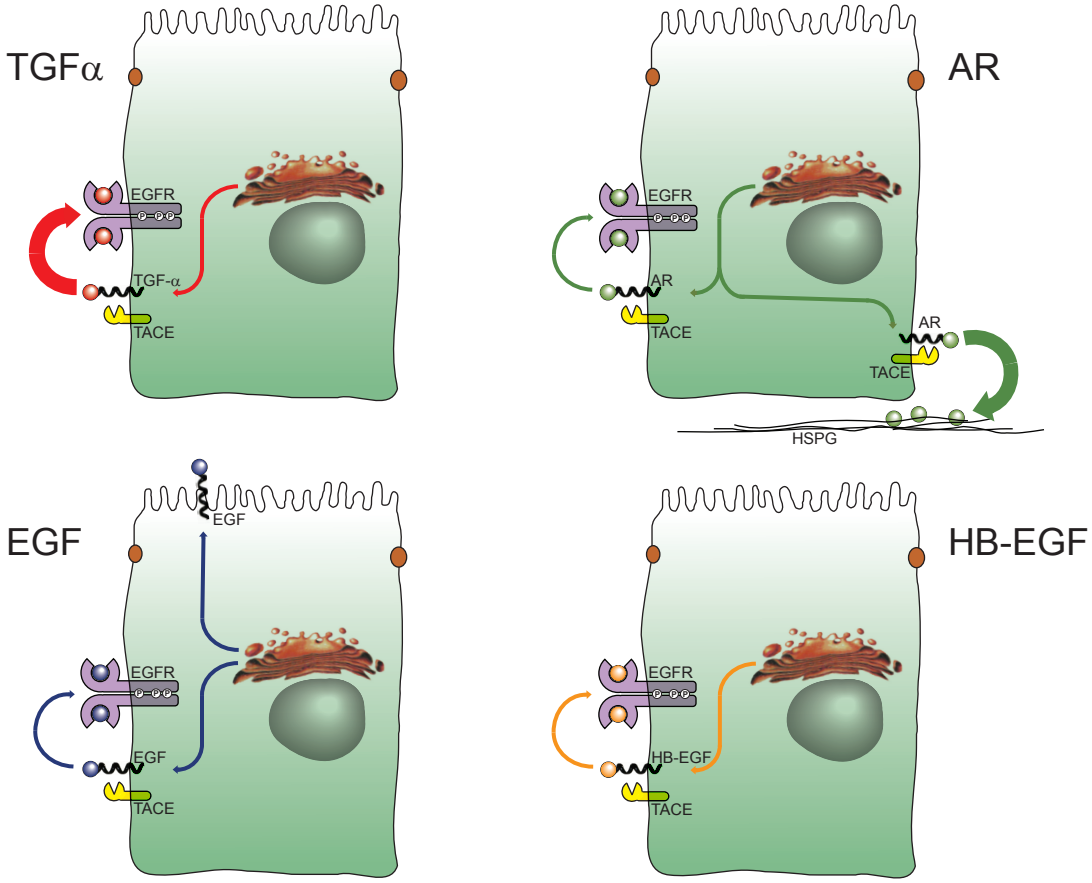
TGFα has been identified as the major endogenous EGFR ligand in the gut, playing key roles in various cell processes, including proliferation, survival and differentiation, to maintain the homeostasis and repair of gut epithelium. However, aberrant overexpression of TGFα is frequently observed in the majority of solid tumors when compared to their normal counterparts (Salomon, Brandt et al. 1995). The involvement of TGFα in autonomous proliferation of carcinoma cells has been verified by previous studies using antisense oligonucleotides and/or neutralizing antibodies against TGFα, which can significantly inhibit *in vitro* growth of various human carcinoma cells (Normanno, Bianco et al. 1996; De Luca, Casamassimi et al. 1999). *In vitro* colony formation assays

Figure 1.4 EGFR ligands and their intracellular trafficking. (A) There are seven mammalian ligands for ErbB receptor family. All of the ligands are made as type I transmembrane proteins that undergo cell surface proteolytic cleavage to release mature soluble growth factors. The EGF-like domain that mediates receptor interactions is conserved in all of the ligands, but heparin-binding domain is only found in AR and HB-EGF (Reprint from Harris, 2003). (B) Differential trafficking of EGFR ligands. Among the four EGFR ligands, only EGF is delivered to both the apical and the basolateral membrane, whereas TGFα, AR and HB-EGF preferentially traffic to basolateral membrane, where they are processed and consumed by the EGFR with different kinetics (See text for details).

A


EGF-like domain
Heparin-binding domain
Ectodomain cleavage
TGFα
AR
HB-EGF
BTC
EPR
EPIGEN
EGF

B


TGFα
EGFR
TGF-α
TACE
AR
EGFR
AR
TACE
AR
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HSPG
EGF
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EGFR
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HB-EGF
EGFR
HB-EGF
TACE

in soft agar and tumorigenicity studies in nude mice also indicate that TGFα can promote transformation of fibroblast cells, such as Rat-1 (Rosenthal, Hulse et al. 1986) and NIH3T3 (Di Marco, Pierce et al. 1989). Genetic studies in engineered mouse models have provided evidence for roles for TGFα in tumor initiation and progression (Matsui, Halter et al. 1990; Halter, Dempsey et al. 1992).

As a proximal event of EGFR axis, regulated processing and cell surface presentation of different EGFR ligands are thought critical for their biological activities and signaling specificities. We have significantly advanced our understanding of the biology of endogenous EGFR ligands by studying trafficking and processing of these ligands in the context of polarized epithelial cells.

When produced by polarized epithelial cells, EGF, TGFα, AR and HB-EGF exhibit differences in trafficking and cell surface delivery, cell surface processing and subsequent binding to the EGFR (summarized in Fig. 1.4B). EGF is delivered equally to both the apical and basolateral plasma membrane (Dempsey, Meise et al. 1997). In contrast, TGFα and AR are sorted preferentially to the basolateral membrane where they are cleaved by TNFα-converting enzyme/a disintegrin and metalloproteinase 17 (TACE/ADAM17) (Brown, Meise et al. 1998; Borrell-Pages, Rojo et al. 2003; Gschwind, Hart et al. 2003; Sahin, Weskamp et al. 2004), a cell membrane metalloproteinase that is restricted to this compartment. Cell surface cleavage of TGFα occurs so rapidly that administration of a selective TACE inhibitor WAY-022 is required for its reliable detection at the cell surface. Released mature soluble TGFα is then avidly consumed by basolateral EGFRs in an

autocrine or local paracrine fashion (Dempsey and Coffey 1994). Although mediated by the same enzyme, the cleavage of AR appears to be less efficient. Mature AR contains an amino-terminal heparin-binding domain (HBD) that interacts with extracellular and cell-associated HSPGs, as well as the tetraspanin, CD9 (Johnson and Wong 1994; Inui, Higashiyama et al. 1997; Nylander, Smith et al. 1998). Interaction with these molecules may facilitate a depot form of AR that, under certain conditions, can bind and activate EGFRs conferring distinct biological properties of AR (Chung, Cook et al. 2005; Chung, Graves-Deal et al. 2005). HB-EGF is also delivered predominantly to the basolateral compartment but remains there in its transmembrane form. The shedding of HB-EGF can be induced by protein kinase C (PKC) activation (Izumi, Hirata et al. 1998) and increased intracellular calcium (Horiuchi, Le Gall et al. 2007). Therefore, in addition to activation of EGFR by soluble HB-EGF, transmembrane HB-EGF can activate EGFRs in adjacent cells in a juxtacrine manner.

Realization that soluble TGFα was avidly captured by basolateral EGFRs emerged from the initial inability to detect TGFα in the apical or basolateral medium of MDCK cells stably expressing a full length TGFα cDNA under control of the CMV promoter (Dempsey and Coffey 1994). One possible explanation was that TGFα was rapidly consumed by the approximately 40,000 EGFRs that are confined to the basolateral surface. In support of this hypothesis, basolateral administration of a monoclonal antibody C225 (an EGFR monoclonal antibody that blocks ligand binding) resulted in a marked increase in TGFα levels in the basolateral medium. This phenomenon is not

merely a tissue culture artifact since a 3-fold increase in serum levels of TGFα was observed one day following administration of C225 to a patient with Ménétrier's disease (Burdick, Chung et al. 2000), a rare disease that is caused by ectopic EGFR activation.

This local capture of TGFα by the EGFR has far reaching biological importance and contributes to such diverse processes as vulva formation in the nematode *C. elegans* and hair follicle formation in mammals. In *C. elegans* vulva development, Lin-3 (a soluble TGFα-like ligand) is released from the anchor cell and is taken up avidly by Let-23 (the EGFR homologue) on the closest vulva precursor cell P6.p to orchestrate the orderly development of the vulva (Sternberg and Horvitz 1986; Kornfeld 1997). Laser ablation of P6.p, as well as genetically reduced LET-23 activity in P6.p (Hajnal, Whitfield et al. 1997), results in diffusion of Lin-3 and its consumption by basolateral Let-23 on neighboring precursor cells (designated P5.p and P7.p). This leads to adoption of a primary cell fate by P5.p and P7.p, and subsequently results in an aberrant multivulva phenotype. Thus, local consumption of Lin-3 (TGFα) by basolateral Let-23 (EGFR) in P6.p is critical to normal vulva development.

Rapid consumption of TGFα by EGFR also appears to be important in mammalian hair follicle development as demonstrated in studies of EGFR null chimeric mice. In these mice, where outer root sheath does not express EGFR but adjacent dermal cells do, TGFα (produced by the inner root sheath) is not rapidly consumed by EGFRs in the outer root sheath, resulting in disorganized hair follicles (David Threadgill, personal communication). In addition, these studies have suggested that when TGFα avoids local

capture by EGFRs in the outer root sheath, it can diffuse and potentially act as a chemotactic factor for adjacent dermal cells. This may account for the acneiform eruptions that are observed with monoclonal antibody blockade of the EGFR where inflammatory cells are found at the base of the hair follicle (Luetteke, Qiu et al. 1993).

The rapid cell membrane cleavage and avid local consumption of TGFα support the hypothesis that TGFα is the major EGFR ligand *in vivo*, and that basolateral delivery is a critical, possibly rate-limiting step in the action of TGFα. Two basolateral sorting determinants have been previously discovered in the cytoplasmic tail of TGFα (Dempsey and Coffey 1994). Recent studies have demonstrated that Naked2, an originally identified Wnt antagonist, binds to the basolateral sorting determinants of TGFα and escorts TGFα-containing vesicles to the basolateral corner of polarized epithelial cells where these vesicles dock and fuse with the cell membrane in a Naked2 myristoylation-dependent manner (Fig. 1.7) (Li, Franklin et al. 2004). This process appears to be specific to TGFα since overexpression of myristoylation-deficient G2A Naked2 disrupts basolateral sorting of TGFα, but not AR.

The two EGFR ligands, TGFα and AR, both are frequently upregulated in colorectal cancer. However, exogenous AR, but not TGFα, disrupts epithelial junctional integrity leading to an epithelial to mesenchymal (EMT)-like transition (Chung, Cook et al. 2005; Chung, Graves-Deal et al. 2005). This effect is abrogated by EGFR blockade. It is possible that an epithelial cell maintains normal homeostasis by proper spatial compartmentalization of the EGFR and TGFα. Perturbations in the Naked2-dependent

trafficking of TGFα may be a cause, and not merely a consequence, of the neoplastic state by creating relatively unoccupied EGFRs that were then bound by AR, inducing an EMT-like transition that contributes to tumor progression.

In addition, a recent report also emphasizes that studying the regulation of EGFR ligand trafficking has important biological relevance. Groenestege et al. discovered that isolated recessive renal hypomagnesemia is due to a mutation in the cytoplasmic tail of EGF that disrupts basolateral sorting of EGF. This leads to insufficient stimulation of basolateral EGFRs in the proximal tubule of the kidney, resulting in impaired activation of the Mg^{2+} channel TRPM6 (transient receptor potential cation channel, subfamily M, member 6) and magnesium wasting in the kidney (Groenestege, Thebault et al. 2007).

I predict that investigation of the regulation and *in vivo* role of Naked2 will provide insights into basic mechanisms of TGFα basolateral trafficking that will eventually lead to a better understanding of the pathogenesis of colorectal cancer (CRC) and more effective ways to diagnose and treat this disease. Moreover, these results may have relevance to other epithelia such as lung, prostate and breast.

Part III: Wnt signaling and its negative regulators

Current model of canonical Wnt signaling

Wnt signaling pathway is a highly conserved pathway that is essential for embryonic development and stem cell maintenance in adult tissues (Fig. 1.5). Deregulation of Wnt pathway has also been closely linked to malignant transformations,

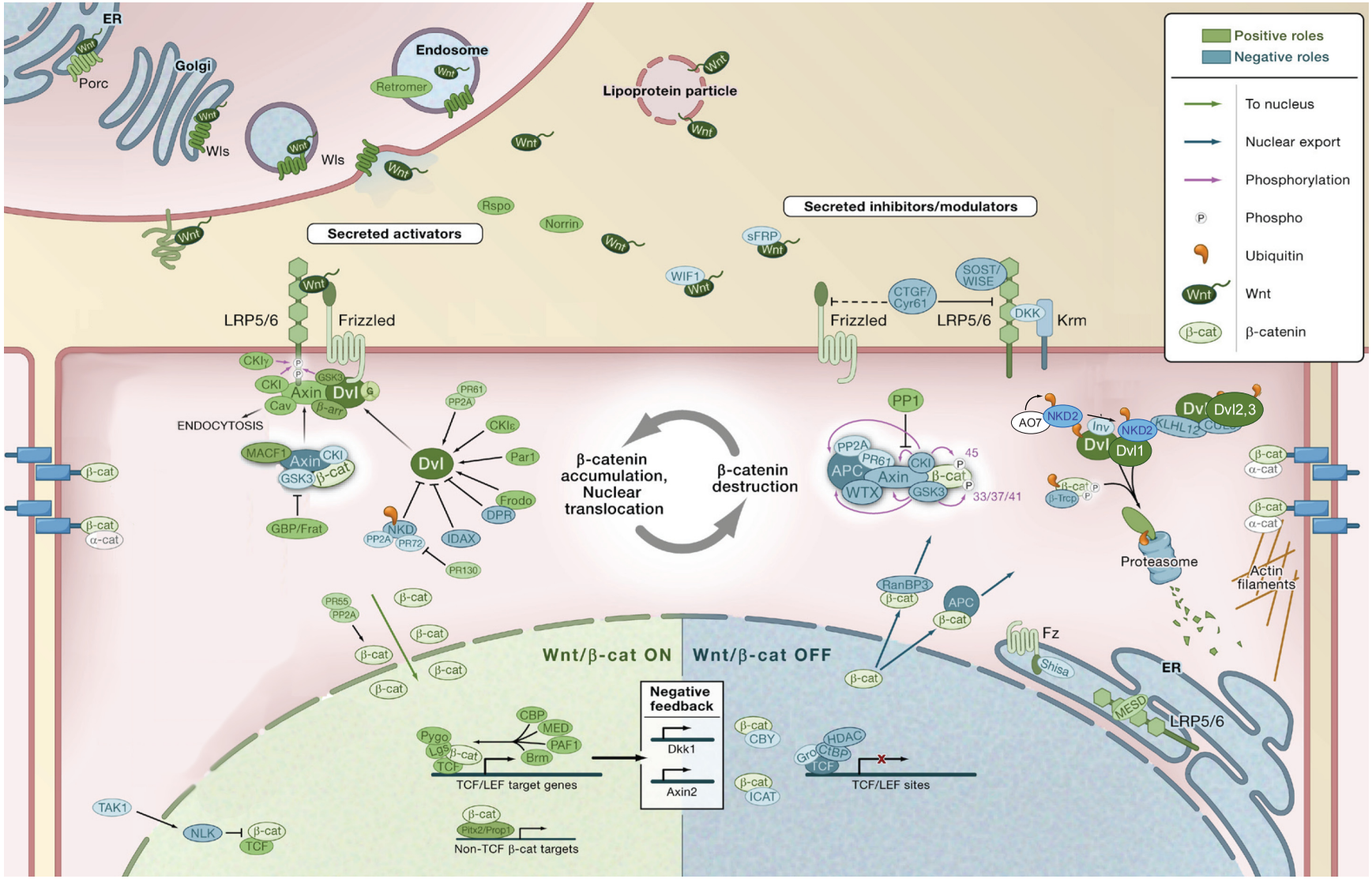


Figure 1.5 Schematic model of canonical Wnt signaling (Adapted from MacDonald, 2007).

and this is best illustrated by the role of Wnt signaling in the initiation of colorectal cancer (Logan and Nusse 2004; Gregorieff and Clevers 2005; Reya and Clevers 2005).

The discovery of the Wnt family originated from *Drosophila wingless* that was identified as a key segmental polarity gene during early development of *Drosophila* embryos. Subsequently, the first mammalian homolog of wingless was discovered as a proto-oncogene *int-1* in mouse, and this was followed by a rapid expansion of the Wnt gene family. To date, 19 Wnt genes are found in the mouse and human genomes, and all encode cysteine-rich glycoproteins. Wnt proteins are secreted as hydrophobic proteins due to palmitoylation at a conserved cysteine residue (Willert, Brown et al. 2003). This post-translational lipid modification not only affects membrane association of Wnt proteins, but it is also required for their biological activity. An enzyme that is encoded by the *Drosophila porcupine* (*porc*) gene (Kadowaki, Wilder et al. 1996), called *mom-1* in *C. elegans* (Rocheleau, Downs et al. 1997), appears to catalyze the addition of palmitate to Wnts; however, direct evidence has not yet been demonstrated.

After secretion from signaling cells, Wnt proteins act on target cells by interacting with membrane-bound receptors, the Frizzled (Fz)/ low density lipoprotein (LDL) receptor-related protein (LRP) complex (Bhanot, Brink et al. 1996; Pinson, Brennan et al. 2000; Tamai, Semenov et al. 2000; Wehrli, Dougan et al. 2000). This interaction causes phosphorylation of the cytoplasmic tail of LRP, which recruits Axin to LRP (Tamai, Zeng et al. 2004). The translocation of Axin, along with a potential mechanism involving Dishevelled (Dsh) through its interaction with Fz, disrupts the β-catenin destruction

complex, a key component of the canonical Wnt signaling cascade (Fig. 1.5). Alternative Wnt cascades that are independent of β-catenin function are categorized as non-canonical Wnt signaling. This alternative pathway(s) is less well understood but involves intracellular calcium fluxes and planar cell polarity (Veeman, Axelrod et al. 2003). As my work does not address non-canonical signaling, only Wnt/β-catenin signaling will be discussed in the thesis.

Under physiological conditions, Dsh, casine kinase I (CKI), GSK-3β, Axin and APC form a β-catenin destruction complex that sequesters cytoplasmic β-catenin. CKI and GSK-3β sequentially phosphorylate a set of Ser and Thr residues within the amino terminus of β-catenin (Rubinfeld, Albert et al. 1996; Amit, Hatzubai et al. 2002; Liu, Li et al. 2002) that results in a recruitment of β-TrCP-containing E3 ubiquitin ligase, which ubiquitylates β-catenin and targets it for proteasomal degradation (Hart, Concordet et al. 1999; Kitagawa, Hatakeyama et al. 1999; Winston, Strack et al. 1999).

In the presence of Wnt, through an unclear signal mediated by Axin and Dsh, activated Wnt receptor Fz/LRP disassembles the β-catenin destruction complex. Stabilized β-catenin then translocates into the nucleus and forms a transcriptional activator complex with TCF transcriptional factors (TCF1, LEF, TCF3 and TCF4) (Behrens, von Kries et al. 1996; Molenaar, van de Wetering et al. 1996; van de Wetering, Cavallo et al. 1997). In the absence of nuclear β-catenin, TCF represses expression of target genes (Brannon, Gomperts et al. 1997) by interacting with Groucho (Cavallo, Cox et al. 1998), a transcriptional repressor that recruits histone deacetylases (HDAC), which

results in a chromatin structure inaccessible to the transcriptional machinery (Chen, Fernandez et al. 1999). Nuclear β-catenin displaces the transcriptional repressor Groucho from TCF and converts TCF to a transcriptional activator by recruiting at least two other factors - histone acetylase CBP/p300 (cyclic AMP response element-binding protein) (Hecht, Vleminckx et al. 2000; Takemaru and Moon 2000) and the SWI/SNF (switching-defective and sucrose nonfermenting) component BRG1 (Barker, Hurlstone et al. 2001). In addition, activation of target genes also depends on Legless (BCL9) and Pygopus (Kramps, Peter et al. 2002; Parker, Jemison et al. 2002; Thompson, Townsley et al. 2002), possibly by recruiting chromatin remodeling factors and/or facilitating nuclear translocation of β-catenin (Townsley, Cliffe et al. 2004).

The biological consequences of canonical Wnt/β-catenin signaling are solely carried out by the expression of its target genes, involving stem cell renewal, cell proliferation, cell survival and cell motility. Among a large number of Wnt targets, c-Myc and cyclin-D1 have been the most intensively studied. They play important roles in cell cycle progression and are the major mediators of the proliferative effects of canonical Wnt signaling. Overexpression of these two genes has been frequently observed in colorectal cancer.

Negative regulators of canonical Wnt signaling

Given its essential roles in normal epithelial homeostasis and stem cell renewal, it is not surprising that Wnt signaling needs to be tightly regulated, and perturbation of this

regulation causes predisposition to various human cancers. A key feature of this tight control is the negative regulators that antagonize Wnt signaling at different levels (Fig. 1.5; Table 1.1). Secreted Wnt inhibitory factor 1 (WIF1) (Hsieh, Kodjabachian et al. 1999) and the sFRP (Moon, Brown et al. 1997; Zorn 1997) protein family resemble the ligand-binding domain of transmembrane Wnt receptors; they inhibit canonical Wnt signaling by sequestering Wnt ligands extracellularly. At the cell surface, another secreted protein, Dickkopf (Dkk) (Glinka, Wu et al. 1998), binds to LRP coreceptor with high affinity (Bafico, Liu et al. 2001; Mao, Wu et al. 2001; Semenov, Tamai et al. 2001) and promotes the internalization of LRP, making it unavailable to bind Wnt ligands. This potent Wnt inhibitor has not been found in invertebrates, but mice and humans have multiple *Dkk* genes (Krupnik, Sharp et al. 1999; Monaghan, Kioschis et al. 1999). Another class of single-transmembrane molecules, the Kremen proteins (Mao, Wu et al. 2002; Mao and Niehrs 2003), act as Dkk receptors and are required for this process, but the effects of Kremen proteins could be context-dependent (Cselenyi, 2008, Science Signaling).

The components of the cytoplasmic β-catenin destruction complex are important intracellular inhibitors of canonical Wnt signaling. The two scaffolding proteins, APC and Axin, are the most extensively studied. They essentially form a scaffold, recruiting additional components to form a functional platform where β-catenin is sequentially phosphorylated by CKI and GSK3β, thus priming β-catenin for poly-ubiquitylation and degradation. An additional component of this complex, WTX, has been recently

Table 1.1 Negative regulators of canonical Wnt signaling

Negative regulator	Localization	Activities
WIF	extracellular	sequester Wnt ligands
sFRP	extracellular	sequester Wnt ligands
Dickkopf	cell surface	promote LRP internalization
Kremen	cell surface	recruit Dickkopf
APC	cytoplasmic	scaffold
	nuclear	export nuclear β-catenin
		recruit transcriptional repressors
Axin	cytoplasmic	scaffold
WTX	cytoplasmic	scaffold
CK1	cytoplasmic	phosphorylate β-catenin
GSK3β	cytoplasmic	phosphorylate β-catenin
β-TRCP	cytoplasmic	ubiquitylate β-catenin
Naked	cytoplasmic	inactivate Dvl
Chibby	nuclear	sequester β-catenin
ICAT	nuclear	sequester β-catenin
Groucho	nuclear	TCF-binding transcriptional repressor
MTGR-1	nuclear	TCF-binding transcriptional repressor
NLK/Nemo	nuclear	phosphorylate TCF
Par5	nuclear	export phosphorylated TCF

identified. Similar to APC, functional analyses demonstrated that WTX promotes ubiquitination and degradation of β-catenin (Major, Camp et al. 2007). Mutations in either APC or the Axin gene have been frequently discovered in colorectal tumors, whereas WTX is mutated in Wilms tumors. Most of these mutations result in truncated proteins that can not mediate interactions with critical molecules. For example, mutant APC usually lacks β-catenin- or Axin-binding domains, and thus it is unable to target cytoplasmic β-catenin for degradation. Recent studies have shown that Axin acts more than just as a scaffolding protein. It is also involved in transducing signals from Wnt receptors and the β-catenin destruction complex. Upon Wnt activation, Dishevelled (Dvl) and Axin are relocated to the plasma membrane through direct interactions with Fz and LRP receptors, respectively. This relocation of Axin causes disassembly of the cytoplasmic protein complex that is required for β-catenin destruction. Stabilized β-catenin then translocates to the nucleus.

In the nucleus, it appears that β-catenin does not necessarily have unrestricted access to TCF. Chibby is a nuclear antagonist that binds to the C terminus of β-catenin (Takemaru, Yamaguchi et al. 2003) and sequesters β-catenin from TCF binding. Another β-catenin-binding protein, ICAT, not only blocks the binding of β-catenin to TCF (Tago, Nakamura et al. 2000) but disassembles complexes between β-catenin, LEF and CBP/p300 (Daniels and Weis 2002; Graham, Clements et al. 2002). Even if β-catenin escapes from these inhibitory factors, it still needs to compete with Groucho that binds to TCF as a transcriptional repressor. Similar to Gourcho, myeloid translocation gene

related-1 (MTGR-1) is another transcriptional repressor that binds to TCF (Moore, Amann et al. 2008). In addition, TCF is also subject to regulation, as it can be phosphorylated by the MAPK–related protein kinase NLK/Nemo (Ishitani, Ninomiya-Tsuji et al. 1999). This phosphorylation diminishes the DNA-binding affinity of the β-catenin/TCF complex, thereby affecting transcriptional regulation of Wnt target genes (Ishitani, Ninomiya-Tsuji et al. 1999; Ishitani, Kishida et al. 2003). Another consequence of TCF phosphorylation, at least in *C. elegans*, is export of TCF from the nucleus (Meneghini, Ishitani et al. 1999), which is carried out by a 14-3-3 protein, Par5 (Lo, Gay et al. 2004). More recently, accumulating data indicate that APC chases nuclear β-catenin and facilitates CtBP-mediated repression of Wnt target genes in normal, but not in colorectal cancer cells (Sierra, Yoshida et al. 2006; Xiong and Kotake 2006).

In summary, physiological levels of canonical Wnt signaling are tightly regulated by a multi-layer network of negative regulators (Table 1.1). Loss of critical negative regulators promotes ectopic Wnt signaling and can lead to cancers or developmental disorders. Elucidation of the regulation of and precise biological roles for Wnt antagonists will provide important insights into the control of Wnt signaling and may identify tractable drug targets.

Part IV: Naked2 in TGFα trafficking and Wnt signaling

Naked protein family

The founding member of the Naked family, *naked cuticle* (*nkd*), was identified as a

segment polarity gene in *Drosophila* (Zeng, Wharton et al. 2000) and was named based on its excess naked cuticle phenotype in *nkd* mutant larvae.

In *Drosophila*, embryonic segmentation begins as early as 3 h after egg laying (AEL). Through positive and negative transcriptional regulation, the pair-rule genes that have already been patterned in a periodically gradient fashion along the anterior-posterior axis initiate the expression of segment polarity genes, such as *wingless (wg), engrailed (en)* and *hedgehog (hh). engrailed* and *hedgehog* are expressed in the same row of cells, whereas *wingless* is expressed in the adjacent stripe of cells. From 3.5 h AEL, as cellularization of the embryo occurs and transcriptional regulation by pair-rule genes fade away, the parasegmental periodicity of the body plan needs to be reinforced through cell to cell signalings between *wg* expressing cells and *en* expressing cells. Secreted *Wg* protein binds to and activates its receptors (D-frizzled-2) (Bhanot, Brink et al. 1996) on the surface of adjacent *en* expressing cells. The downstream signaling of this receptor maintains the transcription of *en* (Siegfried and Perrimon 1994), which itself is a transcription factor that sustains *hh* gene expression in the *en*-expressing cells. When *Hh* protein is secreted and binds to the *Hedgehog* receptors on the adjacent posterior cells, it stimulates *wg* gene expression (Heemskerk, DiNardo et al. 1991; Ingham 1991; Mohler and Vani). This reciprocal signaling loop between neighboring cells stabilizes the transcription pattern of these two types of cells. The boundary between *wg* and *en/hh*-expressing cells forms a temporary landmark of parasegments. In cooperation with other signaling molecules, the diffusion of *Wg* and *Hh* proteins center from the

parasegmental boundary provides gradient signals to specify cell identities across the parasegment during the definitive segmentation.

On the ventral side of the abdominal epidermis, asymmetrical distributions of *Wg*, *Hh* and *EGFR* signalings result in 6 segmental repeats of denticle belts interspersed with naked cuticles. Each denticle belt contains 6 rows of cells secreting unique types of protrusions (denticles) of various sizes and shapes. These cells receive *EGFR* signaling and are composed of a row of cells anterior to the segment boundary and 5 rows of cells posterior to the segment boundary. In contrast, the other 6 rows of cells receive high levels of *Wg* signaling and adopt a smooth or naked cuticle cell fate. In *wg* mutant larva, the cuticles are replaced by mirror images of denticles (Bejsovec and Martinez Arias 1991), while embryos that were exposed to excess *Wg* exhibit a loss of denticle belt phenotype (Noordermeer, Johnston et al. 1992). Naked cuticle phenotype is also seen in embryos that have a mutation in *Wg* antagonists, such as *zestewhite3/glycogen synthase kinase 3*b *(zw3/gsk3*b*)* (ref. 8), *D-axin* (ref. 9) and *D-Apc2* (ref. 10).

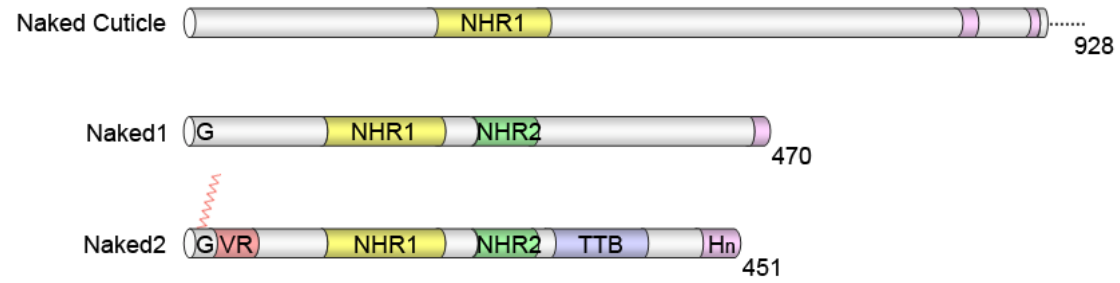
The excess naked cuticle phenotype observed in *nkd* mutant embryos indicates that *Naked Cuticle* acts as a negative regulator of canonical *Wg* signaling (Zeng, Wharton et al. 2000). Epistasis studies have placed *Nkd* activity in a transduction step between *Dsh* and *Zw3* (Rousset, Mack et al. 2001). A direct interaction between *Naked Cuticle* and *Dsh* is required for the antagonistic effect of Naked Cuticle. The interaction is dependent on zinc but not calcium, although Naked Cuticle protein contains an EF-hand domain (Rousset, Wharton et al. 2002). In addition, *nkd* transcription is lost in *wg* mutant embryos,

suggesting that *nkd* is a *Wg* target gene and part of a negative feedback loop of canonical *Wg* signaling through which *nkd* may play critical roles in limiting the duration and distribution of canonical *Wg* signals.

Subsequent studies have identified two mammalian orthologs of *Drosophila Naked Cuticle* in mouse and human (Fig. 1.6), Naked1 and Naked2 (Wharton, Zimmermann et al. 2001; Yan, Wallingford et al. 2001). The most conserved region amongst Naked family members is the EF-hand motif, also known as Naked homologous region 1 (NHR1, residues 107-175 in human Naked2). For Naked1, this motif binds Dsh and Zn^{2+} but not Ca^{2+}; Naked2's affinity for Dsh is weaker (Rousset, Wharton et al. 2002; Wharton 2003). Mice with a targeted deletion of the EF-hand region of Naked1 exhibit reduced spermatogenesis but otherwise appear normal (Li, Ishikawa et al. 2005). The remaining three conserved regions between Naked1 and 2 are as follows: 1) the N-terminal 36 amino acids that include glycine as the second residue that is myristoylated along with adjacent *cis*-acting basic residues; 2) residues 236-265 in human Naked2 (Naked homologous region 2 [NHR2]); and 3) a C-terminal polyhistidine stretch. Naked2, but not Naked1, recognizes a Golgi-processed form of TGFα and binds the cytoplasmic tail of TGFα between residues 300-385 (TGFα cytoplasmic tail-binding domain [TTB]), a sequence in Naked2 that diverges from Naked1 (Li, Franklin et al. 2004; Li, Hao et al. 2007).

Similar to their Drosophila counterpart, mammalian Nakeds directly interact with Dishevelled (Dvl) in yeast two-hybrid, GST-pulldown and co-immunoprecipitation

A



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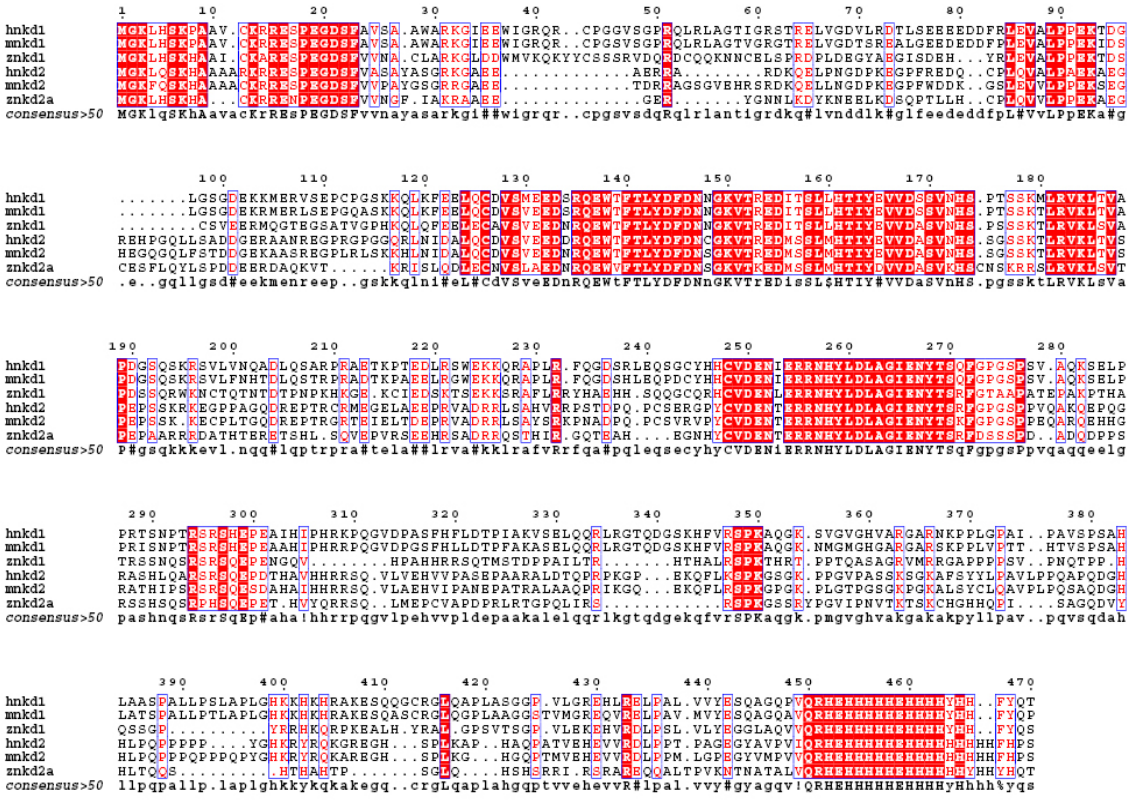

Figure 1.6 Naked protein family. (A) Naked family includes *Drosophila Naked Cuticle* and vertebrate Naked1 and Naked2. The most conserved region amongst Naked family members is the EF-hand motif, also known as Naked homologous region 1 (NHR1). Residues 236-265 in human Naked2 (Naked homologous region 2 [NHR2]), N-terminal myristoylation site (G) and C-terminal polyhistidine stretch (Hn) are conserved in Naked1 but not in *Naked Cuticle*. Other functional domains, such as TGFα-tail-binding (TTB) domain and vesicle recognition (VR) domain, only exist in Naked2. (B) Nucleotide sequence alignment of Naked1 and Naked2 in human, mouse and zebrafish.

assays (Wharton, Zimmermann et al. 2001; Yan, Wallingford et al. 2001). Overexpression of mouse Naked1 inhibits Wnt-1-induced canonical Wnt/β-catenin signaling as demonstrated by TOPflash reporter assays. *In vivo* expression of mouse Naked1 rescues wnt-8-induced secondary axes formation in *Xenopus* embryos. Independent of its roles in canonical Wnt signaling, Naked1 is reported to stimulate Wnt/PCP (planar cell polarity) signaling, resulting in defective convergence extension in *Xenopus* embryos (Yan, Wallingford et al. 2001). Given that Naked directly binds to Dvl, a known regulator of both canonical Wnt/β-catenin and Wnt/PCP pathways, these studies suggest that mammalian Naked may act as a switch of Wnt signaling to direct Dvl toward the PCP pathway and away from the β-catenin pathway.

However, a recent study in *zebrafish* revealed a different scenario. Overexpression of *zebrafish* Naked1 and Naked2 not only antagonizes maternal and zygotic Wnt/β-catenin signaling but also inhibits the Wnt/PCP pathway in the early development of *zebrafish* embryos (Van Raay, Coffey et al. 2007). *Silberblick* is a loss-of-function Wnt11 mutant that exhibits impaired PCP signaling with defects in convergence extension during gastrulation and cyclopia due to abnormal extension of axial tissues. Injection of *naked1* or *naked2* RNA exacerbate the penetrance and expressivity of cyclopia. In a separate experiment, Naked1 or Naked2 abolishes the ability of *wnt11* to rescue this phenotype. These results argue that, in *zebrafish*, Naked1 and Naked2 are sufficient to suppress both Wnt/β-catenin and Wnt/PCP pathways.

Taken together, *Drosophila Naked Cuticle* may strictly function in Wg/Armadillo

signaling, but the roles of vertebrate Nakeds appear to be more complicated. The discrepancy of Naked functions in the Wnt/PCP pathway between the two vertebrate systems may be due to the different assays or organisms employed, or just unique to *silberblick* mutants. It indicates that vertebrate Nakeds play complex roles in Wnt signaling pathways rather than a simple switch between β-catenin and PCP pathways. However, despite all discrepancies, the prevailing notion is that Naked binds to Dvl and antagonizes canonical Wnt/β-catenin signaling in both vertebrates and invertebrates.

Naked2 functions in basolateral trafficking of TGFα

In addition to roles in Wnt signaling, our previous studies have demonstrated a unique function of human Naked2 in sorting and delivering TGFα to the basolateral cell membrane of polarized epithelial cells (Fig. 1.7) (Li, Franklin et al. 2004; Li, Hao et al. 2007).

Using a yeast two-hybrid library that we made from RNA from polarizing HCA-7 cells, we identified that Naked2 interacts with the two BL sorting determinants in the cytoplasmic tail of TGFα. Naked2 interacts with the cytoplasmic tail of a Golgi-processed form of TGFα by coating a novel subset of TGFα-containing exocytic vesicles and escorting these vesicles to the basolateral corner of polarized epithelial cells, where the vesicles dock and fuse in a Naked2 myristoylation-dependent manner (Li, Franklin et al. 2004). TGFα is unable to reach the plasma membrane in myristoylation-deficient (G2A) Naked2-expressing cells. These effects are specific for

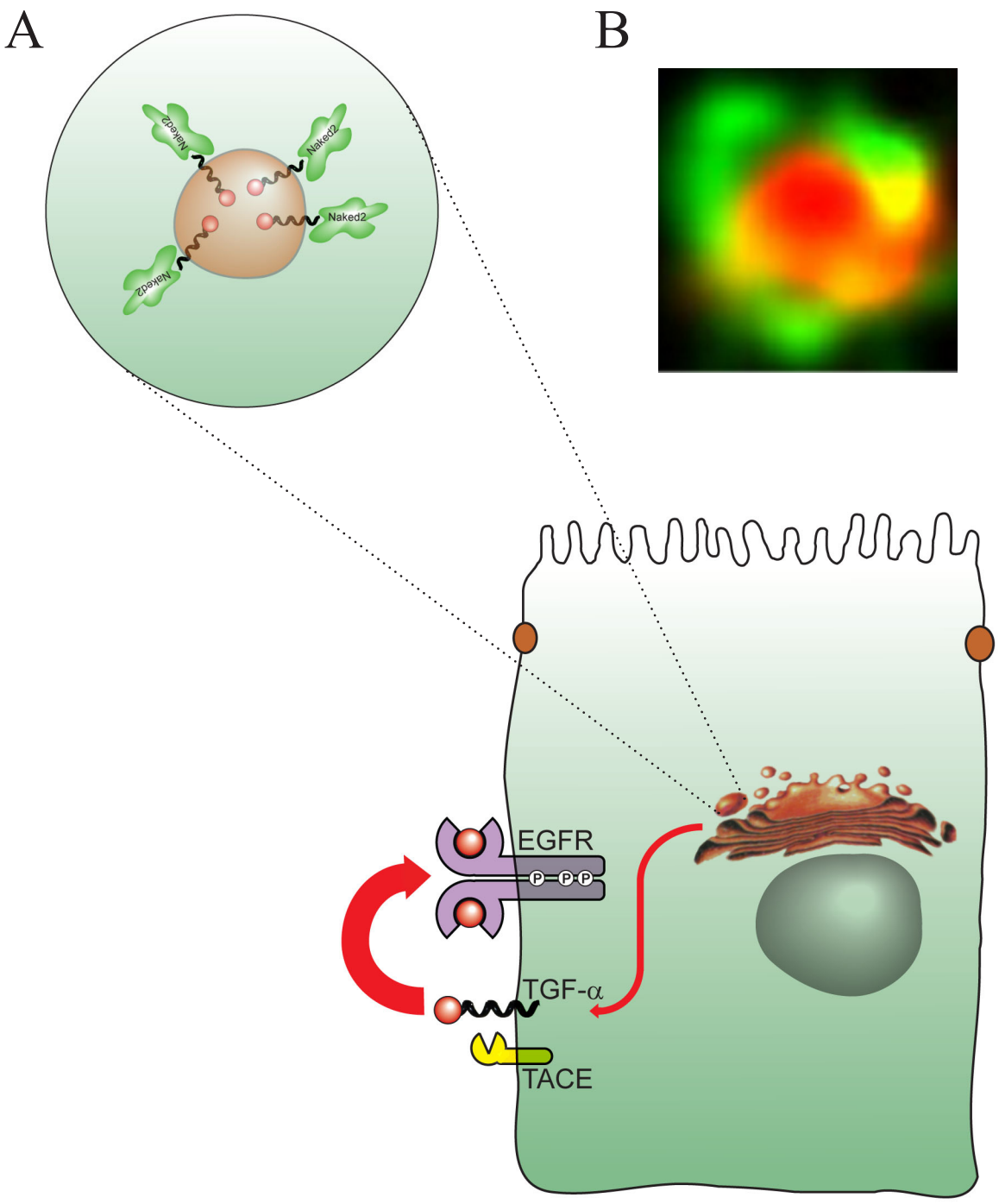

Figure 1.7 TGFα delivery by Naked2-associated vesicles. (A) As TGFα-containing vesicles emerge from the *trans* Golgi network, Naked2 coats and escorts these exocytic vesicles to the basolateral corner of polarized epithelial cells. These vesicles dock and fuse with the plasma membrane in a Naked2 myristoylation-dependent manner. (B) Demonstration of a Naked2-coated TGFα-containing vesicle by fluorescence microscopy. Naked2-EGFP (green) outlines the surface of the vesicle, while HA staining (red) decorates the ectodomain of epitope-tagged TGFα in the vesicle lumen.

TGFα in that basolateral sorting of AR is retained in both wild-type and G2A Naked2-expressing cells.

Myristoylation of Naked2 not only affects cell membrane docking and fusion, but it also stimulates a dynamic behavior between vesicles (Li, Hao et al. 2007). We have shown that discrete residues within the N-terminus of Naked2 direct these vesicles to the basolateral cell surface. Residues 1-173 redirect Na^+/H^+ exchanger regulatory factor (NHERF)-1 from the apical cytoplasm to the basolateral membrane, and internal deletion of residues 37-104 results in apical mislocalization of Naked2 and TGFα. The basolateral trafficking of low-density lipoprotein receptor (LDLR) requires μ1B subunit of the epithelial cell–specific AP-1B adaptor complex (Folsch, Ohno et al. 1999). Sec6/8 exocyst complex beneath the tight junctions is then utilized for basolateral docking and fusion events for LDLR and vesicular stomatitis virus G protein (VSV-G) (Kreitzer, Schmoranzer et al. 2003; Polishchuk, Di Pentima et al. 2003). However, Naked2-associated vesicles are directed to the lower lateral membrane of polarized MDCK cells and do not appear to require μ1B nor utilize the subapical Sec6/8 exocyst complex as a targeting patch for basolateral docking and fusion. We have proposed that Naked2 acts as a specific cargo recognition and targeting (CaRT) protein to ensure proper targeting, tethering and fusion of TGFα-containing exocytic vesicles at the lower lateral membrane of polarized MDCK cells (Li, Hao et al. 2007).

These data suggest that, under normal conditions, TGFα signaling may be regulated at its ligand intracellular sorting step by a Naked2-dependent mechanism; however, in a

pathological state, TGFα can escape this regulated delivery and processing step due to downregulated or mutated Naked2 (e.g. in colon cancer, see Chapter III). As we discussed earlier in this thesis, impaired TGFα trafficking may disorganize epithelial homeostasis and contribute to tumor progression.

Regulation of Naked2

Naked Cuticle has been originally discovered as an inducible antagonist of the *Wg/Arm* pathway in that *nkd* transcription is markedly reduced in *wg* mutant embryos, and misexpression of either *wg* or an activated form of *Armadillo* (*UAS-Arm*^{S10}) results in ectopic transcription of *nkd* in similar patterns (Zeng, Wharton et al. 2000). In vertebrates, a close correlation between Naked1 expression and active Wnt signaling has been observed in zebrafish embryos, chick embryos, mouse liver epithelial cells and human colon tumors (Yan, Wallingford et al. 2001; Yan, Wiesmann et al. 2001; Ishikawa, Kitajima et al. 2004; Schmidt, Otto et al. 2006; Van Raay, Coffey et al. 2007). Moreover, 5 perfectly matched TCF-binding sites are identified in the putative promoter of the human Naked1 gene (Yan, Wiesmann et al. 2001). In contrast, Naked2 is maternally expressed, and its expression appears ubiquitous in zebrafish and mouse embryos (Wharton, Zimmermann et al. 2001; Van Raay, Coffey et al. 2007). More recently, Lei et al. has demonstrated that mouse Naked2 promoter activity is directly repressed by a homeobox gene Hoxc8 (Lei, Juan et al. 2007), which plays essential roles in differentiation and proliferation and is upregulated in various human cancers (Alami,

Castronovo et al. 1999; Miller, Miller et al. 2003; Chen, Gu et al. 2005). Naked2 appears to be differentially regulated among other Naked family members. In fact, we have shown that Naked2 is indirectly inhibited by canonical Wnt signaling in SW480 cells and that down-regulation of Naked2 is a common event in human colorectal cancer (see Chapter III).

As discussed earlier, Naked2 does exhibit unique adaptor-like activities in TGFα exocytic trafficking. As part of the constitutive intracellular trafficking machinery, most adaptor or coat proteins like, AP-1 and AP-2, are ubiquitously expressed and long-lived (Sorkin, McKinsey et al. 1995). It is generally thought that adaptors regulate recruitment and stability of cargo but not vice versa. However, Hirst and co-workers recently reported that degradation of a relatively short-lived clathrin-dependent adaptor, GGA2, was delayed when one of its cargos, CIMPR, was overexpressed in HeLa cells (Hirst, Seaman et al. 2007). The authors concluded that cargo proteins may not be simply passively sorted by adaptors, but they may actively contribute to the formation of coated vesicles by interacting with and regulating coat components, at least in the case of GGA2. In a similar context, we asked whether the stability of Naked2 was regulated by its cargo, TGFα.

Ubiquitin-mediated and proteasome-dependent proteolysis is one of the key mechanisms that regulate protein stability and expression levels in a variety of cellular processes, such as cell cycle progression, signaling transduction and protein transport (Hochstrasser 1996; Pickart 2001; Weissman 2001). Ubiquitylation is a multi-step

post-translational protein modification catalyzed by E1s (ubiquitin-activating enzymes), E2s (ubiquitin-conjugating enzymes) and finally E3s (ubiquitin ligases) (Fig. 1.9). Cycling of these reactions can conjugate poly-ubiquitin chains on substrate proteins and thereby target them to the proteasome for degradation. The highly regulated, exquisitely precise ubiquitylation of particular substrates is conferred by protein interactions involving substrate-specific E3s, of which there are well over 500. Poly-ubiquitylated substrates that contain a ubiquitin chain with more than 3 ubiquitin moieties are targeted for proteasomal degradation.

In my thesis study, I not only identify ubiquitylation of Naked2 but also elucidate the biochemical basis for this phenomenon by identifying a ubiquitin ligase for Naked2. Naked2 is a short-lived protein with a half-life of 60 min due to rapid ubiquitin-mediated proteasomal degradation. AO7, a RING finger protein that exhibits ubiquitin ligase activity (Lorick, Jensen et al. 1999), binds to and ubiquitylates Naked2 *in vitro* and *in vivo*. Increased expression of TGFα dose-dependently reduces AO7 binding to Naked2, thus protecting Naked2 from ubiquitin-mediated proteasomal degradation. This effect of TGFα is EGFR-independent; a physical interaction between the cytosolic tail TGFα and Naked2 is necessary and sufficient to attenuate Naked2 ubiquitylation. These studies identify the first E3 for Naked2 and a novel EGFR-independent action of TGFα (see Chapter II for details).

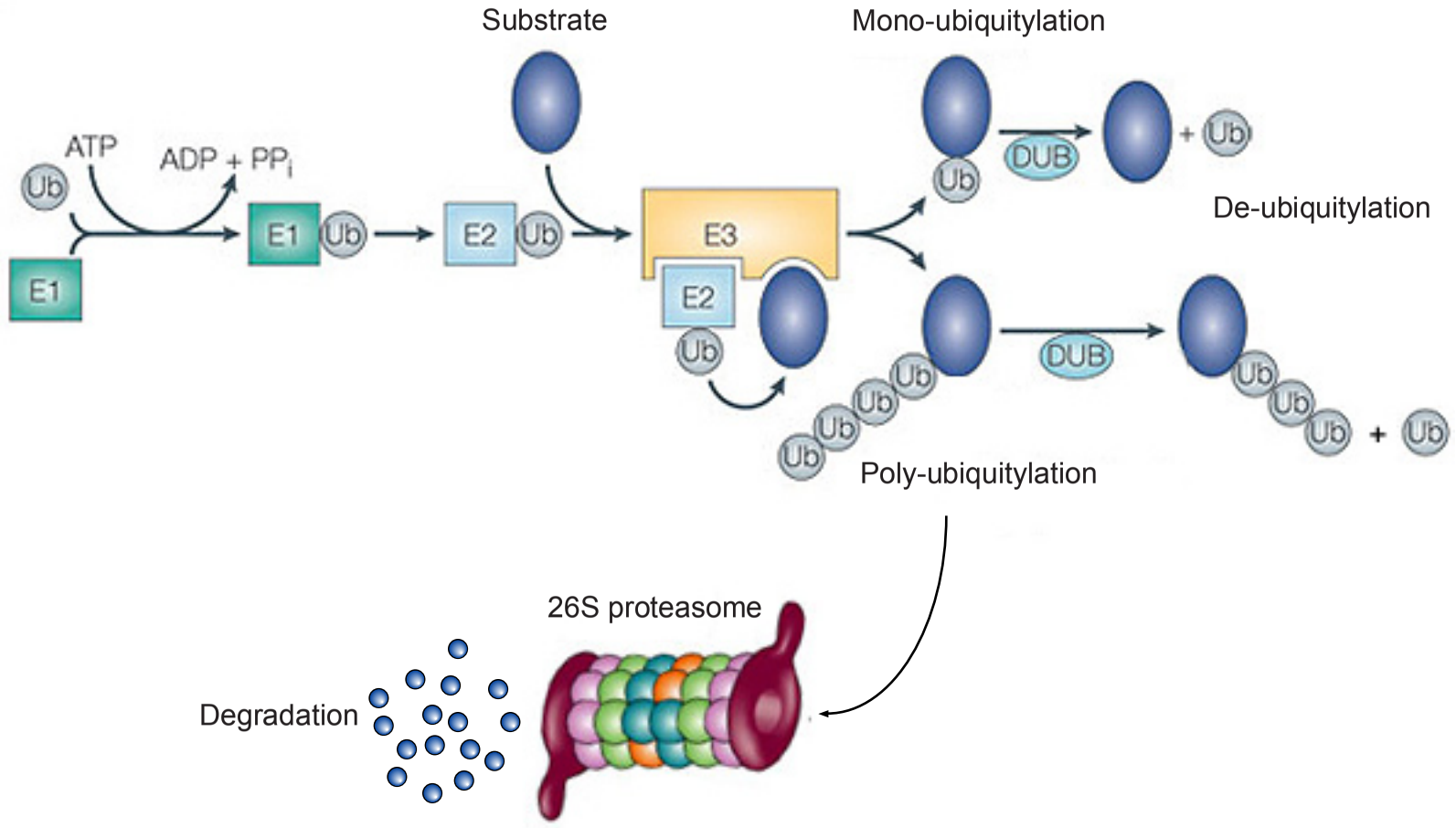

Figure 1.8 Protein ubiquitylation and proteasomal degradation. This post-translational protein modification is initiated by an ATP-dependent activation of ubiquitin (Ub) catalyzed by E1s (ubiquitin-activating enzymes). Activated ubiquitin is passed to E2s (ubiquitin-conjugating enzymes) that can be recruited by E3s (ubiquitin ligases). Substrate-specific E3s recognize substrate proteins and conjugate ubiquitin to lysine residues in the substrates. Following reactions can occur on lysine residues in the previously conjugated ubiquitin, and thereby produce a poly-ubiquitin chain that targets protein for proteolysis mediated by the 26S proteasome (Adapted from Liu, 2005).

CHAPTER II

EGF RECEPTOR-INDEPENDENT ACTION OF TGF-ALPHA PROTECTS NAKED2 FROM AO7-MEDIATED UBIQUITYLATION AND PROTEASOMAL DEGRADATION

Introduction

EGFR signaling is one of the most extensively studied signal transduction pathways and the EGFR itself has proven to be a tractable target in cancer therapy. It is therefore not surprising that EGFR signaling has emerged as an attractive candidate for a systems biology approach (Wiley, Shvartsman et al. 2003; Citri and Yarden 2006). All seven of the mammalian EGFR ligands are produced as transmembrane ligands that are cleaved at the cell surface by proteases to release soluble ligands that then engage the EGFR (Harris, Chung et al. 2003). It is increasingly recognized that a key regulatory node in the initiation of EGFR signaling is transactivation of the receptor by G protein-coupled receptor (GPCR)-mediated, protease-executed cell surface cleavage of pro-ligands (Prenzel, Zwick et al. 1999). An under appreciated aspect of EGFR signaling is the cell surface delivery of the different EGFR ligands. This is especially relevant in the context of polarized epithelial cells where the EGFR is concentrated at the basolateral surface. The importance of this process was underscored by the recent finding that isolated recessive renal hypomagnesaemia is due to a mutation in the cytoplasmic tail of EGF that disrupts its sorting to the basolateral surface of proximal tubular cells of the kidney

(Groenestege, Thebault et al. 2007).

Our laboratory has studied the trafficking of transforming growth factor-α (TGFα) in polarized epithelial cells (Dempsey and Coffey 1994). TGFα is preferentially delivered to the basolateral cell surface where it is rapidly cleaved by TNF-α converting enzyme/a disintegrin and metalloprotease-17 (TACE/ADAM-17) (Dempsey and Coffey 1994; Sunnarborg, Hinkle et al. 2002). Soluble TGFα is then avidly captured by basolateral EGFRs (Dempsey and Coffey 1994). The rapid cleavage and avid capture of TGFα suggest that its cell surface delivery may be a rate-limiting step in the spatial and temporal regulation of endogenous TGFα activities (Harris, Chung et al. 2003). Two basolateral sorting determinants have been identified in the cytoplasmic tail of TGFα (LL and HCCQVRKH) (Dempsey, Meise et al. 2003), both of which contribute to its interaction with Naked2 (Ishikawa, Kitajima et al. 2004), a previously identified negative regulator of Wnt signaling (Rousset, Mack et al. 2001; Yan, Wallingford et al. 2001; Yan, Wiesmann et al. 2001). We have shown that Naked2 binds to the cytoplasmic tail of a Golgi-processed form of TGFα through its TTB domain (residues 300-385). Although Naked2 contains features of an adaptor and a coat (i.e. cargo recognition and selection), it exhibits additional properties (dominant acting basolateral sorting, motor recognition and myristoylation-dependent docking and fusion at the plasma membrane), leading us to designate it a multifunctional cargo recognition and targeting (CaRT) protein for TGFα trafficking (Li, Franklin et al. 2004; Li, Hao et al. 2007). However, the regulation of Naked2 in epithelial cells has not been elucidated.

Ubiquitin-mediated and proteasome-dependent proteolysis is one of the key mechanisms that regulate protein activities in a variety of cellular processes, such as cell cycle progression, signaling transduction and protein transport (Hochstrasser 1996; Pickart 2001; Weissman 2001). Ubiquitylation is a multistep post-translational protein modification catalyzed by E1s (ubiquitin-activating enzymes), E2s (ubiquitin-conjugating enzymes) and finally E3s (ubiquitin ligases). Cycling of these reactions can conjugate poly-ubiquitin chains on substrate proteins and thereby target them to the proteasome for degradation. The highly regulated, exquisitely precise ubiquitylation of particular substrates is conferred by protein interactions involving specific substrate-specific E3, of which there are well over 500.

In this paper, we demonstrate that Naked2 is a short-lived protein with a half-life of 60 min due to rapid ubiquitin-mediated proteasomal degradation. AO7, a RING finger protein that exhibits ubiquitin ligase activity (Lorick, Jensen et al. 1999), binds to and ubiquitylates Naked2 *in vitro* and *in vivo*. Increased expression of TGFα dose-dependently reduces AO7 binding to Naked2, thus protecting Naked2 from ubiquitin-mediated proteasomal degradation (Fig. 5B). This effect of TGFα is EGFR-independent; a physical interaction between the cytosolic tail TGFα and Naked2 is necessary and sufficient to attenuate Naked2 ubiquitylation. These studies identify the first E3 for Naked2 and a novel EGFR-independent action of TGFα.

Materials and Methods

Plasmid construction, antibodies and chemicals

Generation of full-length human Naked2 and TGFα expression plasmids has been described previously (Li, Hao et al. 2007). Truncated Naked2 sequences were obtained by PCR amplification from full-length human Naked2 cDNA and verified by automated sequencing (Perkin Elmer 377, Vanderbilt Ingram Cancer Center DNA Sequencing Shared Resourse). All 5' primers contained EcoRI restriction sites, and all 3' primers contained BamHI sites. The PCR products were cloned into pEGFP-N2 vector (Clontech, Mountain View, CA) between the EcoRI and BamHI sites. TGFα tail expression cDNA contained the TGFα cytoplasmic tail, transmembrane domain and signal peptide, which were obtained by RT-PCR reactions from full-length human TGFα cDNA. PCR fragments were ligated and cloned into pCB7 expression vector between BglII and HindIII sites. The HA-tagged ubiquitin expression plasmid, pHA-Ub (Sekhar, Yan et al. 2002), was a generous gift from Dr. Michael Freeman (Vanderbilt University). Murine AO7, AO7-RM and GST-AO7T expression plasmids have been described (Lorick, Jensen et al. 1999). FLAG-AO7 and FLAG-AO7-RM mammalian expression plasmids were generated by PCR amplification from mouse HA-AO7 and HA-AO7-RM plasmids using a 5' primer encoding an EcoRI site and a FLAG epitope tag, and a 3' primer encoding a NotI site. The PCR products were cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) between the EcoRI and NotI sites.

The monoclonal HA and β-actin antibody were obtained from Sigma (St. Louis, MO).

The rabbit polyclonal Naked2 antibody VU308 was raised against a GST-fused Naked2 peptide (residues 1-217 of human Naked2) in cooperation with Cocalico Biologicals (Reamstown, PA). Horseradish peroxidase-donkey anti-mouse and anti-rabbit IgG were obtained from Jackson ImmunoResearch (West Grove, PA). Protein-G agrose beads were purchased from Invitrogen (Carlsbad, CA). rhTGFα and amphiregulin were purchased from R&D Systems (Minneapolis, MN). MG-132 and CHX were purchased from CALBIOCHEM (San Diego, CA). The selective TACE/ADAM-17 inhibitor WAY-022 was provided by Jay Gibbons (Wyeth Ayerst Laboratories, Pearl River, NY). Human recombinant E1, UbcH5b and HA-ubiquitin were purchased from BostonBiochem. All other chemicals were obtained from Sigma unless otherwise stated.

Cell culture, transfection and stable cell line

Parental HEK293 cells, MDCK cells, Caco-2 cells and all transiently or stably transfected derivatives were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine, nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin (Hyclone, Logan, UT) with or without 500 μg/ml geneticin and/or 200 μg/ml hygromycin (Roche).

Transient transfections were performed in HEK293 cells using FuGENE6 (Roche Applied Science, Indianapolis, IN) transfection reagent according to the manufacturer's instructions. Twenty-four hrs after transfections, cells were harvested or processed for further experiments. Stable transfections were performed using Lipofectamine 2000

(Invitrogen) according to the manufacturer's suggestions. Transfected cells were split and diluted at least 10-fold into selection medium 24 hrs after transfections. For each transfection, multiple antibiotic resistant colonies were picked 4 to 7 days later.

RT-PCR

Total RNA was isolated from Caco-2 cells using the RNeasy mini kit (QIAGEN, Valencia, CA). RT-PCR was performed to determine the relative gene expression using SuperScript™ One-Step RT-PCR kit (Invitrogen) according to the manufacturer's instructions. All amplifications started with a minimal amount of RNA (250 ng of total RNA), and the reactions were limited to 25 cycles to avoid saturating the reaction. GAPDH primers were added into same reaction tubes to serve as an internal control for equivalent starting materials.

Immunoprecipitation and immunoblotting

For immunoprecipitations, cells were lysed in 1X lysis buffer (25 mM Tris HCl, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM DTT and 2% BSA). After preclearing, supernatants were incubated with HA antibody or Naked2 antibody VU308 for 2 h and then with protein G beads for another 2 h. Beads were washed three times with 1X lysis buffer, boiled in sample buffer for 5 min and proteins were resolved on 8% SDS-PAGE for ubiquitylation analysis or 10% SDS-PAGE otherwise and then transferred to nitrocellulose membrane before western blotting. For immunoblotting of

whole cell lysates, the protein concentration of precleared cell lysates was determined using Micro BCA Protein Assay Kit (Pierce, Rockford, IL) before boiling in sample buffer. All straight western blots were reblotted for β-actin to assure equivalent loading and transfer.

Expression and purification of GST-AO7T

A GST fusion of a C-terminal truncation of AO7 at amino acid 363 (GST-AO7T) was utilized in the in vitro ubiquitylation assay as the full length GST fusion is poorly expressed (Lorick, Jensen et al. 1999). GST-AO7T plasmid was transformed into BL-21 strain and grown overnight at 37ºC. The expression of the GST-AO7T was induced by adding 0.2 mM IPTG for 18 h at 30ºC. Cells were then harvested and lysed as previously described (Hu, Krezel et al. 2006). The clear supernatant of bacteria lysate was used to extract GST-AO7T protein using B-PER® GST Fusion Protein Purification Kit (Pierce, Rockford, IL). Finally, 12ml of volume was eluted from glutathione column and 4ml GST-AO7T enriched fractions were dialysed three times against 4L TBS. The purity of the protein was determined by SDS–PAGE and Coomassie blue staining.

***In vitro* ubiquitylation assay**

Ubiquitylation assays were carried out as previously described (Lorick, Jensen et al. 1999), unless indicated. 100ng each of human recombinant E1, UbcH5b (E2), 1µg of HA-ubiquitin and 400ng purified GST-AO7T were used in each 100µl reaction.

Naked2-EGFP expressed in HEK293 cells was immunoprecipitated using GFP antibody and protein G agrose beads followed by 5 times wash in RIPA buffer. Finally, beads were resuspended in 125µl 50mM Tris (PH 7.4). 20µl of agrose-bond Naked2-EGFP were used in each reaction. The reaction products were boiled immediately in SDS sample buffer and resolved by 8% SDS-PAGE followed by western blotting with HA antibody.

Results

Naked2 is ubiquitylated and undergoes rapid proteasomal degradation

Initial studies were designed to determine the stability of Naked2 protein and whether it was regulated by proteasomal or lysosomal degradation. To that end, MDCK cells stably expressing EGFP-tagged Naked2 (Naked2-EGFP) were treated with cycloheximide (CHX) to block protein synthesis and Naked2 protein levels were monitored by immunoblotting. As shown in Fig. 2.1, Naked2 protein was rapidly degraded with a half-life of 60 min. When MDCK cells were pre-treated with a proteasome inhibitor, MG132, for 2 h prior to the addition of CHX, Naked2 degradation was significantly retarded. By contrast, pre-treatment with a lysosomal inhibitor, ammonium chloride (NH_4Cl), had no effect (Fig. 2.1) on Naked2 degradation. Thus, Naked2 is a short-lived protein that undergoes rapid proteasomal degradation.

Since poly-ubiquitylation is a signature for delivering substrates to the proteasome for degradation (Doherty, Dawson et al. 2002; Pickart 2004), we next performed an *in vivo* ubiquitylation assay (Sekhar, Yan et al. 2002) to directly examine the ubiquitylation of Naked2. We transiently transfected HEK293 cells with plasmids encoding non-tagged Naked2 and HA-tagged ubiquitin (Sekhar, Yan et al. 2002); 48 h later, cell lysates were prepared and subjected to immunoprecipitation using a Naked2 antibody VU308 (see Materials and Methods) and blotted with an HA antibody. Clear laddering of poly-ubiquitylated Naked2 was detected when HA-tagged ubiquitin was co-expressed with Naked2 (Fig. 2.2A, lane 4). The lowest band detected by the HA antibody migrates

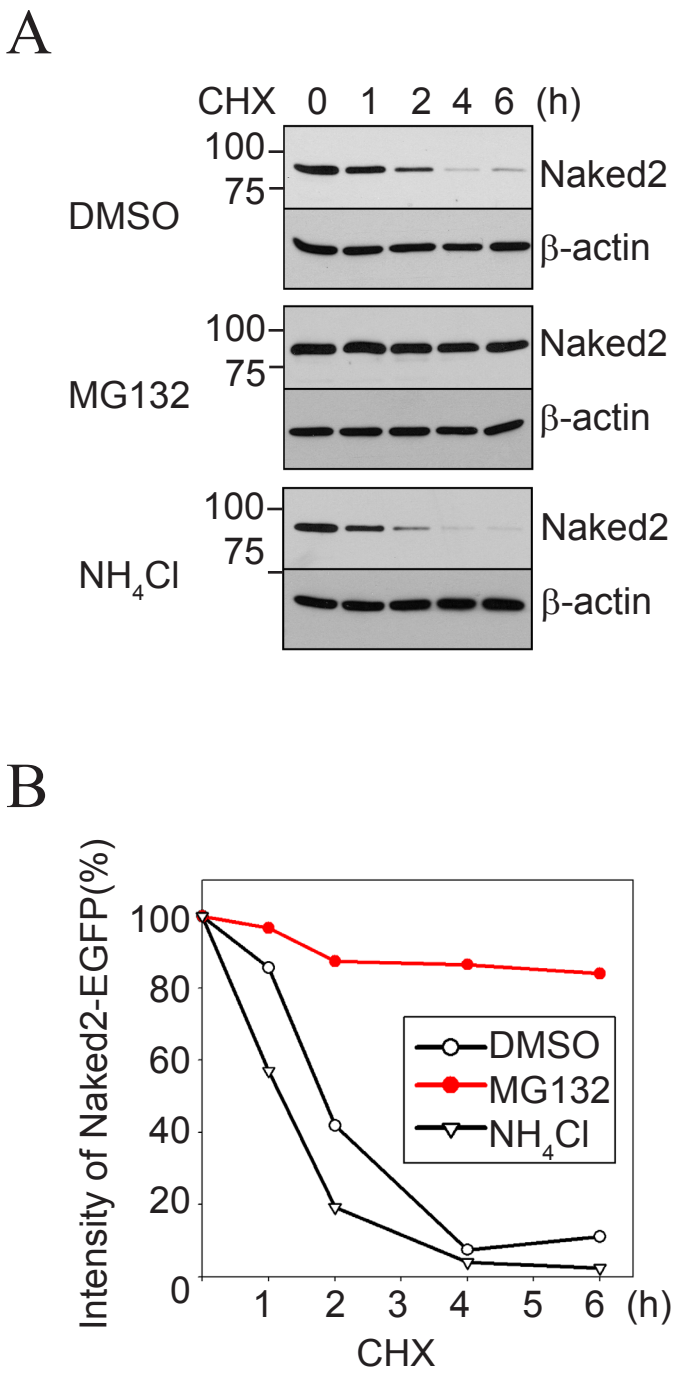

Figure 2.1 Proteasomal degradation of Naked2. (A) MDCK cells expressing Naked2-EGFP were treated with 1 μg/ml cycloheximide (CHX). Cell lysates were collected at the indicated time points and then subjected to Naked2 western blotting. The majority of Naked2-EGFP was degraded within 4 h. Naked2 degradation was blocked by 2 h exposure to a proteasome inhibitor (5 μM MG132) prior to CHX treatment but not by pre-exposure to a lysosomal inhibitor (20 mM NH_4Cl). All blots were reprobed for β-actin as a loading control. (B) Band densities were quantified and normalized to β-actin staining.

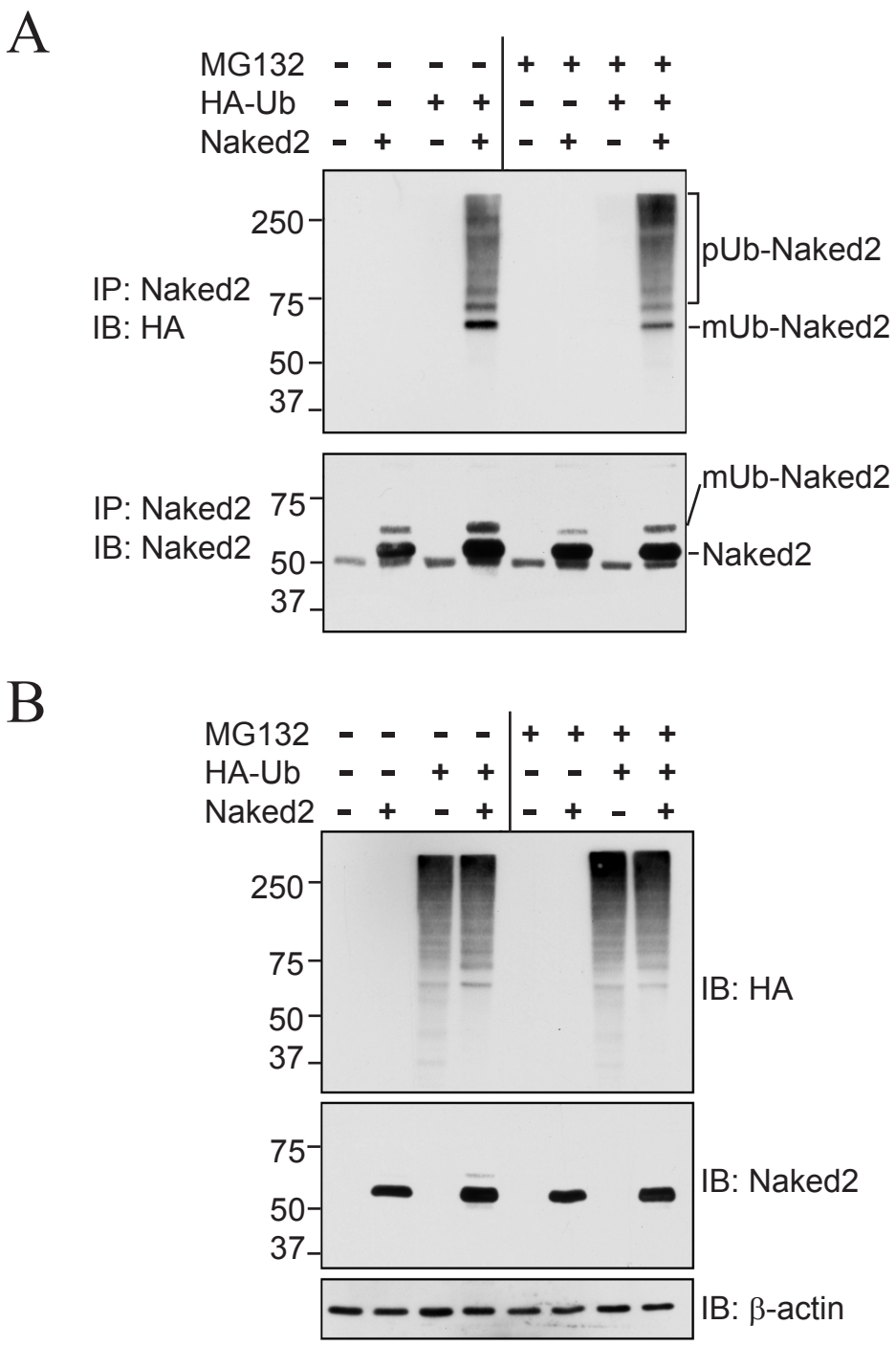

Figure 2.2 Ubiquitylation of Naked2. (A) HEK293 cells transiently expressing Naked2 and HA-ubiquitin were lysed and immunoprecipitated using the Naked2 antibody VU308 (see Materials and Methods) followed by anti-HA western blotting. A ladder of ubiquitylated Naked2 was detected, and the composition of the ladder shifted to higher molecular weight species in the presence of MG132. In the lower panel, the blot was stripped and reprobed with VU308 to demonstrate that comparable amounts of Naked2 were immunoprecipitated. (B) Aliquots of whole-cell lysates were blotted with HA, Naked2 and β-actin antibodies to show their respective expression levels. pUb-Naked2 designates poly-ubiquitylated Naked2; mUb-Naked2 designates mono-ubiquitylated Naked2.

at approximately 9 kDa (the molecular mass of a single HA-ubiquitin molecule) above the Naked2 band, and likely represents mono-ubiquitylated Naked2, which might be an intermediate product of poly-ubiquitylation. The presence of immunoreactive forms migrating at higher molecular weights is consistent with poly-ubiquitylated Naked2, which was confirmed by reprobing with Naked2 antibody (data not shown). The identification of poly-ubiquitylated Naked2 is consistent with it being a short lived protein as demonstrated in the previous degradation assays (Fig. 2.1). Consistent with proteasomal degradation, in the presence of MG132, not only was overall Naked2 ubiquitylation enhanced, but there was also a compositional shift to higher molecular weight forms of ubiquitylated Naked2 (Fig. 2.2A, lane 8). We also observed poly-ubiquitylation of endogenous Naked2 in parental MDCK cells **(**data not shown).

TGFα upregulation of Naked2 is EGFR-independent

We previously demonstrated that Naked2 acts as a CaRT protein for the efficient delivery of TGFα-containing exocytic vesicles to the basolateral surface of polarized epithelial cells (Li, Franklin et al. 2004; Li, Hao et al. 2007). We considered whether the cargo TGFα might regulate its CaRT. We found a marked increase in endogenous Naked2 protein levels in MDCK cells stably overexpressing TGFα (Fig. 2.3A, MDCK-lane 1 and 2). An even more dramatic upregulation of Naked2 protein was observed when TGFα was stably overexpressed in Caco-2 cells, a human colorectal cancer cell line (Fig. 2.3A, Caco-2-lane 1, 2 and 4). Since TGFα is a major EGFR ligand,

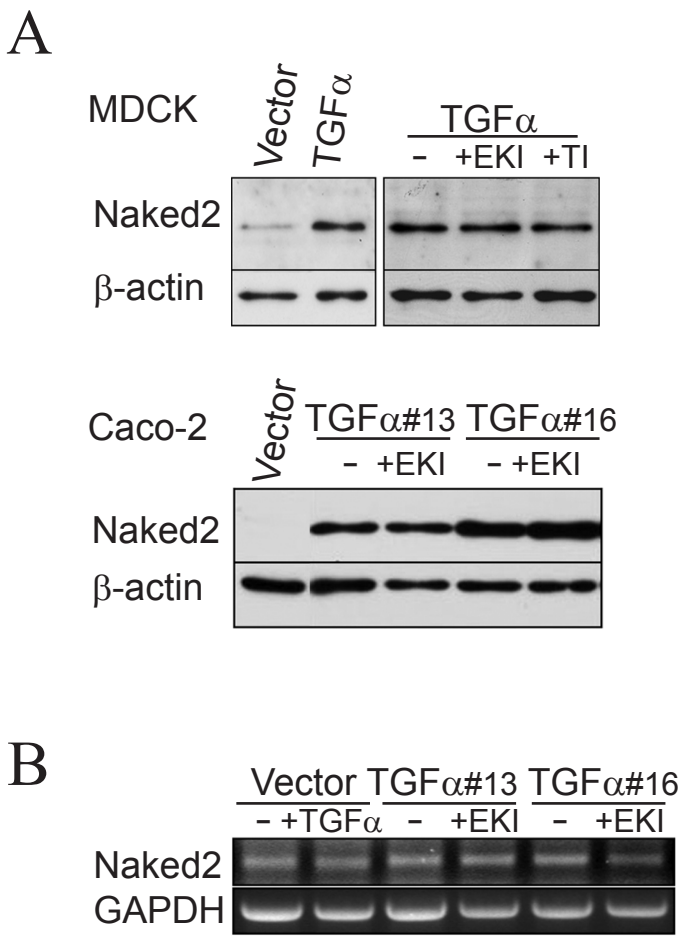

Figure 2.3 EGFR-independent upregulation of Naked2. (A) Compared to vector control cells, MDCK cells stably expressing human TGFα exhibited increased Naked2 levels. Pharmacological blockade of EGFR activation using an irreversible EGFR tyrosine kinase inhibitor (EKI-785, 1 μM) or TGFα cell surface shedding with a selective TACE inhibitor (WAY022, 1 μM; designated as "TI") did not affect Naked2 levels in TGFα transfected MDCK cells. In similar experiments in Caco-2 cells, Naked2 was dramatically upregulated in two independent clones of Caco-2 cells stably expressing TGFα; a longer exposure was required to observe endogenous Naked2 in parental Caco-2 cells (Data not shown). EKI-785 treatment did not affect Naked2 levels in Caco-2 cells. (B) There were no differences in Naked2 expression by RT-PCR analysis of RNA isolated from both vector- and TGFα-transfected Caco-2 cells. GAPDH served as an internal loading control.

we presumed that this effect was mediated by EGFR activation. However, levels of Naked2 in TGFα-expressing MDCK cells were not altered by pharmacological blockade of EGFR tyrosine kinase activity using an irreversible EGFR tyrosine kinase inhibitor (EKI-785) or cell surface cleavage of TGFα using a selective TACE inhibitor (WAY-022) (Fig. 2.3A). Nor did administration of 1 nM recombinant human TGFα (rhTGFα) for 24 h increase Naked2 levels in parental MDCK cells (data not shown). As in MDCK cells, neither blocking EGFR tyrosine kinase activity nor adding rhTGFα affected Naked2 protein levels in Caco-2 cells (Fig. 2.3A and data not shown). To determine whether Naked2 is upregulated at the transcriptional level by TGFα overexpression, RT-PCR for Naked2 was performed in Caco-2 cells. We observed no significant differences in Naked2 transcript levels in TGFα-overexpressing Caco-2 cells compared to vector control cells with or without the EGFR tyrosine kinase inhibitor EKI-785 (Fig. 2.3B). These findings indicate that Naked2 is upregulated by TGFα in an EGFR-independent manner, and this effect occurs at a post-transcriptional level.

Overexpression of TGFα stabilizes Naked2 by inhibiting its ubiquitylation

We next asked whether endogenously produced TGFα upregulated Naked2 protein by retarding its proteasomal degradation. CHX was added to MDCK cells stably expressing Naked2-EGFP with or without co-expressed TGFα. We found that co-expression of TGFα significantly delayed Naked2 turnover compared to vector control cells (Fig. 2.4A), a result consistent with our earlier observation that Naked2

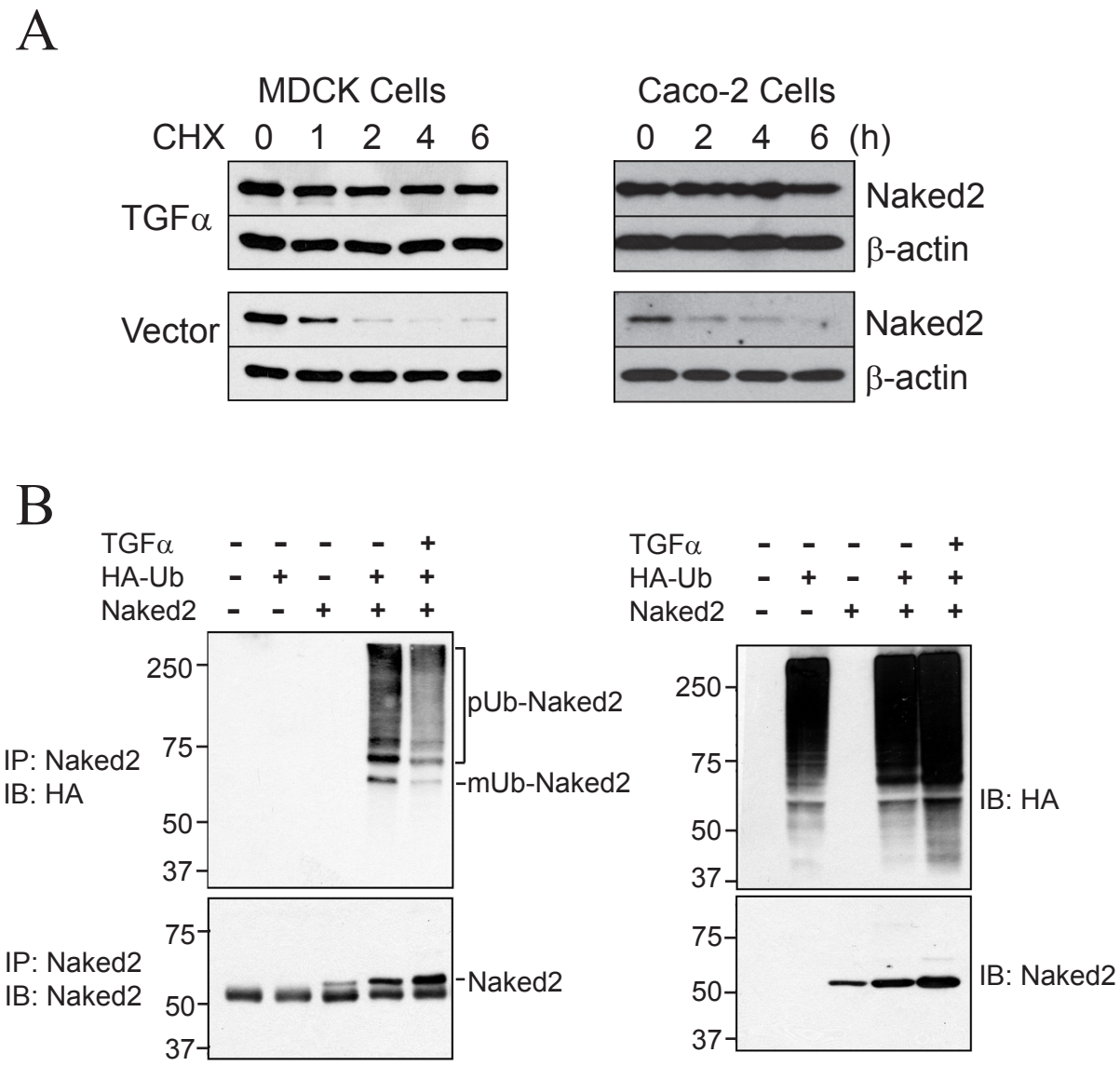

Figure 2.4 Reduced Naked2 ubiquitylation and degradation by TGFα overexpression. (A) MDCK cells stably expressing Naked-2-EGFP and TGFα or Naked-2-EGFP and blank pCB7 vector were incubated with 1μg/ml CHX for the indicated times followed by Naked2 western blotting. Similarly, the degradation of endogenous Naked2 in Caco-2 and Caco-2-TGFα cells was determined. (B) Cell lysates from HEK293 cells transiently expressing Naked2, TGFα and HA-ubiquitin, as indicated, were subjected to immunoprecipitation using VU308 antibody followed by HA (upper panel) or Naked2 (lower panel) western blotting. The ubiquitylation of Naked2 was reduced in the presence of TGFα. In the right panel, WCL were subjected to western blotting using the HA and Naked2 VU308 antibodies to show comparable levels of expression.

expression was increased in MDCK cells and Caco-2 cells stably overexpressing TGFα (Fig. 2.3A). This effect was also observed in Caco-2 cells, the rapid degradation of endogenous Naked2 was dramatically attenuated when TGFα was stably overexpressed (Fig. 2.4A, Caco-2). In line with the stabilization of Naked2 upon TGFα overexpression, the intensity of total ubiquitylated Naked2 was significantly reduced by TGFα in an *in vivo* ubiquitylation assay in transiently co-transfected HEK293 cells (Fig. 2.4B, left panel-lane 4 and 5). As a control, western blotting of whole-cell lysates showed comparable expression of transfected Naked2 and ubiquitin with or without co-expression of TGFα (Fig. 2.4B, right panel). Taken together, these results with both transfected and endogenous Naked2 support our contention that TGFα upregulates Naked2 by inhibiting its poly-ubiquitylation and proteasomal degradation.

Interaction between Naked2 and the cytoplasmic tail of TGFα is required for Naked2 stabilization

Based on our findings that the TTB domain of Naked2 interacts directly with the cytoplasmic tail of Golgi-processed TGFα (Li, Franklin et al. 2004) and that TGFα's upregulation of Naked2 persists despite pharmacological blockade of TGFα cell surface cleavage and EGFR tyrosine kinase activity (Fig. 2.3), we predicted that 1) the TGFα tail alone would be sufficient to stabilize Naked2 and 2) internal deletion of Naked2's TTB domain would abolish this effect (Fig. 2.10C, D).

To test the first prediction (Fig. 2.10C), we constructed a TGFα tail expression plasmid that contains the TGFα cytoplasmic tail (39 residues) preceded by its N-terminal

signal peptide and transmembrane domain; a HA-tag was inserted between the signal peptide and transmembrane sequences for immunoprecipitation studies. Co-immunoprecipitation experiments in transiently transfected HEK293 cells showed that Naked2-EGFP was pulled down as efficiently by the HA-tagged TGFα tail (Fig. 2.5A, upper panel-lane 2) as by full-length TGFα (Fig. 2.5A, upper panel-lane 3). As we predicted, the TGFα tail alone delayed the degradation of Naked2 when stably co-expressed in MDCK cells (Fig. 2.5B).

To examine the second prediction (Fig. 2.10D), an internal deletion of the TTB motif (ΔTTB-Naked2-EGFP) was generated (see Materials and Methods). As expected, co-immunoprecipitation experiments in transiently transfected HEK293 cells showed that the ΔTTB-Naked2-EGFP mutant no longer interacted with HA-tagged TGFα (Fig. 2.5A, upper panel-lane 4). Stably overexpressing full-length TGFα did not protect the ΔTTB-Naked2-EGFP mutant from rapid degradation in MDCK cells (Fig. 2.6A). As we predicted, ubiquitylation of ΔTTB-Naked2-EGFP was not reduced when TGFα was co-expressed (Fig. 2.6B, lane 4) in contrast with the markedly attenuated ubiquitylation of wild-type Naked2 upon TGFα co-expression (Figs. 2.4B and 2.6B). Taken together, these results indicate that the TTB motif of Naked2 and the cytoplasmic tail of TGFα are critical for the Naked2-TGFα interaction, which is required for TGFα-induced Naked2 stabilization but not for Naked2's degradation.

Identification of AO7 as a ubiquitin ligase for Naked2 ubiquitylation

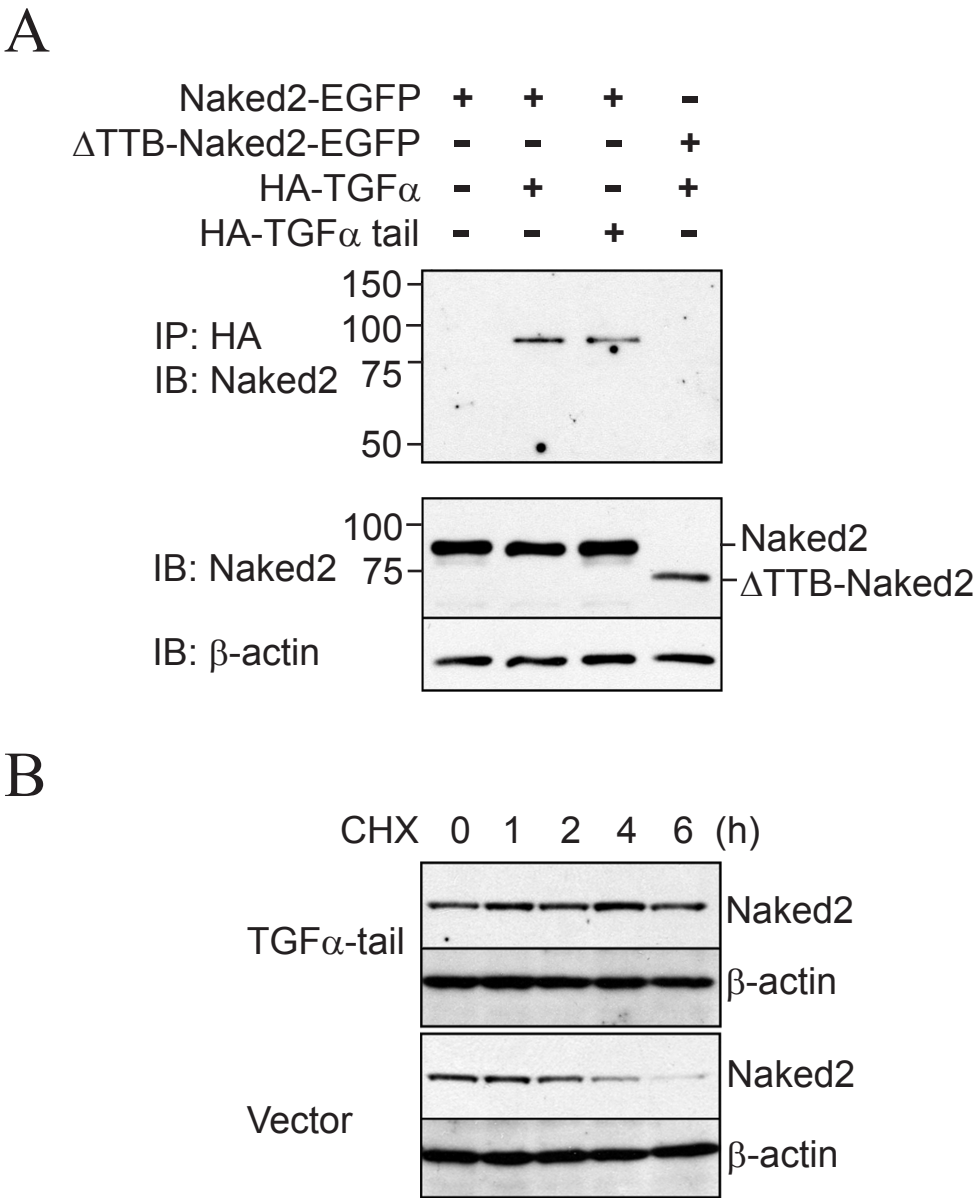

Figure 2.5 A physical interaction between Naked2 and TGFα is required to stabilize Naked2 protein. (A) Validation of binding activities of HA-TGFα tail and ΔTTB-Naked2-EGFP. HEK293 cells were transiently co-transfected with Naked2-EGFP, ΔTTB-Naked2-EGFP, HA-tagged TGFα and a TGFα cytoplasmic tail construct (HA-TGFα tail), as indicated. Cell lysates were immunoprecipitated using HA antibody and then blotted for Naked2. The cytoplasmic tail of TGFα was sufficient to pull down Naked2, but deletion of Naked2's TTB domain abolished the interaction between Naked2 and TGFα. In the lower panel, Naked2 and β-actin western blotting showed equivalent expression and loading. (B) TGFα tail is sufficient to retard Naked2 degradation. MDCK cells stably expressing Naked2-EGFP and HA-TGFα tail (or pCB7 vector control) were exposed to CHX for the times indicated, and the stability of Naked2 protein was monitored by western blotting.

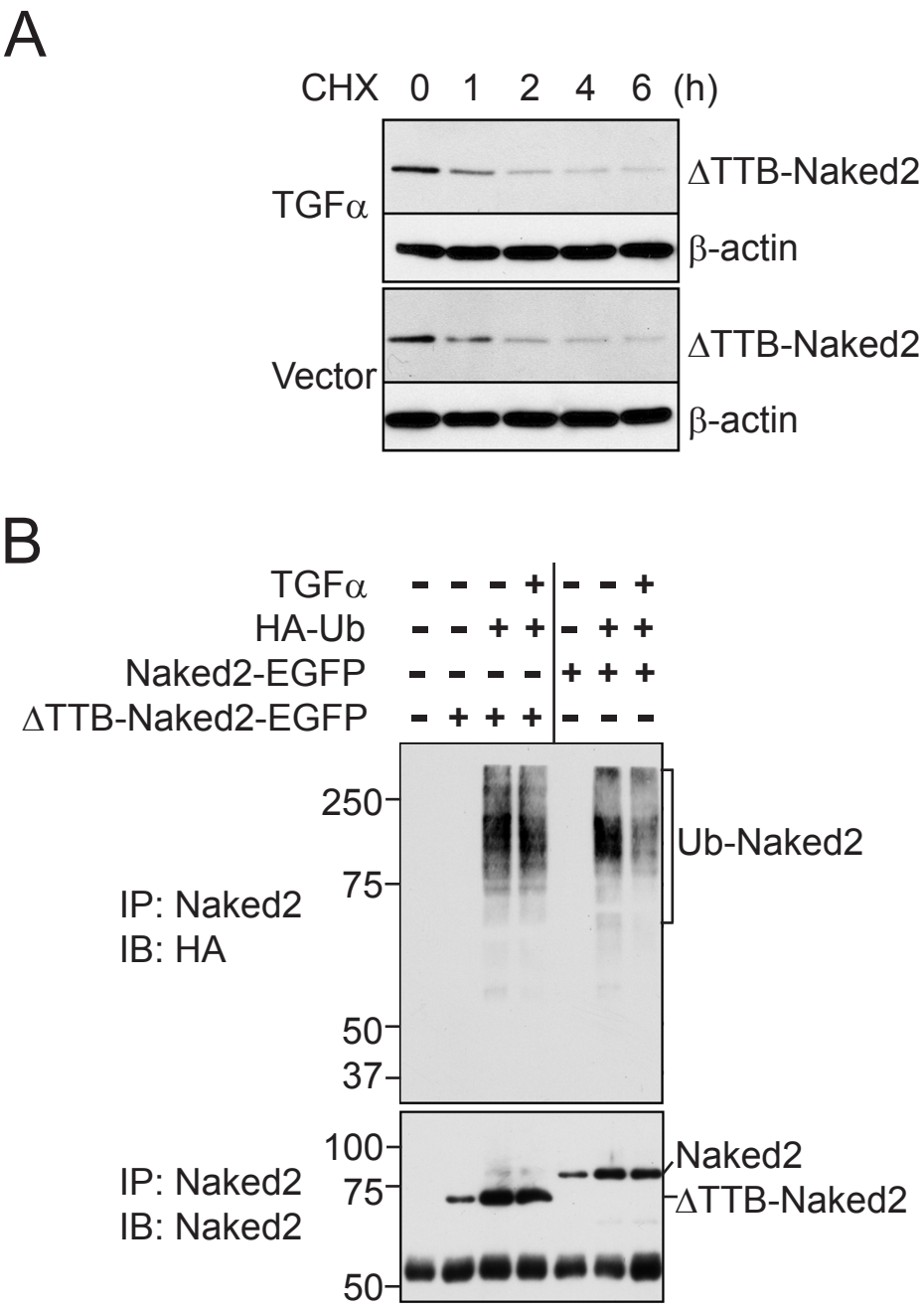

Figure 2.6 TGFα does not inhibit the ubiquitylation and degradation of ΔTTB-Naked2-EGFP. (A) TTB domain of Naked2 is required for Naked2 stabilization. TGFα expression did not prevent degradation of ΔTTB-Naked2-EGFP that is unable to bind TGFα. (D) TGFα overexpression did not affect ubiquitylation of ΔTTB-Naked2-EGFP. HEK293 cells were transiently transfected with ΔTTB-Naked2-EGFP and HA-ubiquitin with or without TGFα. Immunoprecipitation using Naked2 antibody was followed by anti-HA or Naked2 western blotting. In contrast to wild-type Naked2, ubiquitylation of the ΔTTB-Naked2 mutant was not inhibited by TGFα expression.

Because of the direct interaction between TGFα and Naked2, we considered that reduced Naked2 ubiquitylation might result from hindered access of a ubiquitin ligase to Naked2 when TGFα binds. In separate experiments, Naked2 was identified as an AO7-interacting protein by a yeast two-hybrid screen using the N-terminal half of mammalian AO7 (data not shown). AO7, also known as RING finger protein 25 (RNF25), exhibits RING finger-dependent ubiquitin ligase activity (Lorick, Jensen et al. 1999) (Fig. 2.7A). We confirmed this interaction by co-immunoprecipitation in HEK293 cells. HA-tagged AO7 was pulled down by GFP antibody when EGFP-tagged Naked2, but not EGFP alone, was co-expressed (Fig. 2.7B, lane 1 and 2). Two point mutations at C135 and C138 in the conserved RING finger domain of AO7 abolished the E3 activity of AO7 (Fig. 2.7A) (Lorick, Jensen et al. 1999), but this RING mutant AO7 (AO7-RM) retained the ability to interact with Naked2 (Fig. 2.7B, lane 3 and 4). Therefore, we utilized FLAG-tagged AO7-RM as a dominant-negative to examine whether AO7 participates in Naked2 ubiquitylation. Overexpression of AO7-RM dramatically reduced the ubiquitylation level of Naked2 in an *in vivo* ubiquitylation assay in HEK293 cells (Fig. 2.8A, lane 3). In addition, we also conducted an *in vitro* ubiquitylation assay, which employed human recombinant E1, UbcH5B (E2), HA-tagged ubiquitin and a bacterially expressed C-terminal truncation of AO7,GST-AO7T, which is expressed substantially better than the full length protein (Lorick, Jensen et al. 1999). Due to insolubility of full-length Naked2 when expressed in *E. coli* (Hu, Krezel et al. 2006), Naked2-EGFP fusion protein was expressed in HEK293 cells and immunoprecipitated using anti-GFP to

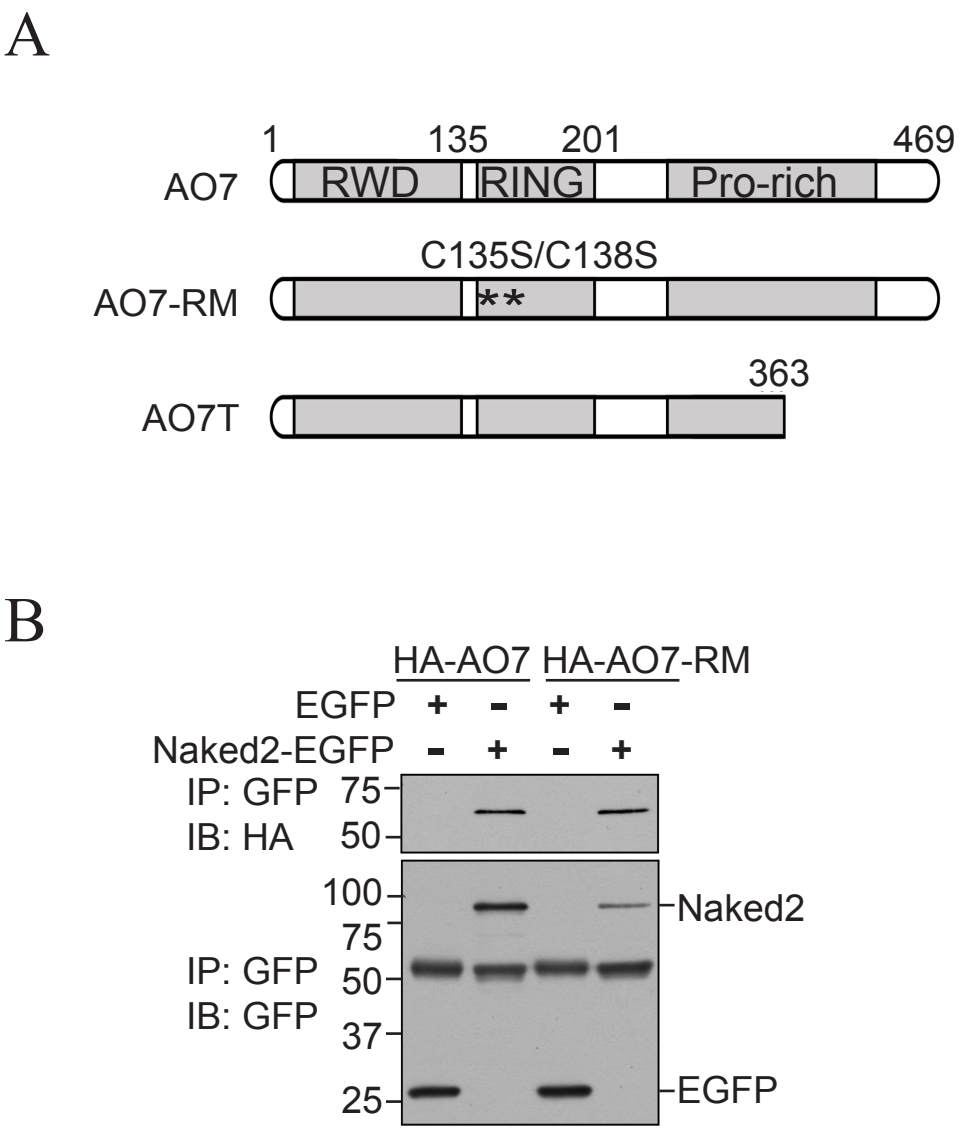

Figure 2.7 Protein interaction between Naked2 and AO7. (A) Schematic illustration of AO7 and its mutants. AO7 contains a RING finger motif and a Pro-rich region. AO7-RM contains two point mutations at C135 and C138 and lacks ubiquitin ligase activity. AO7T is a C-terminal truncation mutant that retains E3 activity and is soluble when expressed in E. coli. (B) Naked2-EGFP and HA-tagged AO7 or AO7-RM were co-expressed in HEK293 cells, followed by co-immunoprecipitation using a GFP antibody. Both forms of AO7 were pulled down by GFP antibody in the presence of Naked2-EGFP, but not EGFP alone. Western blotting using GFP antibody demonstrated the expression levels of Naked2-EGFP and EGFP alone.

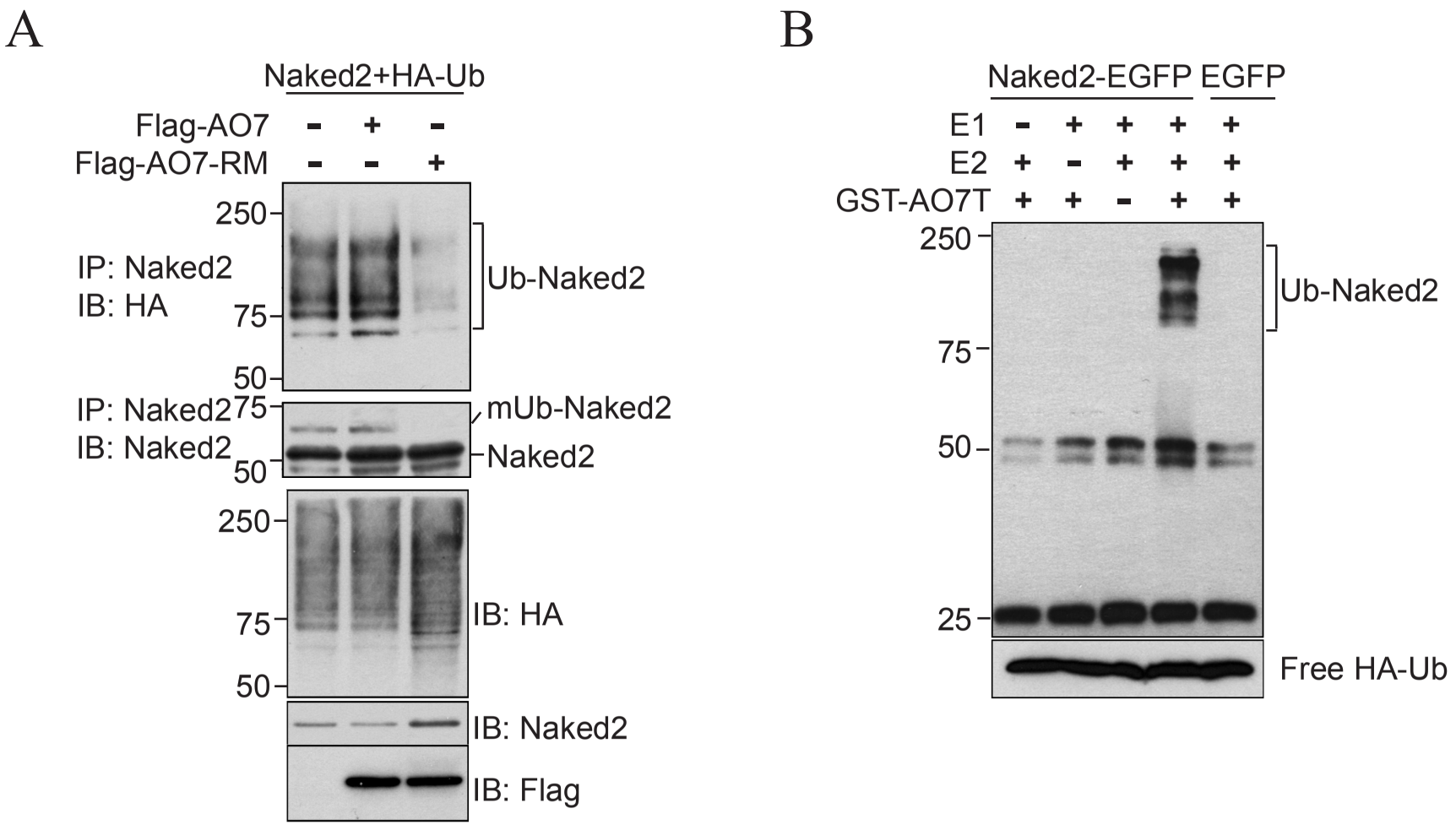

Figure 2.8 AO7 function is required for Naked2 ubiquitylation. (A) Attenuation of Naked2 ubiquitylation by AO7-RM. In vivo ubiquitylation assays were performed with co-expressed FLAG-AO7 or FLAG-AO7-RM. The expression of AO7 only moderately enhanced Naked2 ubiquitylation. In contrast, expression of AO7-RM dramatically reduced ubiquitylation of Naked2. (B) In vitro ubiquitylation of Naked2 by AO7T. Human recombinant E1, UbcH5b (E2), HA-ubiquitin and purified GST-AO7T were used. Naked2-EGFP expressed in HEK293 cells was immunoprecipitated and used as substrate. The reaction products were resolved by SDS-PAGE followed by western blotting with HA antibody. Ubiquitylated Naked2 was detected only when E1, E2 and GST-AO7T were added together.

serve as ubiquitylation substrate. Western blotting using HA antibody revealed ubiquitylation of Naked2-EGFP when E1, E2 and GST-AO7T were all present in the reaction (Fig. 2.8B, lane 4). Ubiquitylated Naked2 was not detected when GST-AO7T was absent from the reaction, ruling out the possibility that the ubiquitylation was mediated by E3s co-purified from HEK293 cells. The specificity of ubiquitylation is demonstrated by the lack of ubiquitylation of EGFP that was not fused to Naked2. These data indicate that AO7 is a ubiquitin ligase for Naked2.

TGFα attenuates AO7-Naked2 binding

Thus far we have shown that TGFα protects Naked2 from ubiquitylation, and that AO7 binds to and ubiquitylates Naked2. We next sought to determine whether TGFα affects Naked2 ubiquitylation by hindering its interaction with AO7. HEK293 cells that were transfected with Naked2 and HA-AO7 were mixed and split into 3 wells, followed by a second transfection with different concentrations of a TGFα expression plasmid. Naked2-bound AO7 was co-immunoprecipitated using Naked2 antibody and revealed by HA western blotting (Fig. 2.9). As the input of TGFα increased, the interaction between AO7 and Naked2 decreased, suggesting that increased levels of TGFα may inhibit Naked2 ubiquitylation by reducing AO7 binding to Naked2.

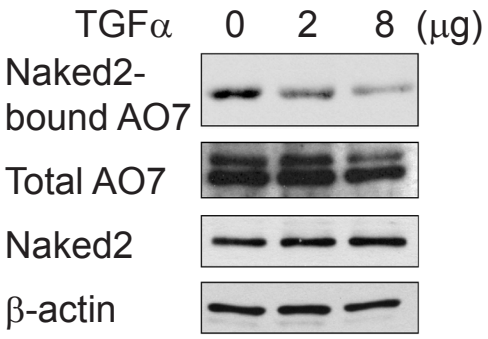

Figure 2.9 Increased TGFα expression reduces AO7 binding to Naked2. Different concentrations of TGFα expression plasmid were transfected into HEK293 cells that express HA-AO7 and Naked2. Co-immunoprecipitation using Naked2 antibody followed by HA western blotting revealed the levels of AO7 that bind to Naked2. The expression levels of total AO7 and Naked2 were examined by western blotting using HA and Naked2 antibody.

Discussion

We previously determined that Naked2 acts as multi-functional protein to ensure the efficient delivery of TGFα to the basolateral surface of polarized epithelial cells. It recognizes basolateral sorting determinants in the cytoplasmic tail of Golgi-processed TGFα, thereby coating TGFα-laden exocytic vesicles (Li, Franklin et al. 2004). Naked2 residues 1-36 confer vesicle recognition (C Li and RJ Coffey, unpublished observation). Naked2 residues 1-173 direct these vesicles to the basolateral corner of polarized epithelial cells, where the vesicles dock and fuse in a Naked2 myristoylation-dependent manner (Li, Hao et al. 2007). In polarized MDCK cells expressing myristoylation-deficient G2A Naked2, Naked2-associated vesicles accumulate at a basolateral corner, and TGFα is unable to reach the plasma membrane. That G2A Naked2 acts as a dominant negative is supported by our recent finding that Naked2 siRNA results in cytosolic accumulation of TGFα and no detectable cell surface TGFα (Li, Hao et al. 2007). These multiple functions carried out by Naked2 to ensure the precise and efficient delivery of TGFα to the basolateral surface of polarized epithelial cells have led us to designate Naked2 a CaRT protein (Li, Hao et al. 2007).

Naked2 does exhibit features of a coat such as cargo recognition and selection. As part of the constitutive intracellular trafficking machinery, most adaptor or coat proteins like AP-1 and AP-2 are ubiquitously expressed and long-lived (Sorkin, McKinsey et al. 1995). It is generally thought that adaptors regulate recruitment and stability of cargo but not vice versa. However, we have found that Naked2 is poly-ubiquitylated and undergoes

rapid proteasomal degradation (Figs. 2.1 & 2.2). Moreover, its stability appears to be regulated by its cargo TGFα (Fig. 2.3 ~ 2.6). This is similar to the recent report of Hirst and co-workers showing that the degradation of a relatively short-lived clathrin-dependent adaptor, GGA2, was delayed when one of its cargos, CIMPR, was overexpressed in HeLa cells (Hirst, Seaman et al. 2007). The authors concluded that cargo proteins may not be just passively sorted by adaptors, but they may actively contribute to the formation of coated vesicles by interacting with and regulating coat components, at least in the case of GGA2. In this paper, we not only identify a similar phenomenon in the context of Naked2-coated TGFα vesicles, but also elucidate the biochemical basis for this phenomenon (Fig. 2.10).

Given the multiple tasks orchestrated by Naked2 (cargo selection, vesicle recognition, basolateral targeting and vesicle fusion), it is perhaps not surprising that it is a highly regulated protein. We show that the upregulation of Naked2 by TGFα is post-transcriptional. Naked2 is a short lived protein with a half-life of 60 min due to ubiquitin-mediated proteasomal degradation (Fig.2.1). We also identified AO7 as a ubiquitin ligase for Naked2. It has been previously shown that AO7 mediates RING finger-dependent auto-ubiquitylation *in vitro* (Lorick, Jensen et al. 1999). A subsequent study implicated AO7 in NF-κB-mediated transcriptional activity (Asamitsu, Tetsuka et al. 2003) through interactions involving the transactivation domain of the p65 subunit. However, no direct ubiquitylation of p65 was detected and, until now, no other substrates for this E3 have been identified. Herein we show that AO7 interacts with and

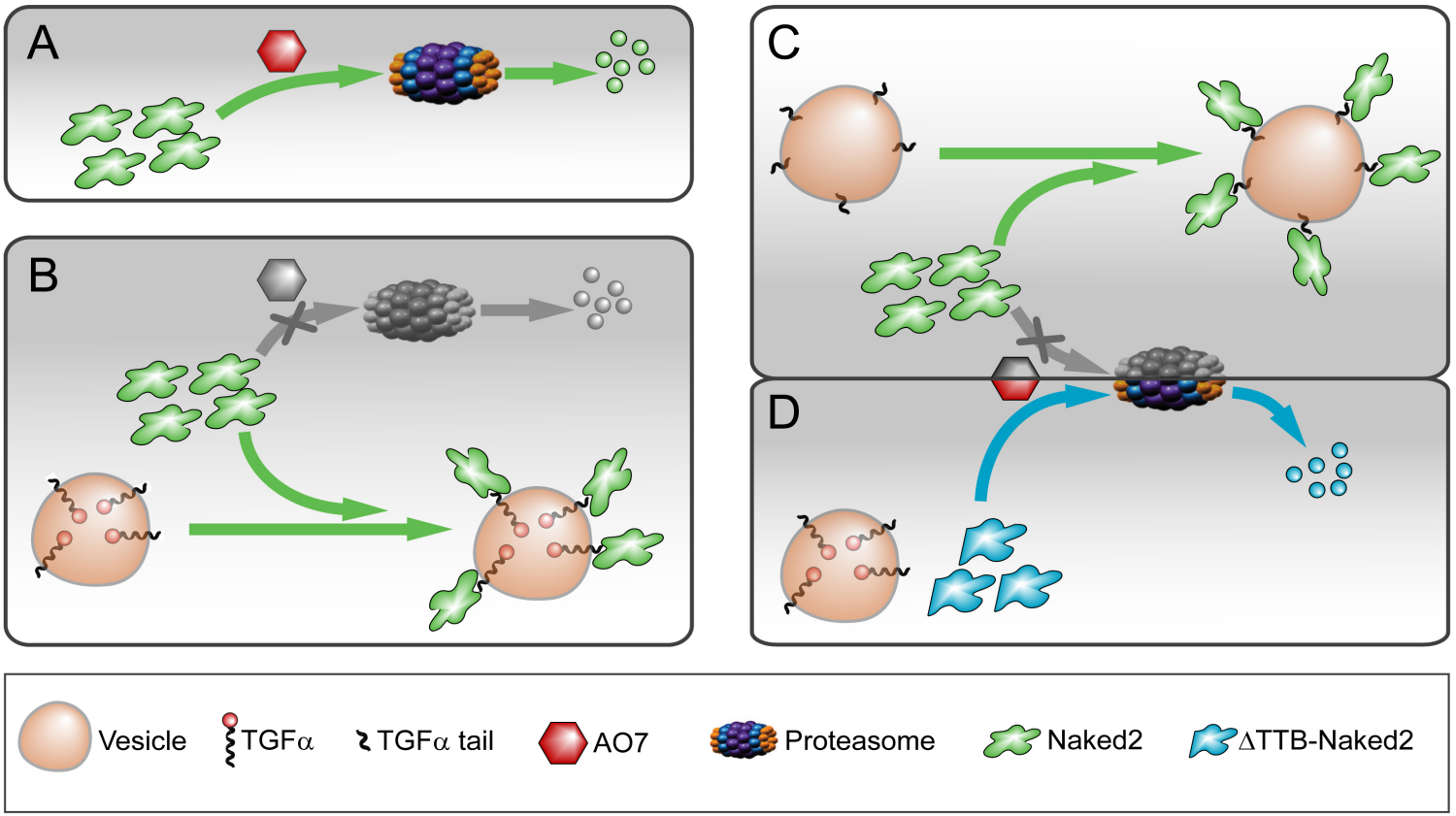

Figure 2.10 A model for TGFα-induced Naked2 stabilization. (A) In the absence of TGFα, Naked2 is ubiquitylated by AO7 and undergoes rapid proteasomal degradation. (B) Naked2 binds to the cytoplasmic tail of TGFα presented on the surface of TGFα-containing vesicles and/or at the plasma membrane. In the presence of TGFα, Naked2 is protected from ubiquitylation and proteasomal degradation. (C) The cytoplasmic tail of TGFα (preceded by its N-terminal signal peptide and transmembrane domain) interacts with Naked2 and thus protects Naked2 from degradation. (D) ΔTTB-Naked2 cannot bind to TGFα and it is not protected from proteasomal degradation by TGFα.

ubiquitylates Naked2. Thus, we have identified the first heterologous substrate for AO7, as well as the first E3 ligase for Naked2.

We previously demonstrated that TGFα binds to Naked2 residues 300-385 (Li, Franklin et al. 2004). Since TGFα protects Naked2 from ubiquitylation and AO7 binds directly to Naked2, we considered whether TGFα binding to Naked2 might hinder access of the ubiquitin ligase to Naked2 or may mask critical lysine residues within Naked2 that undergo ubiquitylation. Indeed, we found that as we increased TGFα input there was reduced binding of AO7 to Naked2 (Fig. 2.9). This result favors the first possibility, but does not exclude the second possibility mentioned above. Thus, studies are underway to map the Naked2-AO7 interaction domains and to identify the ubiquitylation site(s) within Naked2.

The discrepancy between the high efficiency of TGFα in Naked2 stabilization and the partial inhibition of Naked2 ubiquitylation and AO7 binding by TGFα raises the possibility that additional mechanisms may be involved in TGFα-mediated Naked2 upregulation. For example, TGFα-containing vesicles may attract and recruit free Naked2 onto the vesicle surface and facilitate cell membrane trafficking. This process may restrict subcellular localization of Naked2 to membrane structures where it is lacking protein degradation machinery. Therefore, increased level of TGFα may result in effective Naked2 protein stabilization without complete attenuation of Naked2 ubiquitylation. Detailed cellular localization studies will be conducted in order to test this possibility.

TGFα-induced stabilization of Naked2 occurs independently of EGFR activation.

The first EGFR-independent action of TGFα was described by Derynck and co-workers (Shum, Reeves et al. 1994). By addition of a monoclonal antibody to the ectodomain of TGFα, these investigators observed PKC-mediated "reverse signaling." In the present case, the effect is mediated by the cytoplasmic tail of TGFα through a direct protein-protein interaction. Interestingly, the target of this effect, Naked2, plays a critical role in the exocytic delivery of TGFα (Li, Franklin et al. 2004; Li, Hao et al. 2007). Therefore, Naked2 stabilization by TGFα may be a self-regulating mechanism by which TGFα ensures its proper sorting and efficient cell surface delivery.

It has become increasingly clear that regulated cell membrane trafficking of EGFR ligands is essential to their activities and disruption of this process may result in severe physiological consequences. For example, isolated recessive renal hypomagnesaemia is due to a mutation in the cytoplasmic tail of pro-EGF that disrupts basolateral sorting of pro-EGF. This leads to insufficient stimulation of basolateral EGFRs in the proximal tubule of the kidney, resulting in impaired activation of the Mg^{2+} channel TRPM6 (transient receptor potential cation channel, subfamily M, member 6), and magnesium wasting in the kidney (Groenestege, Thebault et al. 2007). We previously demonstrated that Naked2 plays a critical role in escorting TGFα to the basolateral surface of polarized epithelial cells. These effects of Naked2 are specific for TGFα in that overexpression of myristoylation-deficient G2A Naked2 in MDCK cells impairs cell surface delivery of TGFα, but not amphiregulin (AR) (Li, Franklin et al. 2004), another basolaterally targeted EGFR ligand that is also cleaved by cell surface TACE/ADAM17. Thus, TGFα

appears to utilize a unique Naked2-mediated trafficking machinery; it ensures its basolateral cell surface delivery by stabilizing its CaRT Naked2 (Fig. 2.10). Taken together, these results highlight the complexity of basolateral trafficking and underscore the need for considering ligand trafficking and delivery in a systems biology approach to studying EGFR signaling.

Acknowledgements

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CHAPTER III

DOWNREGULATION OF NAKED2 IN HUMAN COLORECTAL CANCER

Introduction

Colorectal cancer is amongst the most common neoplasms in Western society, representing the second leading cause of cancer-related deaths in the United States (Parker, Tong et al. 1996). More than 85% of all sporadic and hereditary colorectal cancers display loss-of-function mutations in the *Adenomatous Polyposis Coli* (*APC*) gene (Kinzler and Vogelstein 1996) that encodes a key negative regulator of canonical Wnt signaling; activating mutations in a Wnt agonist, β-catenin, or inactivating mutations in the *Axin* gene are found in half of the remaining cases (Morin, Sparks et al. 1997). Deregulation of canonical Wnt signaling leads to expansion of the proliferative crypt compartment, and likely additional events, that eventuate in abnormal cellular and tissue architecture known as an adenoma (Oshima, Oshima et al. 1995; Oshima, Oshima et al. 1997; Lamlum, Papadopoulou et al. 2000). Following initiation of a colorectal tumor, additional genetic alterations occur that further enhance proliferation, cell survival and cell motility, all of which promote progression and metastasis (Kinzler and Vogelstein 1996; Polyak 1996; Oshima, Oshima et al. 1997).

From *Drosophila* to human, canonical Wnt signaling is highly conserved, and it plays crucial roles in normal embryonic development and stem cell maintenance in adult tissues

(Reya and Clevers 2005). Dysregulation of canonical Wnt signaling has been closely linked to neoplasia, with the strongest link being in the initiation of colorectal cancer (Logan and Nusse 2004; Gregorieff and Clevers 2005; Reya and Clevers 2005). It is well accepted that canonical Wnt signaling needs to be tightly regulated under normal physiological conditions. A key component of this regulation is the cytoplasmic β-catenin destruction complex that consists of APC, Axin, CKI and glycogen synthase kinase-3β (GSK-3β). Sequential phosphorylations by CKI (Amit, Hatzubai et al. 2002; Liu, Li et al. 2002) and GSK-3β (Yost, Torres et al. 1996) prime β-catenin for poly-ubiquitylation and its constitutive proteasomal degradation (Aberle, Bauer et al. 1997; Latres, Chiaur et al. 1999; Liu, Kato et al. 1999). A signal from an activated Frizzle/LRP (Fz/LRP) receptor complex disassembles this protein complex and prevents degradation of β-catenin. Stabilized β-catenin then translocates into the nucleus, interacts with TCFs (TCF1, LEF, TCF3 and TCF4) by displacing co-repressors and converting TCFs to transcriptional activators (Behrens, von Kries et al. 1996; Molenaar, van de Wetering et al. 1996; van de Wetering, Cavallo et al. 1997). The loss of functional APC that occurs in 80% of colorectal cancers impairs the ability of the destruction complex to degrade β-catenin. However, the signaling mechanism that couples the Fz/LRP receptors and β-catenin destruction complex remains to be fully understood.

Previous studies suggest that recruitment of Dvl and Axin by Fz and LRP, respectively (Cliffe, Hamada et al. 2003; Tamai, Zeng et al. 2004), is required for receptor activation and β-catenin stabilization. *Drosophila Naked Cuticle* directly binds

to *Dsh* and antagonizes the *Wingless/Armadillo (Wg/Arm)* pathway at a step upstream of *Zeste-white 3 kinase (Zw3)*, the *GSK-3β* homolog in *Drosophila* (Rousset, Mack et al. 2001). Vertebrate Naked proteins, Naked1 and Naked2, are recently identified (Katoh 2001; Wharton, Zimmermann et al. 2001; Yan, Wallingford et al. 2001). They both interact with Dvl via a conserved EF-hand domain in a Zn^{2+}-dependent manner (Rousset, Wharton et al. 2002) and retain similar activities in antagonizing canonical Wnt signaling. In addition, vertebrate Nakeds also play a role in the non-canonical Wnt/Planar cell polarity (PCP) pathway. This is not unexpected because Dvl has been shown to be involved in both canonical and PCP Wnt pathways (Theisen, Purcell et al. 1994; Axelrod, Miller et al. 1998; Boutros, Paricio et al. 1998). In *Xenopus* embryos, Naked overexpression inhibits the normal convergent extension by overactivating PCP signals, suggesting that Naked switches Dvl activity from Wnt/β-catenin to the PCP pathway (Yan, Wallingford et al. 2001). However, a recent work by Van Raay et al. has shown an inhibitory effect on both pathways by overexpressing Nakeds in zebrafish embryos (Van Raay, Coffey et al. 2007). Although this discrepancy remains to be elucidated, Naked proteins are indeed important negative regulators of canonical Wnt signaling, which is frequently enhanced in colorectal cancer.

The tight regulation of canonical Wnt signaling is carried out, at least in part, by efficient negative feedback loops through inducible negative regulators, including *Axin*, *Dickkopf (Dkk)* and *Naked Cuticle*. In vertebrates, close correlation between Naked1 transcription and active Wnt signaling has been observed in zebrafish embryos, chick

embryos, mouse liver epithelial cells and human colon tumors (Yan, Wallingford et al. 2001; Yan, Wiesmann et al. 2001; Ishikawa, Kitajima et al. 2004; Schmidt, Otto et al. 2006; Van Raay, Coffey et al. 2007). Moreover, 5 perfectly matched TCF-binding sites were identified in the putative promoter of human *Naked1* gene (Yan, Wiesmann et al. 2001). In contrast, Naked2 expression pattern appears more ubiquitous and distinguishable from Naked1 expression in early development of zebrafish and mouse embryos (Wharton, Zimmermann et al. 2001; Van Raay, Coffey et al. 2007). More recently, Lei et al. has demonstrated that mouse *Naked2* promoter activity is directly repressed by a homeobox gene Hoxc8 (Lei, Juan et al. 2007), which plays essential roles in differentiation and proliferation and is upregulated in many human cancers (Alami, Castronovo et al. 1999; Miller, Miller et al. 2003; Chen, Gu et al. 2005). Thus, *Naked2* appears to be differentially regulated compared to *Naked1*, a known Wnt target gene. In this study, we examined the expression pattern of both Naked1 and Naked2 in SW480 cells, a human colorectal cancer (CRC) cell line with mutant *APC* and active Wnt signaling. Naked2 displays opposite expression pattern of Naked1. In fact, Naked2 expression and promoter activity appeared to be repressed by active Wnt signaling, although with a time lapse. In line with these results, an expression profiling of Naked2 protein in human colorectal tumors has revealed that Naked2 expression is downregulated in carcinomas and metastasis, but not in adenomas. Given the additional roles of Naked2 in maintaining normal basolateral TGFα trafficking (a proximal event of EGFR axis) (Li, Franklin et al. 2004; Li, Hao et al. 2007) and the importance of EGFR

signaling in carcinoma maintenance (Roberts, Min et al. 2002), we proposed that loss of Naked2 expression in the late event of CRC may contribute to the tumor progression by disrupting normal delivery of TGFα and EGFR signaling.

Materials and methods

Plasmids, antibodies and chemicals

Putative mouse *Naked2* promoter sequences were obtained by PCR amplification from a *Naked2*-containing clone identified by the David W. Threadgill lab from a RPCI-22 mouse BAC library (Invitrogen, Carlsbad, CA), which contained the Naked2 locus. PCR primer pairs for pro1.5 (nucleotides -1353 to +105) were 5'-TGTGGATCCTAATCATTGAATC-3' and 5'-GCGAATTCCGAGCTGAGAG-3'; for pro2.3 (nucleotide -1979 to +105) were 5'-TGGATAGATCTGTGTCTTTGG-3' and 5'-GCGAATTCCGAGCTGAGAG-3'. The pro4.5 was a ligation product of a PCR product (by using primers 5'-GCTTACAAAGCTTGATCTTC-3' and 5'-GATTCAATGATTAGGATCCACA-3') and pro1.5 by an intrinsic BamHI site. All PCR fragments were sequenced and confirmed to be free of mutations. The pro1.5, pro2.1 and pro4.5 fragments were then subcloned into pGL3-Basic vector (Promega, Madison, WI) for subsequent luciferase reporter assays. Control vector pGL3-Promoter that contains the SV40 promoter was also obtained from Promega. TOPflash and FOPflash plasmids were purchased from Upstate Biotechnology (Lake Placid, NY). The β-catenin (ΔN89) expression vector (Wagenaar, Crawford et al. 2001) was a generous gift from Lynn M. Matrisian's lab (Vanderbilt University, TN).

The rabbit polyclonal Naked1 and Naked2 antibodies were raised against GST-fused Naked1 (residues 1-229 in human Naked1) and Naked2 (residues 1-217 in human

Naked2) peptides in collaboration with Cocalico Biologicals (Reamstown, PA). The mouse monoclonal β-catenin and β-actin antibodies were obtained from Sigma (St. Louis, MO). The mouse monoclonal α-tubulin antibody was purchased from Oncogene Research Products (San Diego, CA). Horseradish peroxidase-donkey anti-mouse and anti-rabbit IgG were obtained from Jackson ImmunoResearch (West Grove, PA). Alexa Fluor 488 goat anti-mouse antibody was produced from Molecular Probes (Invitrogen, Carlsbad, CA). All other chemicals were obtained from Sigma unless otherwise stated.

Cell culture and transfection

HEK293 cells and all transiently transfected derivatives were grown in Dulbecco's modified Eagle's medium (DMEM) by Hyclone (Logan, UT). SW480 and its derivative cells were generous gifts from Dr. Antony W. Burgess (Ludwig Institute for Cancer Research, Austrilia). SW480 cells were grown in RPMI 1640 medium (Hyclone, Logan, UT). SW480.APC and vector control cells were grown in the RPMI 1640 medium with 1.5mg/ml geneticin (G418) (RPI, Mt. Prospect, IL). All culture media were supplemented with 10% fetal bovine serum (FBS), glutamine, nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin (Hyclone, Logan, UT)

Transient transfections were performed in HEK293 cells using FuGENE6 (Roche Applied Science, Indianapolis, IN) transfection reagent according to the manufacturer's instructions. Twenty-four hrs after transfections in 24-well plates, cells were lysed in 1x Passive Lysis buffer (Promega, Madison, WI) followed by luciferase reporter assays.

RT-PCR

Total RNA was isolated from Caco-2 cells using the RNeasy mini kit (QIAGEN, Valencia, CA). RT-PCR was performed to determine the relative gene expression using SuperScript™ One-Step RT-PCR kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. We used primer 5'-CCACTTCTACCAGACATAGAGC-3' and primer 5'-GTGCTCTCCTGGAAGAGGCTG-3' for *Naked1* PCR and primer 5'-CGATGGGGAAACTGCAGTCG-3' and primer 5'-CAAAGTCATAGAGCGTGAAC-3' for *Naked2*. All amplifications started with a minimal amount of RNA (250 ng of total RNA), and the reactions were limited to 25 cycles to avoid saturating the reaction. RT-PCR for *GAPDH* was performed in the same reaction tubes to serve as an internal control for equivalent starting material. Primers used for *GAPDH* are 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' and 5'-CATGTGGGCCATGAGGTCCACCAC-3'.

Western blotting and immunocytochemistry

Cells were lysed in ice-cold 1X lysis buffer (25 mM Tris HCl, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM DTT and 2% BSA) and precleared by centrifugation. The protein concentration of supernatant was determined using Micro BCA Protein Assay Kit (Pierce, Rockford, IL) before boiling in sample buffer. Then proteins were resolved on 10% SDS-PAGE followed by western blotting. For β-catenin

immunocytochemistry, SW480.APC cells were treated with either 10mM LiCl or NaCl for 24 hrs before fixation in 4% paraformaldehyde. Then cells were permeabilized with 0.1% Triton X-100 for 15 min followed by standard immunofluorescence.

Luciferase reporter assay

The pGL3 vectors that contained different fragments of Naked2 promoter sequence were transiently transfected into HEK293 cells (0.4μg for each well in a 24-well tissue culture plate). Twenty-four hrs after transfection, cells were processed and assayed for luciferase activity using Dual-Glo™ Luciferase Assay System (Promega, Madison, WI). Promoter-less pGL3-Basic and SV40 promoter-containing pGL3-Promoter vectors were used as negative and positive controls, respectively. To test the response to Wnt signaling, the mutant β-catenin (ΔN89) expressing vector was co-transfected with pGL3 derivatives. TOPflash (containing the binding site for the Tcf/LEF family of transcription factors fused to a luciferase reporter gene) or FOPflash (negative control) reporter plasmids (van de Wetering et al., 1997) were utilized to indicate the activation of canonical Wnt signaling by ΔN89 β-catenin. In all luciferase reporter experiments, *Renilla* luciferase vectors were co-transfected with reporter vectors. The resulting reporter luminescence intensities were all normalized to *Renilla* luciferase intensities from the same well.

Results

Opposite Expression pattern of Naked1 and Naked2 in SW480 cells

A direct correlation has been established between canonical Wnt signaling and expression of *Drosophila Naked Cuticle* and vertebrate Naked1(Wharton, Zimmermann et al. 2001; Yan, Wiesmann et al. 2001; Van Raay, Coffey et al. 2007). More directly, several putative TCF-binding sites have been identified in the human Naked1 promoter region (Yan, Wiesmann et al. 2001). To examine whether the other Naked family member, Naked2, is also regulated by canonical Wnt signaling, we analyzed Naked1 and Naked2 expression in SW480 cells and SW480 cells stably expressing full-length APC.

SW480 cells are a human colorectal cancer cell line (Leibovitz, Stinson et al. 1976) that contains a C>T substitution at codon 1338 of the *APC* gene (Nishisho, Nakamura et al. 1991). This mutation results in a truncated APC that lacks the Axin-binding domain and part of the β-catenin binding domain. As a result, β-catenin accumulates in both the cytoplasm and nucleus, and high levels of canonical Wnt signaling are observed as measured by TOPFlash reporter activity. Stable overexpression of wild-type *APC* in SW480 cells (SW480.APC) restores cell surface localization of β-catenin (Faux, Ross et al. 2004) (Fig. 3.1), silences canonical Wnt signaling and reverses the transformed phenotype as assessed by cellular morphology (Fig. 3.1), growth in soft agar and tumors in nude mice (Faux, Ross et al. 2004). Subconfluent SW480, SW480.APC and vector control cells were subjected to both RT-PCR and western blotting to assess relative levels

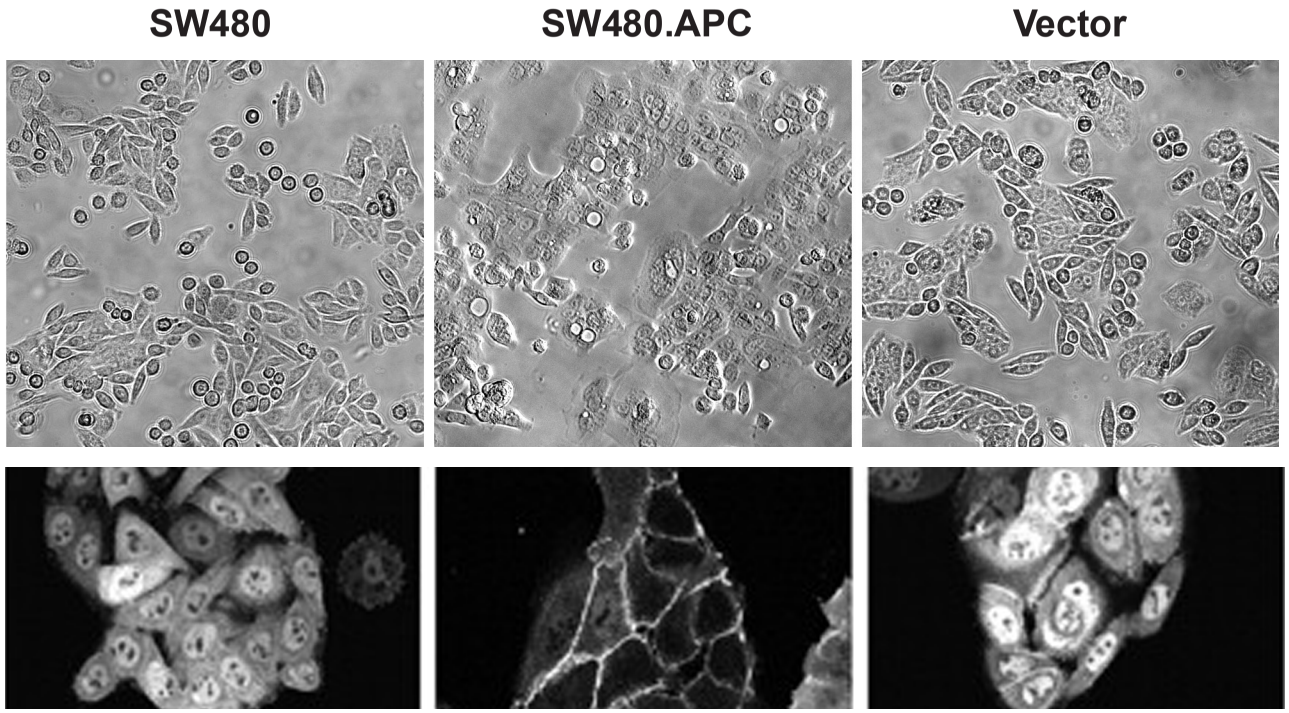


Figure 3.1 Morphologocial changes in SW480 and SW480.APC cells. Stably transfecting wild-type *APC* into *APC*-mutant SW480 cells (SW480.APC) results in a more epithelioid morphological appearance and restoration of cell surface β-catenin (β-catenin staining is adapted from Faux, 2004).

of Naked1 and Naked2 (Fig. 3.2). As expected, Naked1 is highly expressed in parental SW480 cells but levels of *Naked1* mRNA and protein are significantly decreased in the SW480.APC cells (Fig. 3.2). In contrast, Naked2 expression is barely detectable in parental SW480 cells but mRNA and protein levels are markedly increased in SW480.APC cells (Fig. 3.2). Thus, *Naked1*, like *Naked Cuticle*, appears to be a canonical Wnt target gene, whereas *Naked2* acts in an opposite manner.

Active canonical Wnt signaling represses Naked2 transcription

We then examined whether the upregulation of Naked2 in SW480.APC cells is due to repressed canonical Wnt signaling. To activate canonical Wnt signaling, SW480.APC cells were exposed to LiCl, a GSK-3β inhibitor. Twenty-four hr exposure to LiCl resulted in nuclear translocation of β-catenin (Fig. 3.3A), demonstrating activation of canonical Wnt signaling. However, levels of Naked2 protein were unchanged at this time, and it required an additional 24 hr exposure to LiCl before Naked2 protein levels declined (Fig. 3.3B). These results demonstrate a direct correlation between activation of canonical Wnt signaling and downregulation of Naked2 protein, although there was a temporal lag between activation of canonical Wnt signaling and decreased levels of Naked2.

β-catenin inhibits mouse Naked2 promoter activity

Naked1 has been shown to be a direct transcriptional target of canonical Wnt signaling. Five perfectly matched TCF-binding sites have been identified in the putative

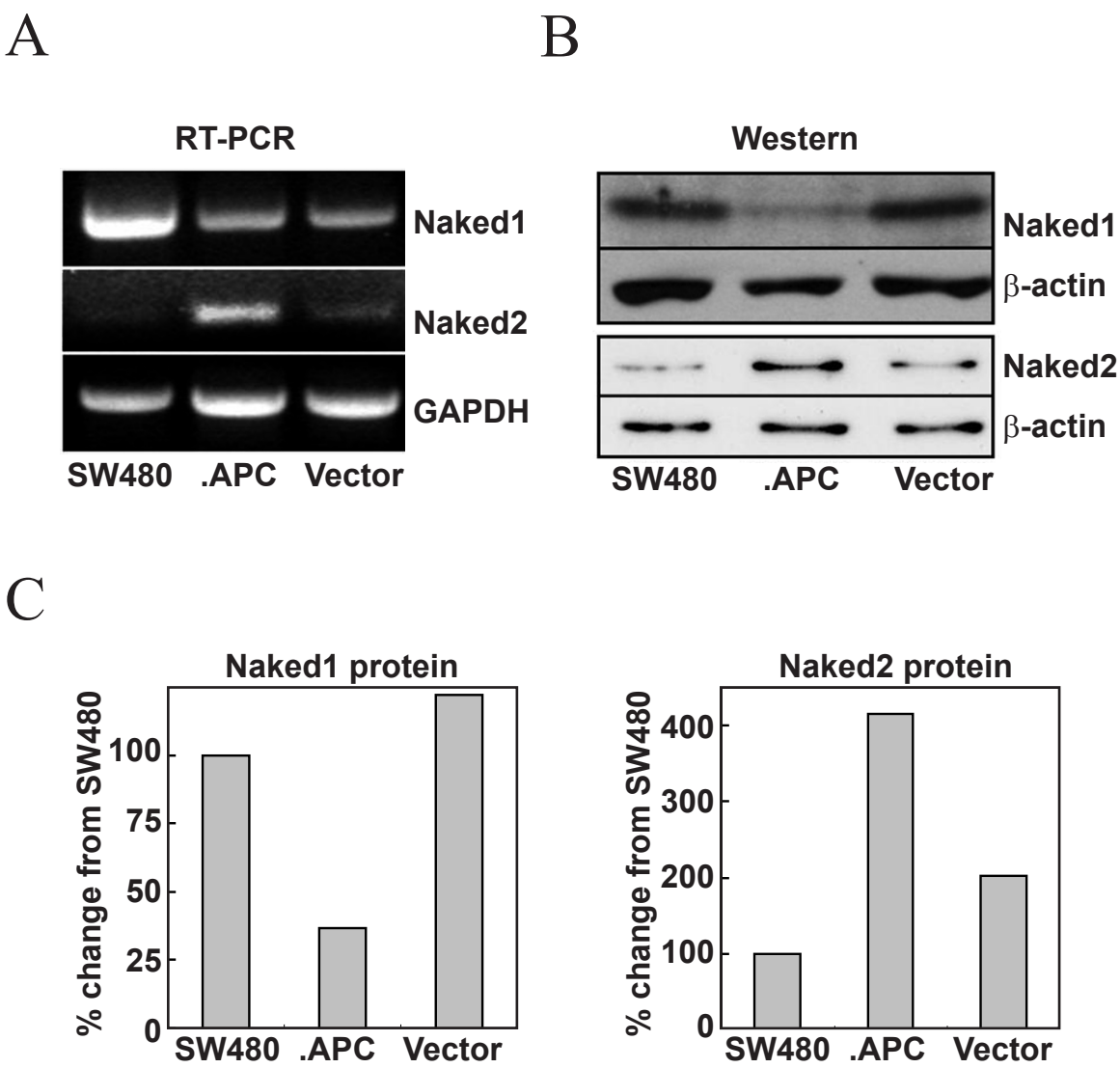
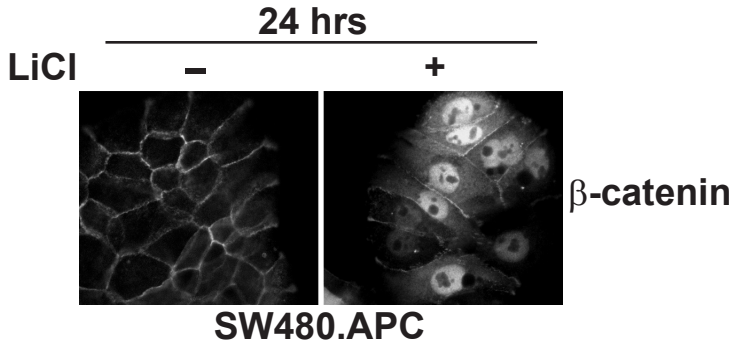


Figure 3.2 Differential expression of Naked1 and Naked2 in SW480 and SW480.APC cells. (A) Relative mRNA expression of Naked1 and Naked2 in SW480 and SW480.APC cells by RT-PCR. RT-PCR for GAPDH was used as internal control for equivalent starting material. (B) Western blotting of Naked1 and Naked2 in SW480 and SW480.APC cells. β-actin blotting was performed to show comparable loading of cell lysates. (D) Quantitation of Naked1 and Naked2 protein levels in SW480 and SW480.APC cells after normalization to β-actin.

A



B

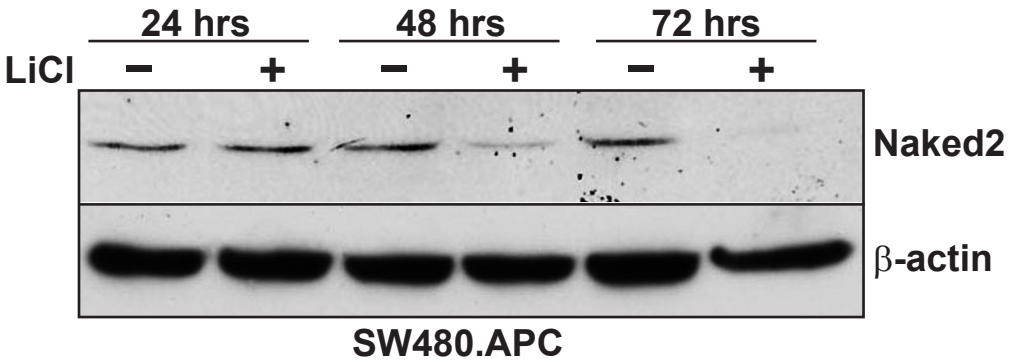


Figure 3.3 Downregulation of Naked2 by canonical Wnt signaling in SW480.APC cells. (A) The GSK-3β inhibitor LiCl induces nuclear translocation of β-catenin in SW480.APC cells. In these APC-transfected cells, β-catenin immunoreactivity is restricted to the plasma membrane, indicating quiescent canonical Wnt signaling. After 24-hr exposure to 10 mM LiCl, there is nuclear and cytoplasmic accumulation of β-catenin, indicating activation of canonical Wnt signaling. As a control, 10 mM NaCl did not affect β-catenin localization. (B) LiCl reduces Naked2 protein levels in SW480.APC cells. Cells were treated with 10mM LiCl or NaCl over a three day time course followed by western blotting for Naked2. It required 48-hr exposure to LiCl for Naked2 levels to decrease and levels further declined at 72 hrs.

human Naked1 promoter and are thought to mediate transcriptional activation by the β-catenin/TCF complex. To test whether canonical Wnt signaling regulates *Naked2* transcription by inhibiting *Naked2* promoter activity, we identified and subcloned the putative mouse *Naked2* promoter that comprised nucleotides from -4,365 to +105 relative to the start of the 5'-untranslated region (UTR) (Fig. 3.4A). In comparison with the mouse *Naked1* promoter, the *Naked2* promoter contains a comparable number of putative TCF-binding sites; however, the orientation of these TCF-binding elements (TBE) differ (Fig. 3.4A). When we subcloned the most proximal 1.5kb (pro1.5) sequence of the putative promoter into the pGL3-Basic (promoter-less) vector, it displayed strong promoter activity (Fig. 3.4B). In contrast, when we extended the sequence to 2.1kb (pro2.1 in Fig. 3.4A), the promoter activity was markedly reduced. Promoter activity was partially restored when the promoter sequence was extended to 4.5kb (pro4.5), but it was still weaker than the pro1.5 fragment. These results suggest that the proximal upstream sequence of *Naked2* 5'-UTR contains basic promoter activity, and the sequence between nucleotides -1,353 and -1,979 contains negative regulatory elements. However, we cannot exclude the existence of long range enhancer elements in further 5' or 3' sequences.

We then tested whether overexpression of β-catenin affected Naked2 promoter activity. Deletion of the N-terminal 89 residues in β-catenin (ΔN89) resulted in loss of phosphorylation sites that are required for its degradation (Munemitsu, Albert et al. 1996; Wagenaar, Crawford et al. 2001). This constitutively active mutant β-catenin was

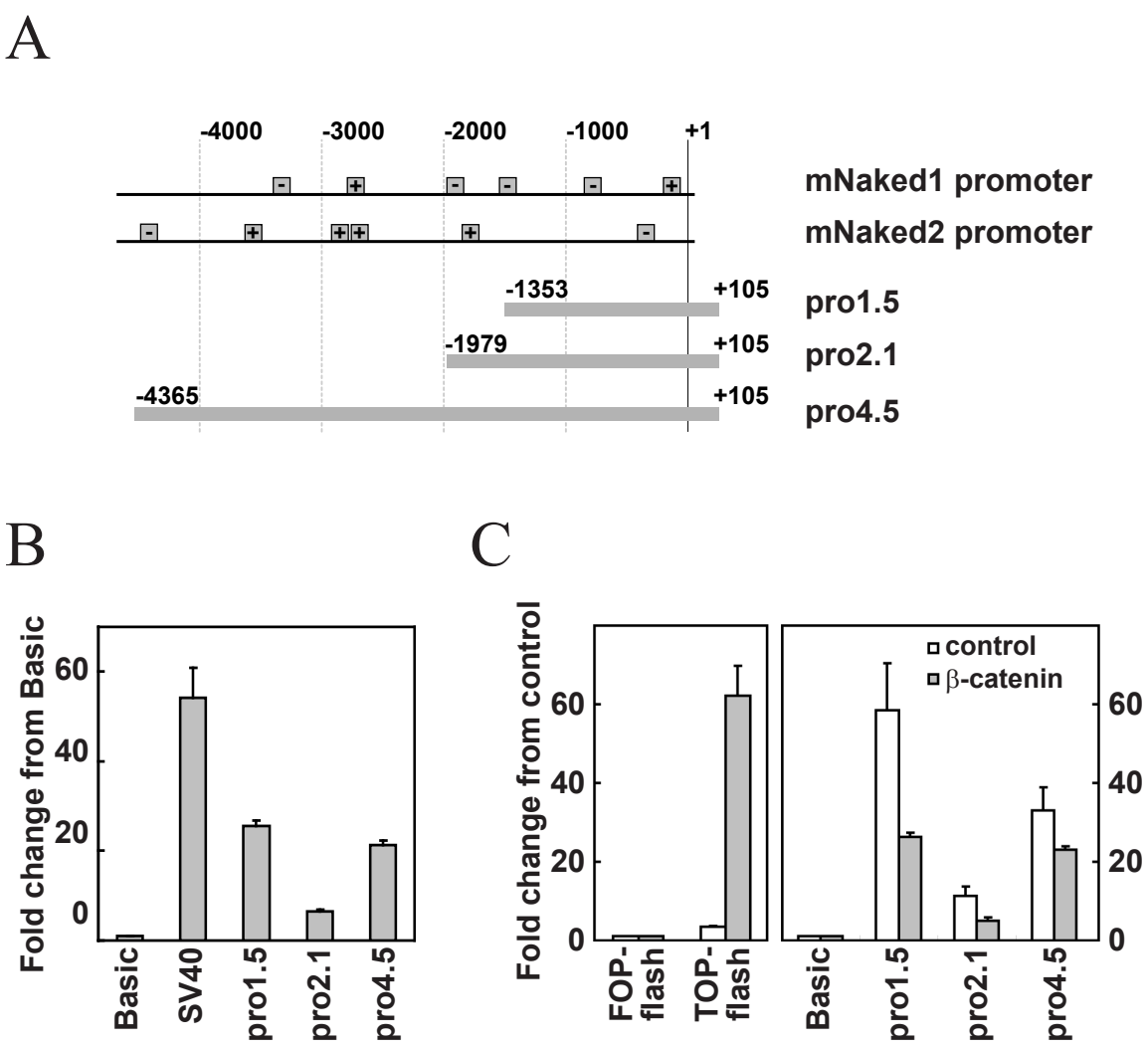


Figure 3.4 Overexpression of β-catenin inhibits mouse Naked2 promoter activity. (A) Schematic depiction of putative mouse Naked1 and Naked2 promoters. Both Naked1 and Naked2 promoters contain multiple core sequences of TCF-binding elements (gray boxes). However, they display different orientations as depicted as "+" for CTTTGA/TA/T and "–" for T/AT/ACAAAG. Different fragments of the Naked2 promoter (shown as gray bars) were subcloned from the mouse genome. The numbers labeled on the promoter sequences represent the nucleotide length. (B) Basic Naked2 promoter activity. Luciferase reporter (pGL3) driven by different Naked2 promoter fragments or the control SV40 promoter were expressed transiently in HEK293 cells. Luminescence intensities were calculated as the fold increase relative to the pGL3-Basic blank reporter. (C) β-catenin represses Naked2 promoter elements. ΔN89 β-catenin was co-expressed with reporter vectors containing Naked2 promoter fragments or with TOPflash/FOPflash reporter plasmids. The induction of TOPflash reporter indicated an activation of Wnt signaling by ΔN89 β-catenin. In contrast, all pro1.5, pro2.1 and pro4.5 activities were greatly attenuated upon ΔN89 β-catenin co-expression.

co-transfected with TOPflash/FOPflash reporters or pGL3 vectors containing different *Naked2* promoter sequences. Strong induction of TOPflash reporter activity demonstrated a substantial activation of β-catenin/TCF transcription factors by ΔN89 β-catenin (Fig. 3.4C). Subsequent luciferase assays detected a reduction in promoter activity in all three *Naked2* promoter fragments (Fig. 3.4C), suggesting that the *Naked2* promoter is repressed by canonical Wnt signaling. These findings may provide a molecular basis for the differential regulation of *Naked2* expression by canonical Wnt signaling compared to *Naked Cuticle* and *Naked1*.

Naked2 expression is downregulated in colorectal cancer but not in adenomas

The majority of human colorectal cancers are initiated by mutations in the *APC* gene, resulting in activation of canonical Wnt signaling. As a Wnt target gene, *Naked1* mRNA was found to be elevated in 65% of laser microdissected human colon tumors compared to adjacent normal colonic mucosa (Yan, Wiesmann et al. 2001). We also observed increased Naked1 expression in parental SW480 cells that harbor a mutant *APC* gene, and it was decreased in SW480.APC cells in which canonical Wnt signaling was silenced. In contrast, Naked2 expression was suppressed in parental SW480 cells but it was restored upon introduction of wild-type *APC*.

These results led us to assess Naked2 protein levels in human colorectal tumors. We observed reduced Naked2 protein levels in 10/16 (62%) of colorectal cancer specimens compared to their adjacent normal colonic mucosa (Fig. 3.5A). In one individual, Naked2

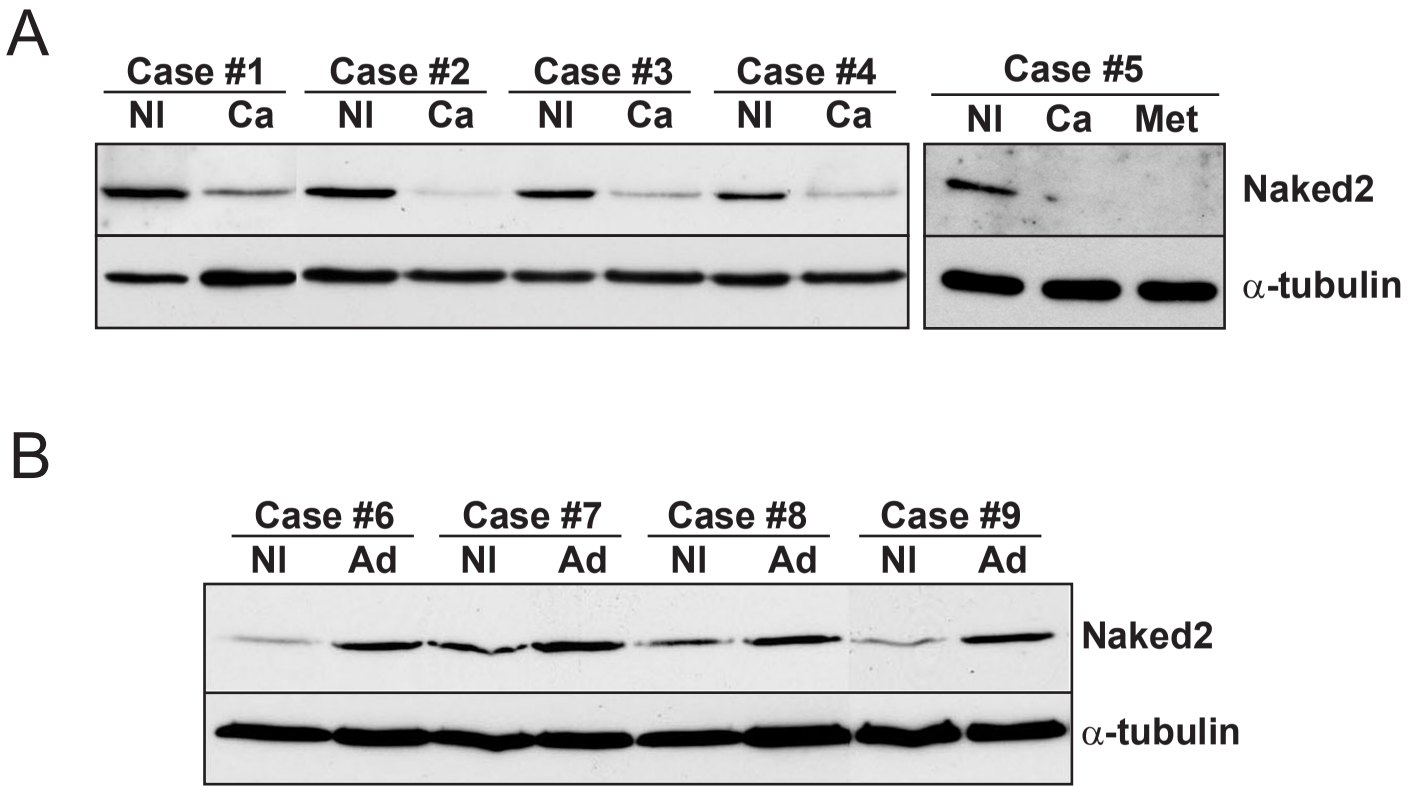


Figure 3.5 Reduced Naked2 expression in human colorectal cancer. (A) Naked2 expression in colorectal carcinomas. Downregulation of Naked2 protein by western blotting was observed in 10 of 16 colorectal carcinomas (Ca) harvested from 16 individuals compared to their adjacent normal colonic mucosa (Nl). In one case, a liver metastasis was collected together with the primary lesion and adjacent normal colonic mucosa. Five representative cases are shown. (B) Naked2 expression in colonic adenomas. Six adenoma specimens (Ad) with matched adjacent normal mucosa tissues were blotted using the Naked2 antibody. Naked2 protein was not downregulated in any of the six adenomas compared to adjacent normal mucosa. Four representative cases are shown in the figure. All western blots were reblotted for endogenous α-tubulin as a loading control.

levels were low in both the primary lesion and liver metastasis compared to adjacent normal colonic mucosa. In contrast, Naked2 protein levels were not decreased in any of 6 adenomas compared to their matched normal colonic tissue (Fig. 3.5B). Thus, decreased Naked2 expression appears to be a late event in colorectal cancer, and further supports an indirect action of canonical Wnt signaling on Naked2 expression.

Discussion

Given its importance in so many biological processes, it is imperative that canonical Wnt signaling be tightly regulated. In fact, a large number of negative regulators act at almost every step of the pathway. Deregulation of this tight control can lead to cancers (Oshima, Oshima et al. 1995; Oshima, Oshima et al. 1997; Lamlum, Papadopoulou et al. 2000). Naked family members, *Drosophila Naked Cuticle* and vertebrate Naked1 and Naked2, are firmly established as effective Wnt antagonists (Zeng, Wharton et al. 2000; Katoh 2001; Wharton, Zimmermann et al. 2001; Yan, Wallingford et al. 2001; Van Raay, Coffey et al. 2007) by interacting with and inactivating Dishevelled, a critical signaling molecule that mediates signals from Fz receptor to the cytoplasmic β-catenin destruction complex (Zeng, Huang et al. 2008). The role of Naked2 is being re-assessed in light of the recently identified, additional function for this molecule, that is, its ability to act as a CaRT for the basolateral trafficking of TGFα in polarized epithelial cells(Li, Franklin et al. 2004; Li, Hao et al. 2007).

The C-terminus of Naked2 contains a TGFα-tail-binding (TTB, residues 300-385) domain that is not conserved in Naked1 and *Naked Cuticle* (Li, Franklin et al. 2004; Li, Hao et al. 2007). As a result, human Naked2, but not Naked1, binds to the cytoplasmic tail of Golgi-processed TGFα and escorts TGFα-containing exocytic vesicles to a basolateral corner of polarized epithelial cells. These vesicles then dock and fuse with the plasma membrane in a Naked2 myristoylation-dependent manner. Overexpression of myristoylation-deficient mutant (G2A) Naked2 in polarized MDCK cells causes

abnormal accumulation of TGFα-containing vesicles that fail to dock and fuse with cell membrane due to much reduced social activity of G2A-coated vesicles (Li, Hao et al. 2007). These studies establish that Naked2 may act as a double agent between TGFα trafficking and Wnt signaling, while other family members are dedicated to the latter pathway.

In this paper, we demonstrate that Naked2 is not only functionally distinct from other family members, but it is also differentially regulated by canonical Wnt signaling. In contrast to *Naked Cuticle* and *Naked1*, *Naked2* transcription is repressed upon activation of the Wnt/β-catenin pathway (Fig. 3.2 and 3.3); this effect correlates with a reduction in *Naked2* promoter activity (Fig. 3.4). However, we observed a 24-hr temporal lag between nuclear translocation of β-catenin and downregulation of Naked2 protein after LiCl treatment of SW480.APC cells (Fig. 3.3). Given that Naked2 is a short-lived protein due to poly-ubiquitylation and rapid proteasomal degradation (Ding, submitted), the delayed downregulation of Naked2 may not be a direct effect of the β-catenin/TCF transcriptional complex. This finding may explain why decreased Naked2 expression is observed in carcinomas, but not in adenomas (Fig. 3.5), whereas loss of functional APC and activation of canonical Wnt signaling is a common early event in colorectal neoplasia.

Most Wnt target genes contain multiple consensus TCF-binding sites in their promoter regions. In the absence of nuclear β-catenin, TCF associates with transcriptional repressors, like Groucho (Cavallo, Cox et al. 1998; Hecht, Vleminckx et al. 2000) and MTGR-1 (Moore, Amann et al. 2008), to repress target gene expression. Upon

Wnt activation, β-catenin translocates to the nucleus where it displaces transcriptional repressors from TCF and results in transcriptional activation of Wnt target genes. Until recently, the β-catenin/TCF complex has not been thought to directly repress transcription of target genes. However, canonical Wnt signaling may repress gene transcription through other transcription factors. For example, one of the key events during the initiation of the epithelial-mesenchymal transition (EMT), a process exploited by invasive cancer cells, is Wnt-dependent downregulation of E-cadherin by the Snail transcription factor (Yook, Li et al. 2005; Yook, Li et al. 2006; Ko, Kim et al. 2007). It has been shown that Snail contains a β-catenin-like motif and undergoes GSK-3β-dependent phosphorylation, β-TrCP-mediated ubiquitylation and proteasomal degradation (in a manner similar to β-catenin). Wnt signaling inhibits Snail phosphorylation and degradation. As a result, Snail translocates into the nucleus and represses transcription of E-cadherin, one of the Snail target genes. Thus, through a mechanism similar to the regulation of β-catenin, canonical Wnt signaling activates Snail and represses gene transcription. A similar mechanism may also be utilized to downregulate Naked2 expression in colorectal cancer, although it may not necessarily be through the Snail transcription factor. A recent study reported that mouse Naked2 gene is a direct transcriptional target of Hoxc8, a member of the homeobox gene family, through a long-range enhancer region (Lei, Juan et al. 2007). Hoxc8 is a known target gene of caudal protein Cdx4, and *Cdx4* is a direct transcription target of β-catenin/TCF transcription factor (Pilon, Oh et al. 2006). Thus, canonical Wnt signaling may repress

Naked2 expression by a cascade of sequential transcriptional events through Cdx4 and Hoxc8. Upregulation of Hoxc8 expression has been observed in prostate, cervical and colorectal cancer (Alami, Castronovo et al. 1999; Miller, Miller et al. 2003; Chen, Gu et al. 2005), but levels of Naked2 have not been examined in this context. Studies are underway to determine whether Cdx4 and Hoxc8 are required for Wnt signaling-induced Naked2 downregulation.

The frequent downregulation of Naked2 in colorectal carcinomas raises an additional question: is it just a passive consequence of active Wnt signaling or does it actively contribute to tumor progression? Given that 90% of colorectal cancers contain mutations in either *APC*, *Axin* or *β-catenin* genes, all acting downstream of Naked2, one may argue that loss of Naked2 may not impact on canonical Wnt signaling in colorectal cancer. However, it has been shown that a Wnt binding protein SFRP attenuates canonical Wnt signaling in colorectal cancer cells with loss of functional APC (Suzuki, Watkins et al. 2004).

Moreover, loss of Naked2 may impact on colorectal cancer through impaired cell surface delivery of TGFα. Upon binding of different ligands, EGFR initiates complex signaling cascades that regulate diverse biological responses, including proliferation, differentiation, cell motility and survival (Marmor, Skaria et al. 2004). The ligands that bind to the receptor may determine the specificity of downstream signaling (Hackel, Zwick et al. 1999; Prenzel, Fischer et al. 2001; Yarden and Sliwkowski 2001). TGFα is the major ligand for EGFR and plays an important role in maintaining epithelial

homeostasis in the gut. In polarized epithelial cells, TGFα is delivered to the basolateral cell surface where it is cleaved by TACE and the soluble ligand is then avidly taken up by the EGFR (Dempsey and Coffey 1994; Dempsey, Meise et al. 2003). Another EGFR ligand, AR, is also preferentially delivered to the basolateral membrane and cleaved by the same enzyme (Sunnarborg, Hinkle et al. 2002; Gschwind, Hart et al. 2003). However, soluble AR binds more efficiently to HSPGs than to the EGFR and thus may exist in a depot form (Schuger, Johnson et al. 1996). Loss of Naked2 may result in impaired cell surface presentation of TGFα. One possible consequence of this loss would be to create relatively unoccupied EGFRs to which AR or other ligands can now bind. We reported recently that AR, but not TGFα, causes a EMT-like phenotype in MDCK cells (Chung, Cook et al. 2005; Chung, Graves-Deal et al. 2005). Taken together, these data lead us to propose a model in which loss of Naked2 expression impairs cellular trafficking of TGFα and contributes to colorectal cancer progression.

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CHAPTER IV

GENERATION AND CHARACTERIZATION OF NAKDED2 KNOCK-OUT MICE

Introduction

Mammalian Naked2 was identified as a *Naked Cuticle* homolog by its conserved EF-hand motif. Similar to Naked1, Naked2 antagonizes canonical Wnt signaling in various vertebrate species, such as *Xenopus*, zebrafish, mouse and human (Wharton, Zimmermann et al. 2001; Yan, Wallingford et al. 2001; Van Raay, Coffey et al. 2007). It also plays a role in the non-canonical Wnt/PCP pathway as elucidated in the early development of *Xenopus* and zebrafish embryos (Yan, Wallingford et al. 2001; Van Raay, Coffey et al. 2007). In addition, our recent cell culture-based studies have demonstrated unique functions of Naked2 in the basolateral cell surface delivery of TGFα (Li, Franklin et al. 2004; Li, Hao et al. 2007), a major EGFR ligand in the gastrointestinal epithelium. However, *in vivo* roles for Naked2 have not been examined in the mouse.

EGFR belongs to the ErbB family of receptor tyrosine kinases; there are seven mammalian ligands to EGFR: EGF, TGFα, HB-EGF, AR, betacellulin (BTC), epiregulin (EPR), and epigen (Shoyab, McDonald et al. 1988; Massague 1990; Higashiyama, Lau et al. 1992; Toyoda, Komurasaki et al. 1995; Strachan, Murison et al. 2001). These ligands are made as type I transmembrane proteins that are inserted into the plasma membrane where they undergo proteolytic cleavage to release mature, soluble growth factors, which

in turn bind to and activate ErbB receptors. TGFα, EGF, AR and epigen bind solely to EGFR, whereas HB-EGF, BTC and EPR can also bind ErbB4.

Upon binding by different ligands, EGFR forms a homo-dimer with itself or a hetero-dimer with other ErbB receptor members — ErbB-2 (HER2), ErbB-3 (HER3) and ErbB-4 (HER4). The dimerization of the ectodomains results in activation of intrinsic tyrosine kinase acitivities that reside in the cytoplasmic tail of the receptors, with the exception of HER3 which lacks intrinsic tyrosine kinase activity. Subsequent auto-phosphorylation at specific tyrosine residues within the cytoplasmic tail of the receptor creates docking sites for binding of adaptor proteins or signaling molecules (such as GRB-2, Shc, PLC-γ and Src) to transduce a subset of intracellular signaling spectrum, regulating diverse biological responses including proliferation, differentiation, cell motility and survival (Marmor, Skaria et al. 2004). The specificity of downstream signaling pathways is determined by which ligand engages the receptor, the composition of the receptor pairs and the particular tyrosine residues that are auto-phosphorylated.
It is increasingly recognized that cell surface delivery and presentation of EGFR ligands are critical steps in regulating EGFR activities.

Each of the EGFR ligands exhibits distinct features as to cell surface delivery, cell surface cleavage and binding to the EGFR (Fig. 1.4B). In polarized epithelial cells, EGF is equally delivered to both the apical and basolateral plasma membrane (Dempsey, Meise et al. 1997). In contrast, TGFα and AR are sorted preferentially to the basolateral membrane where they are cleaved by TACE/ADAM17 (Brown, Meise et al. 1998;

Borrell-Pages, Rojo et al. 2003; Gschwind, Hart et al. 2003; Sahin, Weskamp et al. 2004), a cell membrane metalloproteinase that is restricted to this compartment. The cell surface cleavage of TGFα occurs so rapidly that the cell membrane immunoreactivity of TGFα can only be detected after administration of a selective TACE inhibitor, WAY-022 . Soluble, mature TGFα is then avidly consumed by basolateral EGFRs in an autocrine or local paracrine fashion (Dempsey and Coffey 1994). Cell surface cleavage of AR by TACE appears to be less efficient than that of TGFα. Mature AR contains an amino-terminal heparin-binding domain (HBD) that interacts with extracellular and cell-associated HSPGs as well as the tetraspanin CD9 (Johnson and Wong 1994; Inui, Higashiyama et al. 1997; Nylander, Smith et al. 1998). Interaction with these molecules may facilitate a depot form of AR that, under certain conditions, can bind and activate EGFRs, conferring distinct biological properties to AR (Chung, Cook et al. 2005; Chung, Graves-Deal et al. 2005). HB-EGF is also predominantly delivered to the basolateral compartment but remains as a transmembrane form. The shedding of HB-EGF can then be induced by protein kinase C (PKC) activation (Izumi, Hirata et al. 1998) and increased intracellular calcium (Horiuchi, Le Gall et al. 2007).

The rapid cell surface cleavage and avid local consumption of TGFα suggest that cell surface delivery is a critical, possibly rate-limiting, step in regulating the action of endogenous TGFα. Two basolateral sorting determinants have been found in the cytoplasmic tail of TGFα (Dempsey, Meise et al. 2003). Naked2, an originally identified Wnt antagonist, binds to the basolateral sorting determinants of TGFα and escorts

TGFα-containing vesicles to the basolateral corner of polarized epithelial cells where these vesicles dock and fuse with the plasma membrane in a Naked2 myristoylation-dependent manner (Li, Franklin et al. 2004; Li, Hao et al. 2007). This process appears to be specific to TGFα, since overexpression of myristoylation-deficient G2A Naked2 disrupts basolateral sorting of TGFα but not that of AR.

In the normal gut, TGFα is the major EGFR ligand that binds basolateral EGFRs, and it plays a critical role in maintaining epithelial homeostasis. Loss of Naked2 expression in colorectal cancer will perturb Naked2-dependent basolateral delivery of TGFα and thus create relatively unoccupied EGFRs to which AR can now bind. This substitution of AR for TGFα may have significant biological consequences. Previous work from the Coffey lab has shown that recombinant human AR, but not TGFα, disrupts epithelial junctional integrity leading to an EGFR-dependent epithelial to mesenchymal (EMT)-like transition (Chung, Cook et al. 2005; Chung, Graves-Deal et al. 2005). Therefore, Naked2 may play an important role in maintaining epithelial homeostasis in the gastrointestinal tract. Loss of Naked2 expression may perturb TGFα trafficking, and this may have physiological consequences *in vivo*. To test this possibility, I have generated a conditional targeted allele of Naked2 gene in mice, and the initial characterization of these mice is underway.

Materials and Methods

Plasmids and bacteria strains

The *Naked2*-containing BAC clone was identified by the David W. Threadgill lab from the RPCI-22 mouse BAC library (Invitrogen, Carlsbad, CA).

The PL253, PL451 and PL452 vectors (Liu, Jenkins et al. 2003) were obtained from the Neal Copeland lab (NCI, Frederick, MA). The PL253 is a pBluescript-derived plasmid for retrieval of DNA from a BAC clone. This plasmid contains a *Mc1*-driven *Thymidine Kinase* (*TK*) cassette for negative selection in ES cells. The PL451 plasmids contains a neo cassette flanked by two *frt* sites and one *loxP* site (*Frt-Pgk-em7-Neo-Frt-loxP*). The PL452 contains a neo cassette flanked by two *loxP* sites (*loxP-Pgk-em7-Neo-loxP*). The neo gene in these two plasmids is expressed both from a prokaryotic promoter (*em7*) and a eucaryotic promoter (*Pgk*).

The *exo, bet* and *gam* containing *E.coli* strains, EL250 and EL350, are used in this study. They are derived by transferring the defective λ prophage present in DY330 cells (Yu, Ellis et al. 2000) into DH10B cells to create DY380 cells (Lee et al. 2001). An arabinose-inducible *cre* gene (P_{BAD}-*cre*) is then introduced into the defective λ prophage present in DY380 cells to create EL350 cells (Lee et al. 2001). Instead, EL250 is a DY380 derivative containing an arabinose-inducible *flpe* gene (P_{BAD}-*flpe*).

Antibodies and other reagents

The Naked2 antibody was generated as described in Chapter II. The α-tubulin

antibody was purchased from Oncogene Research Products (San Diego, CA). Horseradish peroxidase-donkey anti-mouse and anti-rabbit IgG were obtained from Jackson ImmunoResearch (West Grove, PA). Endonucleases and cloning enzymes were purchased from New England Biolabs (Ipswich, MA). All other chemicals were obtained from Sigma (St Louis, MO) unless otherwise stated.

PCR and RT-PCR

PCR amplification (*PfuTurbo*, La Jolla, CA) was performed by setting up the reaction mixture containing 0.5 μl of dNTP (10 mM), 10 ng of BAC DNA, 0.5 μl (10 μM) of each primer, 5 μl of 10X *Pfu* DNA polymerase reaction buffer, 1 μl of *PfuTurbo* (2.5 U/μl) and water to a total volume of 50 ul. PCR was performed using a PE-9700 PCR machine with the following settings: 94°C for 2 min, then 25 cycles of 94°C for 15 sec, annealing for 30 sec, and 72°C for 1 min. Annealing temperatures were adjusted for each primer pair. PCR products were ligated into the pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) followed by DNA sequencing and subcloning. Genotyping PCR was performed using Taq DNA polymerase (Roche, Indianapolis, IN) with following primers: 5'- GACACGCCTTGGGTCTCC-3' and 5'-TTTTTCCACTGCCCATTTGT-3'. The wild-type allele will generate a 708 bp product; the Naked2 null allele will generate a 113 bp product.

Total RNA was extracted from mouse intestinal tissues using the RNeasy mini kit (QIAGEN, Valencia, CA). RT-PCR was performed using the SuperScript™ One-Step

RT-PCR kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. We used primer (5'-CACCCGTCCTAGCGCCACTG-3') and primer (5'-CTTCTTGCTAAGTCTCAGTG-3') that encompass exon 1 and exon 5 of the mouse *Naked2* gene.

Southern blotting

Phenol-chloroform extracted ES cell genomic DNA (>10 μg) was digested with EcoRV (4 units / μg DNA) at 37ºC overnight. Digested DNA was then resolved by 0.8% agrose electrophoresis in 1x TAE buffer at 1 v/cm gel for about 16 hrs. The next day, the gel was soaked in 5 volumes of 0.2M HCl for 6min, rinsed briefly with H_2O twice, soaked in denature buffer for 30min with constant, gentle agitation (Denaturing buffer: 1.5M NaCl, 0.5M NaOH) and then rinsed again with H_2O twice. DNA was then transferred to HybondN+ membrane (Amersham Life Science) overnight. The next day, membrane was rinsed with 2x SSC buffer and incubated at 65ºC for 2 hrs in prehybridization solution (6XSSC + 1% SDS + 5X Denhardt + 0.1mg/ml boiled SSDNA). Meanwhile, southern probes were synthesized using pre-purified templates, Prime-It II Random Primer Labeling Kit (Strategene #300385) and ^{32}P-dCTP. Gel-purified PCR products, using following primer pairs, were used as templates for probe synthesis.

3' probe primers	5'-CTGAAGGGTACAGAGAGCAAG-3'
	5'-GCTTGCTGTGAGGCAGGTTAG-3'
5' probe primers	5'-GTCTTGGTAAGCAAGAAGTGA-3'

5’-GCATGCGCCACCACCTCCAG-3’

neo probe primers 5’-CTTCAAAAGCGCACGTCTGC-3’

5’-GTGCTCGCTCGATGCGATGT-3’

^{32}P-labeled probes were boiled and then cooled on ice before adding to prehybridization solution. The hybridization was incubated overnight at 65°C in hybridization bottles. On the last day, the membrane was washed with 2XSSC + 0.5% SDS, at 65°C for 10min, followed by washes in preheated 1XSSC +0.5% SDS at 65°C for 10min and 3 times in preheated 0.1XSSC +0.1% SDS at 65°C for 30min. Then autoradiography was conducted at -80°C in a sealed cassette.

Western blotting

Mouse ileum tissues were dissected and homogonized in ice-cold lysis buffer (25 mM Tris HCl, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM DTT and 2% BSA) in the presence of protease inhibitor cocktail (Sigma, St Louis, MO). Lysates were centrifuged at 13,000 rpm for 15 min at 4ºC, and clear supernatants were saved. The protein concentration of the supernatant was determined using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL) before boiling in sample buffer. Approximately 50 μg proteins of each sample were resolved on 10% SDS-PAGE followed by regular western blotting using the Naked2 antibody.

***In situ* hybridization**

The synthesis of digoxigenin (DIG)-labeled probes was performed according to the manufacturer's protocol (Roche). Briefly, one *in vitro* transcription reaction (20µl) contained: 1µg of linearized DNA template (normally 2-4µl); 1X DIG RNA labeling mix (1mM ATP, CTP and GTP; 0.65mM UTP; 0.35mM DIG-11-UTP pH7.5); 1X transcription buffer; 40U RNasin (RNase inhibitor, Promega) and 50U of the appropriate RNA polymerase (T7,T3 or SP6). The transcription reaction was performed at 37°C for 2 hours and stopped by adding RNase-free DNaseI (20U) for 15 minutes at 37°C to destroy the template DNA. The labeled probes were precipitated with 3M sodium acetate (pH 5.2) and 100% ethanol, washed in 70% ethanol, resuspended in diethyl pyrocarbonate (DEPC)-treated water and stored at -80°C. The *Naked1* (739 bp) and *Naked2* (824 bp) template sequences were PCR amplified from the mouse genomic DNA using the following primer pairs: *Naked1* probe (5'-CGGAATTCTCCTTCCACCTCCTTGACAC-3') and (5'-CGGGATCCGGTTTGGGGAGACCTGTGTA-3'); *Naked2* probe (5'-CGGAATTCGAAAACCTCTTGGGACACCA-3') and (5'-CGGGATCCTCTGGCCTCTTTGTTTTGCT-3'). PCR products were inserted into pBluescript KS(+) in EcoRI and BamHI. After linearization with EcoRI, anti-sense probes were synthesized with T7 RNA polymerase.

Section *in situ* hybridization was carried out as described (Hogan et al,, 1994) with some modifications. After dissection, mouse intestines were Swiss-rolled, directly embedded in Tissue-Tek® OCT compound in a cold ethanol-dry ice bath and stored at -80°C. Cryosections, at 15µm thickness, were collected on Superfrost Plus slides and

dried in a 37°C incubator for 40 minutes before being fixed in 4% PFA for 20 minutes at RT. Slides were then washed twice in DEPC-PBS for 5 minutes each time, followed by proteinase K treatment (2μg/ml in 50mM Tris pH7.5 and 5mM EDTA) at RT for various lengths of time depending on the embryonic stage. After a brief rinse in DEPC-PBS, samples were post-fixed in 4% PFA for 15 minutes at RT. To enhance signaling, sections were treated for acetylation with 250ml 0.1M triethanolamine-HCl (pH 8.0) containing 0.625ml acetic anhydride. After two 5 minute washes in DEPC-PBS at RT, slides were incubated with hybridization buffer (same as in whole-mount hybridization) for at least 2 hours at 60°C; DIG-labeled probes were then added onto the slide at 1-2μg/ml and incubated overnight at 60°C. Unbound probes were removed by a series of washes in 1XSSC (60°C, 10 minutes), 1.5XSSC (60°C, 10 minutes), 2XSSC (37°C, 20 minutes, twice), 2XSSC containing 0.2μg/ml RNaseA (37°C, 30 minutes), 2XSSC (RT, 10 minutes), 0.2XSSC (60°C, 30 minutes, twice), PBTw (60°C, 10 minutes, twice; RT, 10 minutes), and PBT (PBS containing 0.1% TritonX-100 and 0.2% BSA) (RT, 15 minutes). Slides were incubated in blocking buffer (PBT containing 20% goat serum) for at least 2 hours at RT, before anti-DIG antibody conjugated to AP was added into the blocking buffer at 1:2000 dilution for overnight incubation at 4°C. After three 30 minute washes in PBT at RT, sections were equilibrated in NTM without or with 5mM levamisole for 5 minutes each time and incubated in BM Purple at 37°C until specific signals were detected. The color reaction was stopped by rinsing slides in PBS.

Results

Generation of targeting vector using BAC recombineering

Given the early expression of Naked2 in various tissues of the mouse embryo (Wharton, Zimmermann et al. 2001), we employed a conditional knock-out strategy to avoid potential embryonic lethality.

The mouse *Naked2* is a single-copy gene on chromosome 13C1, and it contains ten exons (Fig. 4.1). The first three exons are short and condensed in a <700-bp region, followed by a large 20-kb intron. Since intron one is only 97-bp long, insertion of a 34-bp loxP site in this sequence may alter the transcriptional initiation or RNA splicing of the wild-type *Nkd2* gene. Therefore, we targeted the 5'-loxP site into intron 2, which is 253-bp long (Fig. 4.1). To avoid the disruption of splicing acceptor and potential branch point of intron 2, the loxP site was inserted 70-bp upstream of exon 3. In intron 3, the second loxP site was introduced with a *pgk-neo* cassette flanked by Frt sites. In the presence of active Cre recombinase, the floxed fragment will be excised, resulting in deletion of exon 3 and creating a pre-mature stop of the *Naked2* transcript, expressing only the first 22 residues encoded by the first two exons.

To precisely and efficiently target the loxP sequences and *neo* cassette in the *Naked2* locus, I utilized a phage-based *E.coli* homologous recombination system, which allows plasmid-based subcloning and DNA modification without the need for restriction enzymes or DNA ligases.

The first step was to retrieve the 10 kb mouse genomic sequence encompassing the first 3 exons of *Naked2* gene and their contiguous regions from a BAC clone RPCI-22-164-N12 (BAC N12) and place it into PL253 vector by a process known as gap repair. The gap repair is a homolog recombination reaction that requires recombinases encoded by two *Red* genes, *exo* and *det*. The functions of these two recombinases are further assisted by the Gam protein. We first electroporated BAC N12 DNA into overnight-cultured EL350 cells that contained *exo, det* and *gam* genes, and the BAC-containing cells were selected using the chloramphenicol resistance (Cam^r) gene that is carried in the BAC vector backbone. Two short homologous arms, A-B and Y-Z (~450 bp), were PCR amplified and ligated into PL253 between NotI and SpeI sites. The retrieval vector was subsequently linearized with HindIII at the junction of the two arms to create a DNA double-strand break for gap repair. We electroporated one μg of the linear retrieval vector into BAC N12-containing EL350 cells, which had been induced for *exo, det* and *gam* expression by prior growth at 42ºC for 15 min. Resultant ampicillin resistant (Amp^r) colonies were diagnosed by digestion with NotI and SpeI. This retrieved plasmid containing the 10.1 kb genomic sequence spanning exon 3 of the *Naked2* gene was designated as pWT000.

The second step was the introduction of a loxP site into intron 2 of the subcloned *Naked2* genomic DNA. Mini-targeting arms that were homologous to the targeting site were PCR amplified using primers C-D and E-F from BAC DNA and inserted into EcoRI and BamHI sites in the PL452 plasmid to flank the loxP-neo-loxP cassette. The floxed

Table 4.1 Primers used in generating the Naked2 targeting vector

Primer	Sequence 5'-3'	Purpose
A	TAGCGGCCGCTATGTTGAGGACATGCTTG	Retrieval, 5' arm
B	GTAAGCTTGATTCACAGAACACATGTC	
C	AGTCGACGGGGAAATTTCAGTCC	1st loxP targeting, 5' arm
D	CGAATTCACCCAGAGCCCCGAGG	
E	AGGATCCGGGTGGGCGCTGCAAG	1st loxP targeting, 3' arm
F	AGCGGCCGCTTCTGCAAGGCCCTT	
G	AGTCGACTCTAAGGCAFAFATGGGACT	2nd loxP targeting, 5' arm
H	CGAATTCCGCTGTATTAAGCTCTCCAA	
I	AGGATCCGGAGCATCCCTGGACAAATGG	2nd loxP targeting, 3' arm
J	AGCGGCCGCTCTGCTCTTAAACACAGCAC	
Y	GTAAGCTTATGTATGTGGAGGCCAGAGG	Retrieval, 3' arm
Z	GTACTAGTCAGAAACCACCAATCCCCTGT	

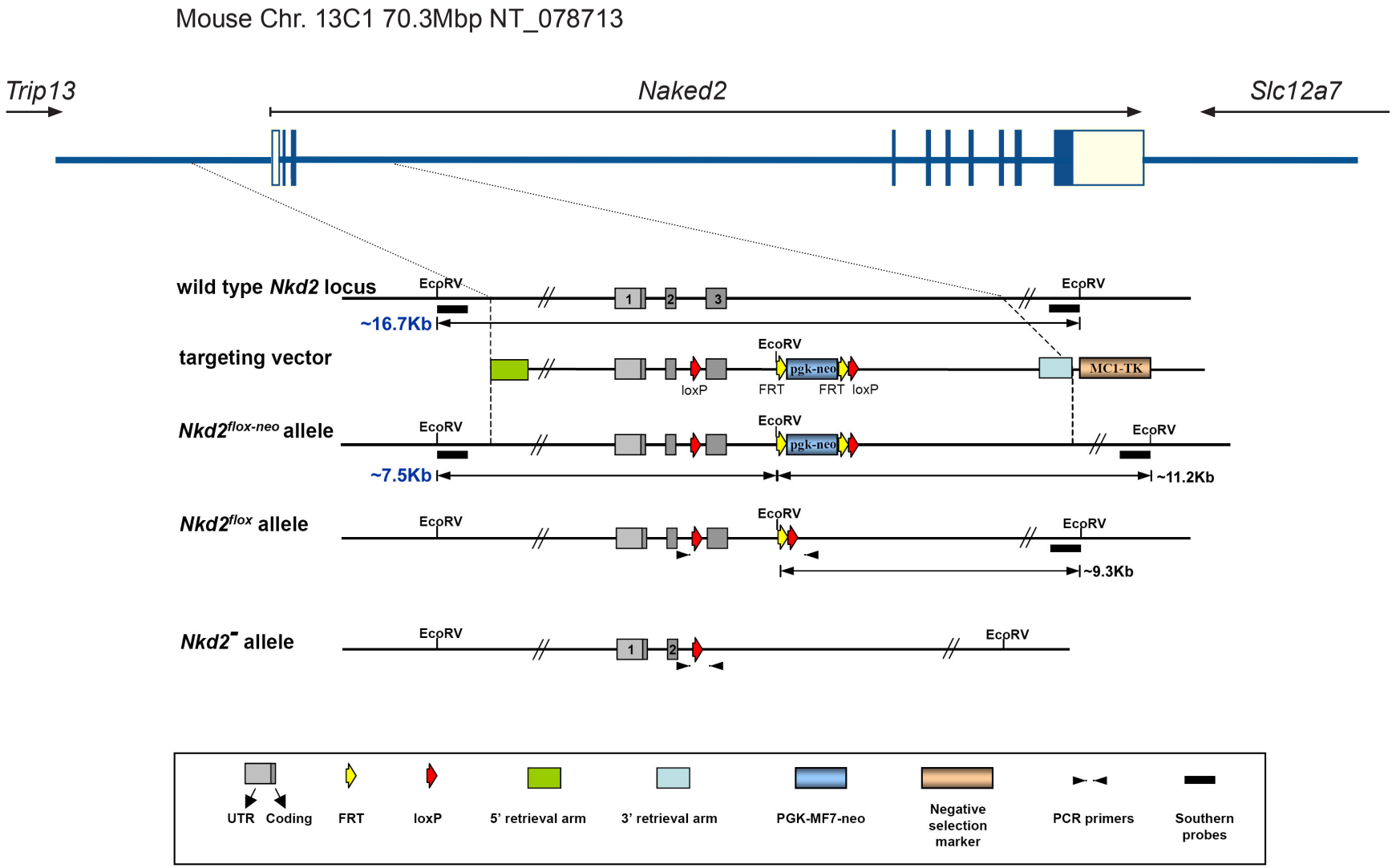

Figure 4.1 Schematic depiction of mouse Naked2 locus and targeted alleles.

Neo cassette together with the mini-targeting arms was excised by SalI and NotI and electroporated to gap repair-competent EL350 cells (see Materials and Methods) that contained the pWT000 plasmid. Kanamycine resistant (Kan^r) colonies were examined by KpnI digestion, and the correct recombination product that had the floxed neo cassette targeted into intron 2 was designated as pWT006. Excision of the neo cassette from the pWT006 was accomplished by inducing Cre expression by prior growth in arabinose-containing media for 1 hr. As a result, a single loxP site was left in the targeting site of intro 2, and this plasmid was designated as pWT008.

The last step was to target a second *loxP* site together with a *Frt-neo-Frt* cassette into the 5' end of intron 3. The *Frt-Pgk-em7-neo-Frt-loxP* cassette in the PL451 plasmid was flanked by mini-targeting arms that were amplified using PCR primer pairs G-H and I-J. A similar gap repair reaction was conducted and produced the pWT006 plasmid that had the second loxP site correctly targeted. This final targeting vector was subsequently linearized with DraIII and electroporated into TL-1 ES cells.

Screening targeted ES cell clones by Southern blotting

After electroporation, 106 G418-resistant and TK-negative ES cell colonies were picked, and genomic DNA was extracted. The clones were digested with EcoRV endonuclease and resolved by agrose gel followed by Southern blotting. A 5' probe outside of the 5' targeting arm recognized a 16.7 Kb fragment in the wild-type allele and a 7.5 Kb fragment in the targeted $Naked2^{flox\text{-}neo}$ allele (Fig. 4.1). Six of 106 clones were

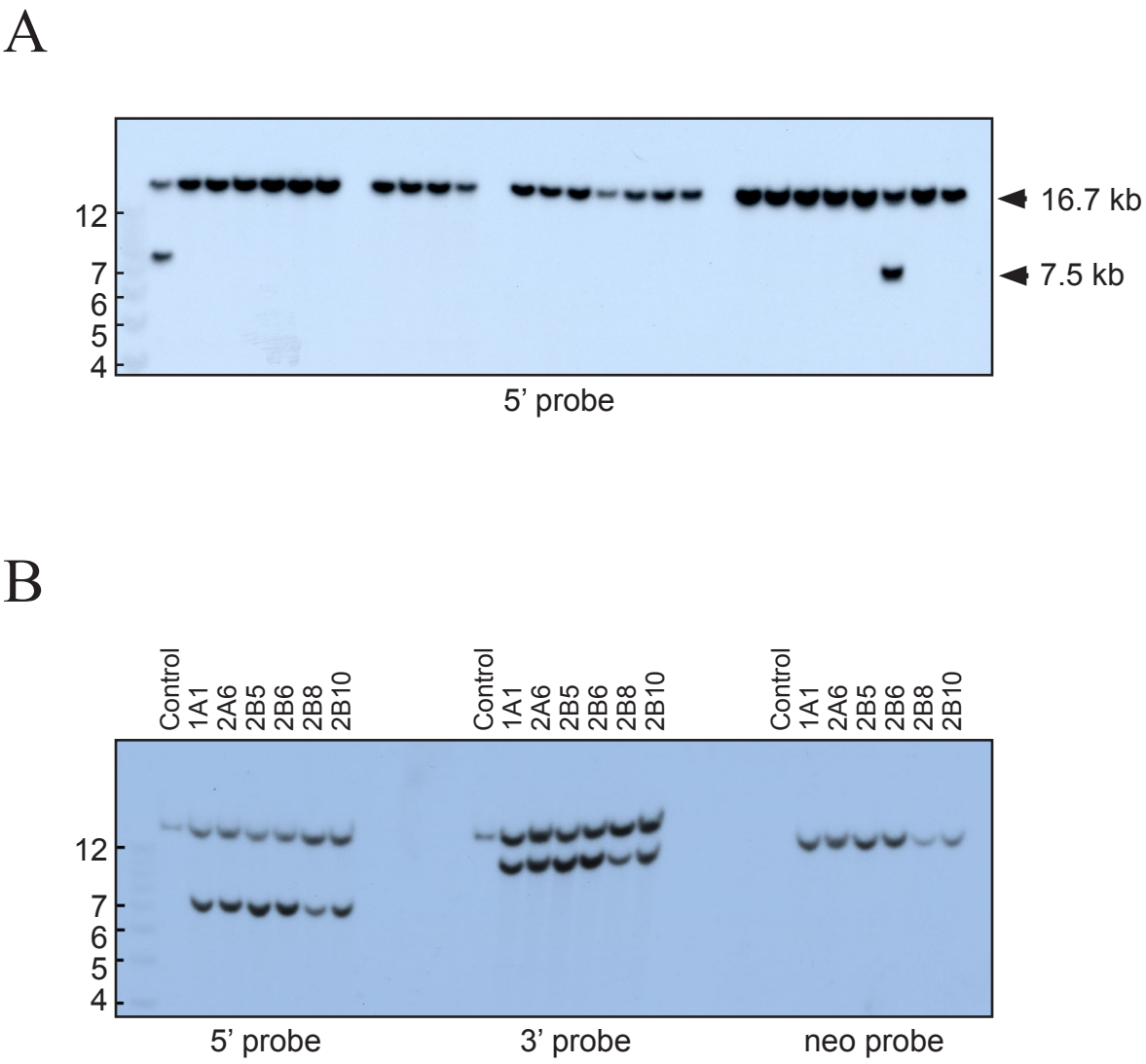

Figure 4.2 Southern blot for ES cell screening. (A) One hundred and six G418 resistant and TK-negative ES cell clones were screened by Southern blotting using the 5' probe. A 7.5 kb band that represents the targeted allele was detected in six clones. The wild-type allele produced a 16.7 kb band using the 5' probe. (B) All six targeted clones displayed correct band sizes by additional probes (3' and neo).

positive for the 7.5 Kb band by 5' probe screening (Fig. 4.2A). The targeted ES cell clones were also confirmed by Southern blotting using a 3' probe and neo probe (Fig. 4.2B).

Given the possibility that homologous recombination may also occur by utilizing the 633 bp sequence between the 5' loxP site and *Frt-neo-Frt* cassette as a short recombination arm (Fig. 4.3B), we conducted a further examination using PCR. The neomycin resistance displayed by these targeted clones indicated that potential mis-recombination may only happen between the short middle sequence and the long 3' targeting arm, which will result in loss of the 5' loxP site (Fig. 4.3A, B). Thus, we designed a pair of primers that encompassed the 5' loxP site. The presence of targeted 5' loxP site will produce a PCR product 46 bp longer than un-targeted sequence. To our surprise, 2 of the 6 clones were missing the 5' loxP site, indicating a high frequency of recombination mediated by the short arm (Fig. 4.3C). PCR products were sequenced to confirm the accuracy of 5' loxP sites, and three correctly targeted clones (1A1, 2B6 and 2B10) were chosen for blastocyst injection.

Genotyping of targeted Naked2 alleles in mice

Only injections using 1A1 and 2B6 clones resulted in high percentage agoutis, which were then directly crossed to *Flpe* female mice for germline transformation and removal of the neo cassettes. Resultant F1 pups were genotyped and germline transformation of *Naked2*^{flox} alleles was confirmed. Heterozygous *Naked2*$^{flox/+}$ male mice were crossed with

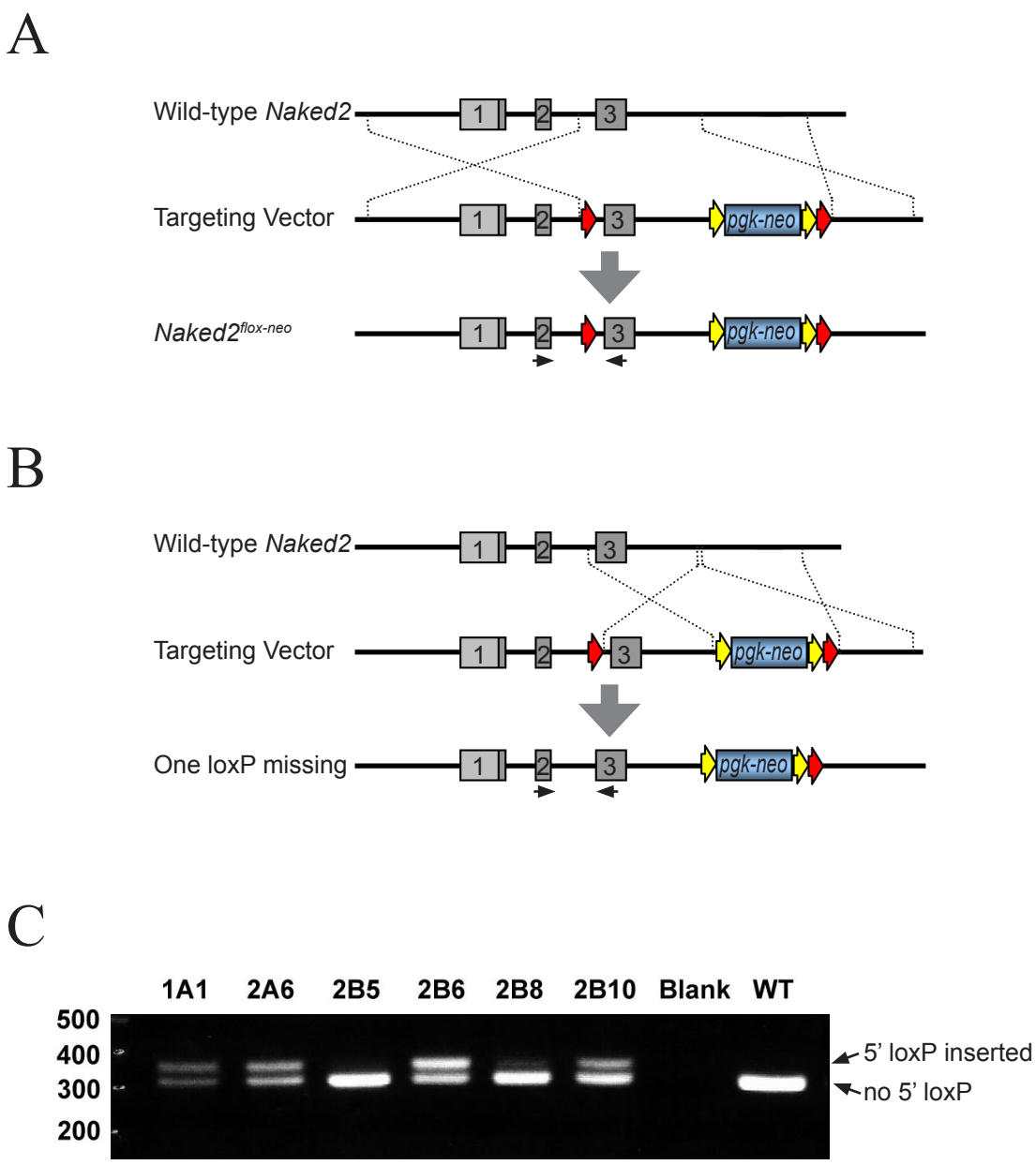

Figure 4.3 Possible homologous recombination events that may result in loss of one loxP site. (A) Prefered homologous recombination event that targets both loxP sites (red arrows) encompassing exon 3 of the mouse Naked2 gene. (B) The 5' loxP site was lost due to unwanted homologous recombination. (C) PCR diagnosis for the six targeted ES cell clones using primers that flank the 5' loxP sites.

Sox2-cre females to generate Nake2-null alleles. To test for embryonic lethality, *Naked2* $^{+/-}$ mice were intercrossed, resulting in all wild-type, heterozygous and homozygous Naked2 mice. The genotypes were verified by PCR genotyping (Fig. 4.5A); the number of each genotype that appeared adhered to Mendellian rules of inheritance (data not shown). Therefore, embryonic lethality was not observed in *Naked2*$^{-/-}$ mice.

Molecular confirmation of Naked2 expression in normal and knock-out mice

In wild-type intestinal tissues of 6-week old mice, endogenous *Naked2* expression was detected by in situ hybridization using a 3' probe (see Materials and Methods). The expression of *Naked2* was found in both small intestine and colonic epithelial cells but not in stromal cells (Fig. 4.4). Along the crypt-luminal axis, *Naked2* was highly expressed at the luminal surface and displayed a decreasing gradient of expression towards the crypt base. The majority of crypt epithelial cells were negative for *Naked2* staining except a small niche of cells that expressed moderate levels of *Naked2* at the base of the crypt. In mouse colon, *Naked2* was also detected at the top of crypt, but it exhibited a weak but much broader pattern of expression in the bottom half of the colonic crypt.

We verified the loss of Naked2 expression in the young adult intestinal tissues of *Naked2*$^{-/-}$ mice. Both RT-PCR and western blotting confirmed partial and complete loss of Naked2 expression in hetero- and homozygote *Naked2* mutant mice (Fig. 4.5B, C).

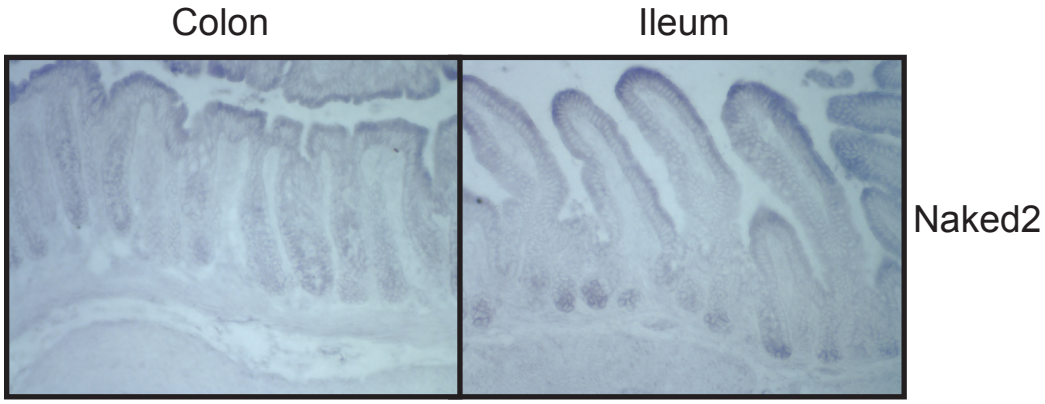

Figure 4.4 Naked2 *in situ* hybridization in normal mouse intestine tissues. Naked2 is expressed at the luminal surface of the crypt epithelium in both colon and small intestine of wild-type mice. Moderate Naked2 expression is also detected at the base of intestinal crypt but it is much weaker in colonic crypt.

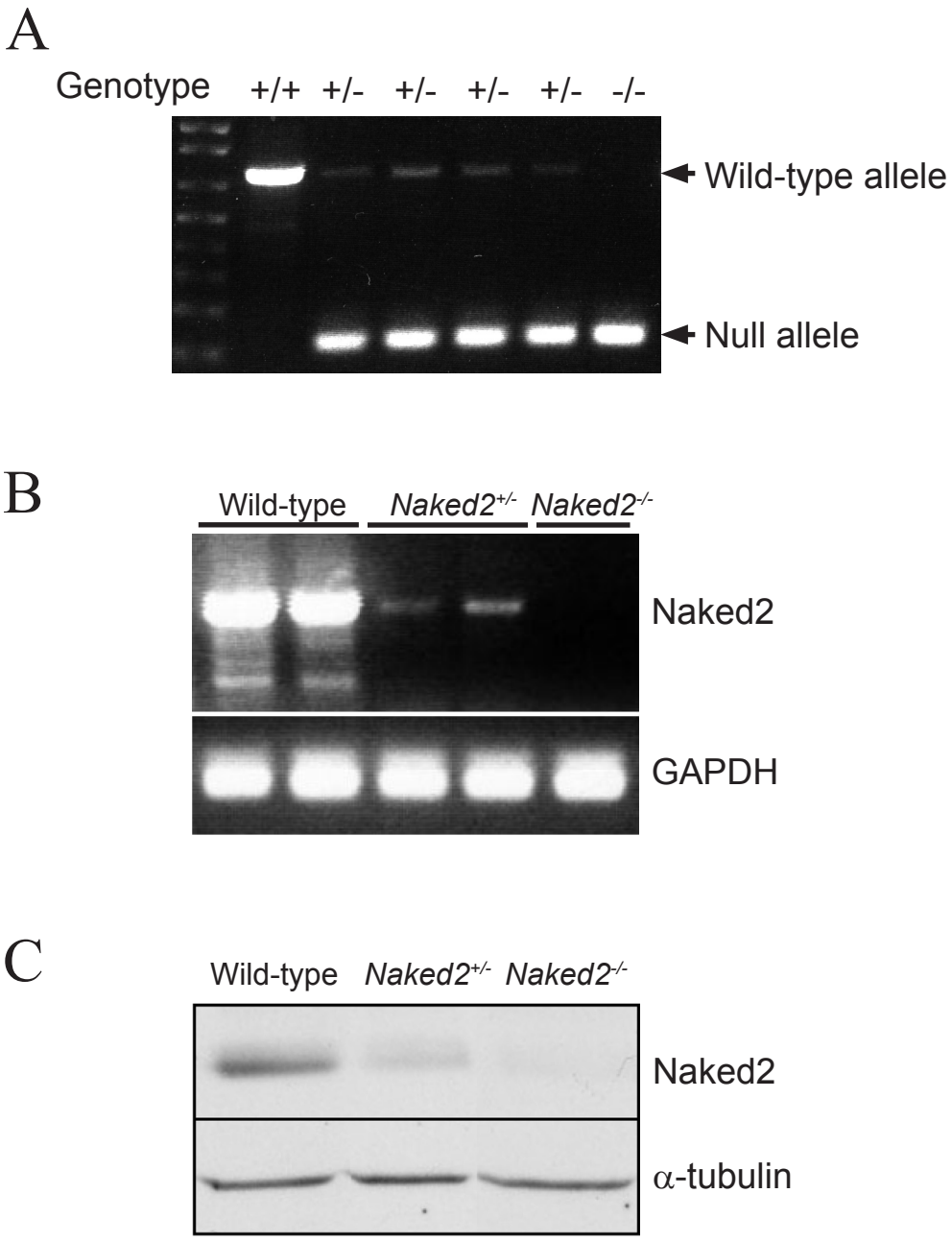

Figure 4.5 Molecular confirmation of *Naked2* **knock-out mice.** (A) PCR genotyping of tail DNA from wild-type and *Naked2* mutant mice. (B) Naked2 mRNA expression in wild-type and *Naked2* mutant mice by RT-PCR. Total RNA of intestinal tissues was extracted from young adult mice and subjected to One-step RT-PCR using primers that encompass exon 1 and exon 5. (C) Western blotting of intestinal tissues from wild-type and *Naked2* mutant mice. Naked2 mRNA and protein levels are reduced in the heterozygous mice and lost in *Naked2*$^{-/-}$ mice.

Discussion

Despite previous Naked2 studies using overexpression and RNA interference in epithelial cell culture and zebrafish embryos, *in vivo* characterization of Naked2 function has not been characterized in the mouse due to the lack of genetically engineered *Naked2* mutant mice. Recently, a *Naked2* mutant mouse was generated by replacing the exons that encode Dvl-binding motif with *IRES-lacZ/neomycine* cassette (Zhang, Cagatay et al. 2007). However, homologous mutant *Naked2*^{lacZ} mice are viable with slightly reduced litter sizes. Surprisingly, the double knock-out of both *Naked1* and *Naked2* using a similar strategy did not produce an overt phenotype, except subtle alterations in cranial bone morphology that resemble the phenotype of a mutation in *Axin2*, another Wnt/β-catenin antagonist. Based on these data, the authors concluded that Naked2 function is dispensable for embryonic development.

However, the above study may be flawed. First, we have identified an additional Dvl-binding domain located at the C-terminus of human Naked2 (unpublished data) that is not replaced by *IRES-lacZ/neomycine* cassette. Resultant mutant Naked2 may still be able to affect Dvl and Wnt signaling. Second, Naked2 protein without the EF-hand domain retains TTB motif (residue 300-385) that is sufficient to interact with the cytoplasmic tail of TGFα. The mutant Naked2 generated in the previous study may have intact functions in delivering TGFα to the basolateral cell surface. Therefore, elimination of the entire *Naked2* gene or the C-terminus functional TTB and the additional Dvl-binding domains may produce a more accurate phenotype of Naked2 function.

In this study, we floxed exon 3 of the mouse *Naked2* gene. Deletion of exon 3 resulted in premature termination of the *Naked2* transcript that only encodes the N-terminus 22 residues of Naked2 protein. This design produced a more complete elimination of functional Naked2 gene. RT-PCR using primers that encompass exon 1 to exon 5 and western blotting using Naked2 antibody that was generated against residues 1 to 217 have confirmed the loss of Naked2 expression in homologous *Naked2*$^{-/-}$ mouse intestinal tissues (Fig. 4.5). However, we have not observed any developmental defects upon initial examination.

Besides molecular confirmation, functional examinations are underway. To test the effect of *Naked2* ablation on Wnt signaling, the *Top-Gal* transgene will be introduced into *Naked2*$^{-/-}$. The level of Wnt signaling can be determined by lacZ staining. To test whether the loss of Naked2 disrupts basolateral trafficking of TGFα, TGFα immunohistochemsitry will be performed in *Naked2*$^{-/-}$ mouse tissues. We expect to observe loss of cell surface TGFα in *Naked2*$^{-/-}$ epithelial cells in the presence of a TACE inhibitor.

We may detect phenotypic abnormalities by a more comprehensive histological examination of *Naked2*$^{-/-}$ mice, but it is not too surprising that ablating *Naked2* alone is not enough to produce a simultaneous phenotype. Naked2 exhibited weaker interaction with Dvl compared to Naked1 in yeast two-hybrid assays (Wharton, Zimmermann et al. 2001), indicating that Naked2 may not be dedicated to regulating Wnt signaling and loss of Naked2 function in inhibiting Wnt signaling can be compensated for by Naked1. This

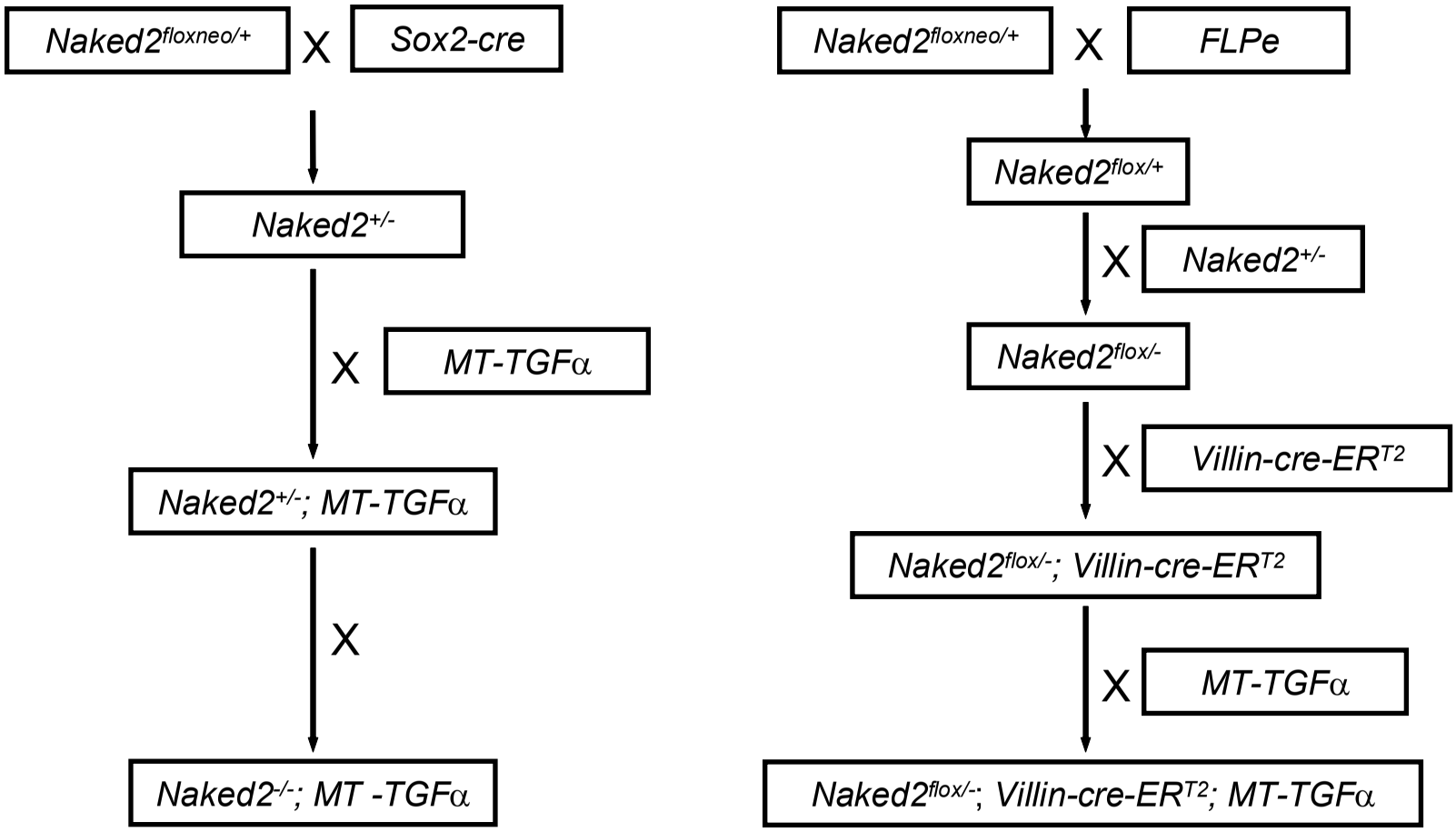

Figure 4.6 Generation of traditional and conditional *Naked2* null alleles in mice.

possibility can be tested by introducing Dr. Keith Wharton's *Naked1* mutation in our *Naked2*$^{-/-}$. In addition, Naked2 expression appears to be repressed by canonical Wnt signaling (see Chapter III), a manner that is opposite to Naked1 expression. This difference suggests that Naked2 is not part of the negative feedback loop of self-regulated Wnt signaling. Moreover, because normal epithelial cells express low levels of TGFα and thus have low dependency of Naked2, disruption of TGFα trafficking by *Naked2* ablation in these cells may not cause biological consequences. We suspect that in the presence of high levels of TGFα, *Naked2*$^{-/-}$ mice may result in a severe phenotype (Fig. 2.6).

Although further characterization is needed, the *Naked2* null mice generated in this study provide a valuable tool to determine *in vivo* functions for Naked2.

Acknowledgements

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CHAPTER V

GENERAL DISCUSSION

Tumorigenesis is a complex process requiring the accumulation of alterations in multiple genes affecting multiple pathways. In particular, human colorectal cancers represent a paradigm for the molecular and genetic mechanisms underlying tumor formation and progression (Fearon 1999). More than 85% of colorectal tumors have mutations in the *APC* gene, and loss of APC function results in constitutive activation of canonical Wnt signaling (Powell, Zilz et al. 1992). Overexpression of EGFR is found in more than one third of colorectal carcinomas; its overexpression may be linked to advanced stages (Gross, Zorbas et al. 1991) and may predict metastatic disease (Radinsky, Risin et al. 1995). This indicates the importance of EGFR signaling in the pathogenesis of colorectal cancer. Although the importance of Wnt and EGFR signaling pathways in tumorigenesis is well established, the convergence of these two pathways in colorectal cancer has not been generally appreciated.

Naked2 has been shown to act both as a negative regulator of Wnt signaling and as a CaRT protein for basolateral delivery of TGFα, a major EGFR ligand in the gut. In my thesis work, I have focused on the regulation of Naked2 expression in the context of TGFα trafficking and canonical Wnt signaling. Naked2 is not only required for proper TGFα exocytosis, but it is also regulated by TGFα through a direct protein-protein interaction. TGFα binds Naked2 and inhibits proteasomal degradation of Naked2 in an

EGFR-independent manner. This effect is mediated by a reduction in the association of Naked2 and AO7, a RING finger protein that acts as a ubiquitin ligase for Naked2. I show that, unlike *Naked Cuticle* and Naked1 that are induced by canonical Wnt signaling, Naked2 mRNA expression is repressed by activation of canonical Wnt signaling in human colorectal cancer cells. However, this repression appears to be indirect. Loss of Naked2 expression is a common event in colorectal carcinomas but not in adenomas. My work sheds new light onto the regulation of Naked2, a unique member of the Naked family, and the work provides a convergence point between canonical Wnt signaling and an EGFR-related event, that is, basolateral trafficking of TGFα in polarized epithelial cells. In addition, I have successfully generated a *Naked2* knock-out mouse by utilizing BAC recombineering. This mouse will be a valuable tool to examine physiological roles for Naked2 *in vivo*.

Ubiquitylation of Naked2 and its E3 ligase

I have discovered that Naked2 is a short-lived protein that undergoes rapid proteasomal degradation. This was an unexpected finding because most classic adaptor or coat proteins involved in exocytic and endocytic trafficking are long-lived and ubiquitously expressed, such as AP-1 and AP2 (Sorkin, McKinsey et al. 1995). When Naked2 is viewed from the perspective of canonical Wnt signaling and the need for its tight regulation, it is not surprising that Naked2 protein is regulated dynamically. The robust poly-ubiquitylation of Naked2 that was detected in *in vivo* and *in vitro* assays

substantiates its proteasomal degradation. However, the critical lysine residues that are required for Naked2 ubiquitylation and degradation remain to be determined. In addition, it will be of interest to determine the nature of the Naked2 ubiquitin chains, although poly-ubiquitin chains that are extended through the K48 residue of the ubiquitin moiety target protein for proteasomal degradation.

In my thesis work, AO7 has been shown to be a ubiquitin ligase for Naked2, and the binding of AO7 to Naked2 is affected by levels of TGFα, suggesting a role for AO7 in the regulation of Naked2 by TGFα. My results do not exclude the possibility that other ubiquitin ligases for Naked2 may exist and regulate the expression of Naked2 in other circumstances. However, due to the large number of existing ubiquitin ligases, identification of other ubiquitin ligases for Naked2 could be technically challenging. Similarly to phosphatases that counteract corresponding kinases, deubiquitinases (DUBs) hydrolyze the ubiquitin moieties from ubiquitylated proteins. Thus, DUBs can be involved in the cellular processes that are regulated by ubiquitylation. For example, activation of NF-κB requires proteasomal degradation of IκB, a negative regulator of NF-κB in cytoplasm. Cylindromatosis (CYLD) acts as a DUB for IκB and thereby inhibits NF-κB signaling (Kovalenko, Chable-Bessia et al. 2003; Trompouki, Hatzivassiliou et al. 2003; Courtois 2008). It will be important to identify DUBs for Naked2 and to understand how ubi/deubiquitylation balances the regulation of Naked2.

It is worth noting that a strong mono-ubiquitylated Naked2 species was frequently observed in ubiquitylation assays. Is it a specific mono-ubiquitylated Naked2 or just an

intermediate product of the poly-ubiquitylation reaction? Mono-ubiquitylation regulates a variety of cellular processes ranging from transcriptional regulation to cell signaling and membrane trafficking (Hicke 2001; Mukhopadhyay and Riezman 2007). As an important trafficking protein for TGFα delivery, mono-ubiquitylated Naked2, if it exists, is likely to have biological significance.

The regulation of Naked2 degradation

The prevailing concept is that adaptor or coat proteins play active roles in cargo selection and vesicle assembly but not vice versa. However, a recent study has reported that the degradation of a relatively short-lived clathrin-dependent adaptor, GGA2, was delayed when one of its cargos, CIMPR, was overexpressed in HeLa cells (Hirst, Seaman et al. 2007). My work not only identifies a similar phenomenon in the context of Naked2-coated TGFα vesicles, but it also elucidates the biochemical basis for this phenomenon. In a separate study utilizing fluorescence-activated vesicle sorting (FAVS**)**, we have identified 389 proteins that are associated with Naked2 vesicles. These include other potential cargos for Naked2-associated vesicles like Na^+/K^+-ATPase α1. It will be interesting to examine whether Naked2 degradation is regulated by cargos other than TGFα.

My observation - that increased expression of TGFα reduces the interaction between AO7 and Naked2 - may provide molecular insight as to how TGFα stabilizes Naked2. To test whether this effect is through a direct competition for Naked2 binding between TGFα

and AO7, we sought to determine the AO7-binding sites in the Naked2 sequence. Preliminary data suggests that AO7 binds to multiple sequences in Naked2 excluding the TGFα tail-binding domain (residue 300-385). Thus, direct competition between TGFα and AO7 for binding to Naked2 is unlikely. A recent structural study found that the N-terminal half of Naked2 behaves as an intrinsically unstructured protein (IUP) (Hu, Krezel et al. 2006). $p27^{Kip1}$ is also an IUP and it assumes a secondary structure when it binds to the cyclin A-Cdk2 complex (Verkhivker 2005). I propose that TGFα binding may confer an ordered protein structure to Naked2 that makes it less accessible to other proteins such as AO7. Structural studies of Naked2 in the presence of TGFα will be needed to examine this possibility.

Besides ubiquitylation, another posttranslational modification, myristoylation, has been identified previously to be critical for Naked2 function (Li, Franklin et al. 2004). Myristoylation deficient-mutant (G2A) Naked2 no long localizes to the plasma membrane when expressed in polarized MDCK cells. By immuno-electron microscopy, G2A-associated vesicles accumulate in the cytoplasm (Li, Hao et al. 2007). Of interest, an independent study has shown that G2A mutant Naked2 exhibits delayed protein degradation compared to wild-type Naked2 (unpublished data). Based on these results, we suspect that Naked2 is stabilized upon recruitment to the surface of vesicles, whereas cell membrane and/or cytosolic Naked2 is subjected to active protein degradation. This localization-dependent degradation of Naked2 begs the question as to the cellular distribution of AO7, which has not been characterized.

The transcriptional regulation of Naked2 by Wnt signaling

In addition to stabilization of Naked2 protein by TGFα, Naked2 transcription is repressed by active Wnt signaling as we demonstrated in Chapter III. This result not only adds additional complexity to Naked2 regulation but also distinguishes Naked2 from other Naked family members that are inducible target genes of canonical Wnt signaling (Rousset, Mack et al. 2001; Yan, Wiesmann et al. 2001; Van Raay, Coffey et al. 2007). Whereas there are a number of examples of inducible antagonists to canonical Wnt signaling such as Axin2 and Dkk1, there are few, if any, examples of a negative regulator that is inhibited by Wnt signaling. This may be a way that Wnt activation amplifies its own signal. Given the role of Naked2 in TGFα trafficking, we propose a model in which active Wnt signaling undermines epithelial homeostasis by disrupting cell surface delivery of TGFα. Transcriptional repression of *Naked2* has been described previously by Lei, H et al (Lei, Juan et al. 2007). The transcription factor, Hoxc8, a member of the homeobox gene family, directly binds to a distal enhancer element at 25 kb upstream of human *Naked2* promoter. The enhancer activity was significantly repressed by Hoxc8 expression in NIH3T3 cells that did not express endogenous Hoxc8. There is considerable evidence that the canonical Wnt/β-catenin pathway induces expression of caudal (Cdx) proteins (Pilon, Oh et al. 2006) that impact on skeletal patterning by direct regulation of Hox gene expression. These observations provide a possible cascade in which Naked2 is repressed by active Wnt signaling. In addition to the distal enhancer

element that is regulated by Hoxc8, we have characterized the proximal sequence of mouse *Naked2* promoter as shown in Chapter III. This 4.5 kb sequence exhibited strong promoter activity that was repressed by overexpression of ΔN89 β-catenin, suggesting that Naked2 promoter can be repressed by canonical Wnt signaling. Future efforts should further dissect this promoter sequence. Proximal regulatory elements may be identified, and the synergistic effects of proximal and distal enhancer elements in *Naked2* transcriptional regulation may be found. However, given the temporal lag between Wnt activation and Naked2 repression, it is possible that transcriptional regulation may not be a direct effect of the β-catenin/TCF complex.

Naked2 in normal epithelial homeostasis and colorectal tumorigenesis

The role of ErbB receptor signaling in both normal development and malignant progression has been extensively studied over the past decade. The increasing complexity of ErbB signaling pathways has led to systems biology approaches. Cell surface cleavage of the ligands, activation of receptor tyrosine kinase activity and MAPK phosphorylation have been proposed to be three critical nodes in ErbB signaling (Wiley, Shvartsman et al. 2003; Citri and Yarden 2006). In these systems biology approaches, cell surface delivery of ligands has been neglected. However, evidence is accumulating that ligand engagement of the EGFR may determine the specificity of downstream signals, and that differential cell surface delivery and presentation of each ligand appear to be critical steps for the accessibility of different ligands to the EGFR. Moreover, disruption of regulated

exocytic sorting of EGFR ligands may have important biological consequences as demonstrated by a recent study in isolated recessive renal hypomagnesaemia (Groenestege, Thebault et al. 2007), a disease caused by a mutation in the cytoplasmic tail of EGF that impairs its basolateral sorting.

In normal epithelial cells, Naked2 ensures efficient delivery of TGFα to the basolateral surface of polarized epithelial cells where it is then rapidly cleaved by TACE, and soluble ligand is avidly taken up by the EGFR. Coordination of these events maintains local signaling by TGFα in an autocrine and/or local paracrine manner. Occupation of the EGFR by TGFα prevents engagement by other basolaterally delivered ligands such as AR. Maintaining the preeminence of TGFα in engaging the EGFR may be essential for epithelial homeostasis, because it has been shown that exogenous recombinant human AR, but not TGFα, results in an EMT-like phenotype in polarized MDCK cells (Chung, Cook et al. 2005; Chung, Graves-Deal et al. 2005). Thus, alteration in normal TGFα trafficking may have important biological consequences, such as EMT, that may contribute to tumor progression. To ensure its efficient basolateral trafficking, TGFα stabilizes its CaRT protein Naked2 by inhibiting its proteasomal degradation (see Chapter II).

Naked2 is also a known antagonist of canonical Wnt signaling by inactivating Dvl, although the mechanism underlining this inactivation is unclear. In recent studies, the Coffey lab has identified that Naked2 and Dvl-1 form a mutual degradation complex (Hu, in preparation).

These two independent roles of Naked2 suggest that Naked2 may provide a convergence point between EGFR-related events (i.e., cell surface delivery of TGFα) and canonical Wnt signaling. In separate studies, we have observed Naked2 interactions with either TGFα or Dvl-1, but we have not detected TGFα, Naked2 and Dvl-1 together in a ternary complex (C Li and RJ Coffey, unpublished observation). In addition, we have not identified Dvl among the 389 proteins that have been identified in Naked2-associated vesicles by LC/MS-MS (J Cao and RJ Coffey, unpublished observation). These findings lead us to entertain the possibility that TGFα may compete Naked2 from binding to Dvl and thereby enhance Wnt signaling. However, it is also possible that Naked2 may act in a sequential manner. Naked2 first interacts with TGFα and facilitates efficient delivery of TGFα-containing exocytic vesicles to the plasma membrane. Upon discharging this function, Naked2 now binds and degrades Dvl so as to repress canonical Wnt signaling. In fact, we have detected decreased TOPflash activity in Caco-2-TGFα cells compared to parental Caco-2 cells, and this reduction is attenuated by Naked2 siRNA in Caco-2-TGFα cells (Fig. 5.1).

Based on these results, we proposed a two-step model by which Naked2 participates in both normal epithelial homeostasis and colorectal tumor progression (Fig. 5.2). In normal intestinal epithelium, as cells migrate up from the stem cell niche at the base of the crypt, they start to differentiate and express TGFα. Decreased canonical Wnt signaling in the differentiating cells allows expression of Naked2 mRNA to facilitate TGFα trafficking. Constitutive delivery of TGFα-containing vesicles stabilizes Naked2

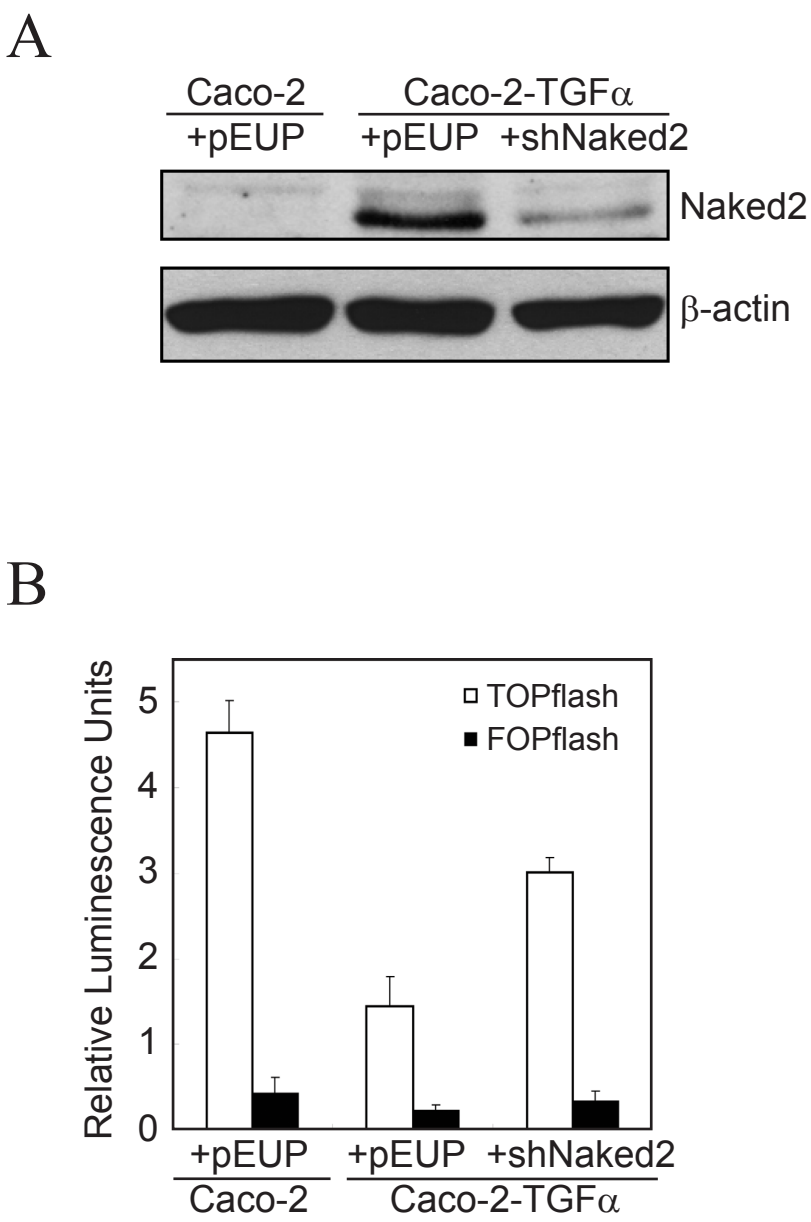

Figure 5.1 Naked2-dependent inhibition of canonical Wnt signaling in Caco-2-TGFα cells. (A) Knocking-down Naked2 in Caco-2-TGFα cells by Naked2 shRNA. The pEUP vector expressing 29-mer small hairpin RNA for human Naked2 or the empty vector was transfected in TGFα-expressing Caco-2 cells. Subsequent western blotting using the Naked2 antibody reveals a significant reduction of Naked2 expression by Naked2 shRNA. (B) TOPflash assay in Caco-2 and Caco-2-TGFα cells. Compare to Caco-2 cells, Caco-2-TGFa cells display reduced TOPflash activity , which is partially restored by Naked2 shRNA. shNaked2 refers to Naked2 shRNA.

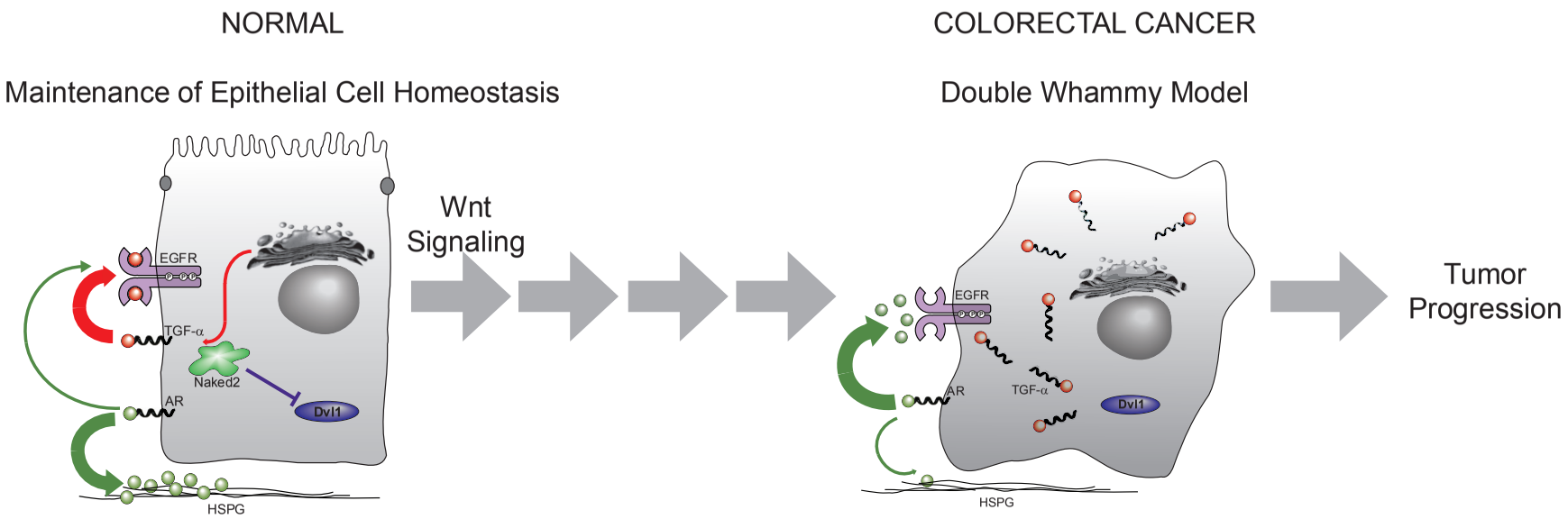

Figure 5.2 A model for the function of Naked2 maintaining normal epithelial homeostasis and how its loss promotes tumor progression. In normal epithelial cells, Naked2 facilitates basolateral cell surface delivery of TGFα. Constitutive delivery of TGFα-containing vesicles stabilizes Naked2 protein, which in turn inactivates Dvl and thereby maintains tight control of canonical Wnt signaling. In colorectal cancer, Naked2 transcription is repressed. TGFα and AR are often upregulated in colorectal tumors. If Naked2 mRNA expression is markedly suppressed, there may be insufficient levels of Naked2 protein to escort TGFα to the cell surface. As a consequence, AR will now bind to the relatively unoccupied EGFRs and predispose to tumor progression.

protein, which in turn inactivates Dvl and thereby maintains tight control of canonical Wnt signaling.

In contrast, upon activation of the canonical Wnt signaling in early stage of colorectal cancer, Naked2 transcription is repressed. TGFα and AR are often upregulated in colorectal tumors. If Naked2 mRNA expression is markedly suppressed, there may be insufficient levels of Naked2 protein to escort TGFα to the cell surface. As a consequence, AR will now bind to the relatively unoccupied EGFRs and predispose to tumor progression.

Taken together, Naked2 is regulated by both TGFα and canonical Wnt signaling but at different levels. Through regulating Naked2, the inhibitory relationship between the two pathways contributes to both epithelial homeostasis and colorectal tumor progression.

Proposed studies with Naked2 knock-out mice

Genetically engineered *Naked2* mutant mice have been reported to exhibit no severe developmental defects (Zhang, Cagatay et al. 2007). However, the lack of a phenotype may be due to the incomplete elimination of the *Naked2* gene, as I have detected an immunoreactive Naked2 band in these purportedly Naked2 null mice. I have generated a targeted Naked2 mutation that produces a pre-mature transcript of Naked2 encoding only 22 residues of the N-terminal sequence. These Naked2 knock-out mice will allow us to explore the *in vivo* significances of Naked2 in the context of both Wnt signaling and

TGFα trafficking.

We have achieved germline transformation of the targeted allele and the floxed exon 3 of the *Naked2* gene has been deleted by crossing to the *Sox2-cre* transgene. Loss of Naked2 expression in the Naked2$^{-/-}$ mice has been confirmed by both RT-PCR and western blotting (Fig. 4.5). However, no overt defects have been observed in these Naked2$^{-/-}$ mice.

In vitro assays have established that mammalian Naked2 inhibits canonical Wnt signaling, but we have not yet examined alterations in canonical Wnt signaling in these mice. I suspect that ablation of *Naked2* may not result in significant increase of canonical Wnt signaling because Naked2 is not a strong inhibitor of Wnt signaling due to its weaker interaction with Dvl compared to Naked1. In addition, loss of Naked2 function in antagonizing the canonical Wnt pathway can be compensated for by Naked1. Moreover, other negative regulators that are downstream of the Nakeds may also attenuate the effects of Naked2 ablation. These might also explain why Naked1 mutant mice are mostly normal except for minor defects in spermatogenesis and cranial bone morphology. Therefore, one of the first functional examinations of *Naked2*$^{-/-}$ mice will be a determination of canonical Wnt signaling levels by introducing a *TOPGAL* reporter gene. Similar to the *in vitro* TOPflash assay, the *in vivo* levels of canonical Wnt signaling will be assessed by the levels of lacZ staining.

The *in vivo* roles of Naked2 in TGFα trafficking will also be examined in *Naked2*$^{-/-}$ mice. We anticipate an accumulation of cytoplasmic TGFα and reduced cell surface

TGFα; this latter analysis will be performed after systemic administration of a TACE inhibitor. In addition, potential alterations in EGFR signaling will be studied. However, based on initial observations, we anticipate that additional provocative maneuvers may be needed to elicit phenotypic defects in the *Naked2*$^{-/-}$ mice. Previous studies have demonstrated that overexpression of TGFα in mouse by the *metallothionein-TGFα* (*MT-TGFα*) transgene promoted hyperplasia in the intestinal epithelium without otherwise causing major alterations in the tissue architecture (Sandgren, Luetteke et al. 1990). Although *MT-TGFα* mice showed higher sensitivity to carcinogenesis in various tissues, such as skin (Shibata, Ward et al. 1997), pancreas and liver (Sandgren, Luetteke et al. 1993), they only exhibited enhanced tumor formation in the duodenum once crossed to *APC*^{Min} mice for colorectal cancer (Williams Dove, personal communication). This difference may be due to the high levels of Naked2 expression along the intestinal track except the duodenum (Li, Franklin et al. 2004). To test the effects of Naked2 loss in the setting of high levels of TGFα, we intend to cross the *MT-TGFα* transgenic mice to the *Naked2*$^{-/-}$ mice (Fig. 4.6).

Since TGFα overexpression is not sufficient to cause colorectal neoplasia, additional events may need to be introduced to test the two step model of Naked2 function in promoting tumor progression. *APC*^{Min} mice produce intestinal polyps at three months of age due to loss of the wild-type APC allele. Because the APC is a Wnt antagonist downstream of Naked2, *APC*^{Min} mice are an ideal model to study the effects of Naked2 that are solely mediated through disrupting the TGFα cell surface delivery but not

through Wnt signaling. Based on the model I proposed above, loss of Naked2 in APC^{Min} mice may promote tumor establishment and progression.

As we have retained the conditional mutant *Naked2* allele, we can achieve a spatiotemporal removal of Naked2 expression by crossing floxed *Naked2* to tissue-specific *cre-ER*^{T2} transgenes (Fig. 4.6). *Villin-cre-ER*^{T2} is expressed specifically in the gut epithelium and kidney. It therefore appears to be a useful tool to study Naked2 function in the intestinal epithelium. We may also consider administering the colon cancer carcinogen azoxymethane (AOM) in these models.

Taken together, the successful generation of *Naked2* knock-out mice provides a valuable reagent to test the two-step model of Naked2 that we have proposed based on previous *in vitro* studies. This study will shed new light on the idea that efficient cell surface delivery of TGFα by Naked2 helps to maintain normal epithelial homeostasis and that disruption of this process promotes tumor progression.

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