EGF receptor-independent action of TGF-α protects Naked2 from AO7-mediated ubiquitylation and proteasomal degradation

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Naked family members (*Drosophila Naked Cuticle* and mammalian Naked1 and Naked2) have been identified as inducible antagonists of canonical Wnt signaling. We recently reported that Naked2, but not Naked1, interacts with the cytoplasmic tail of TGF-α, thereby coating TGF-α-containing exocytic vesicles and directing these vesicles to the basolateral corner of polarized epithelial cells. Here, we show that Naked2 is a short-lived protein with a half-life of 60 min caused by its rapid ubiquitin-mediated proteasomal degradation. Overexpression of TGF-α stabilizes Naked2 protein in an EGF receptor (EGFR)-independent manner; a physical interaction between the cytoplasmic tail of TGF-α and Naked2 is necessary and sufficient for this protection. We have identified a RING finger protein, AO7/RNF25, as a ubiquitin ligase for Naked2, and we have shown that overexpression of TGF-α reduces binding of AO7 to Naked2. These results identify an EGFR-independent action of TGF-α, in which it protects Naked2 from proteasomal degradation, thus ensuring its delivery to the basolateral surface of polarized epithelial cells.

AO7/RNF25 | E3 ligase | Wnt antagonist

EGF receptor (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. 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 (1). It is increasingly recognized that a critical regulatory node in the initiation of EGFR signaling is protease-executed cell surface cleavage of proligands. An underappreciated aspect of EGFR signaling is 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 hypomagnesemia is caused by a mutation in the cytoplasmic tail of EGF that disrupts its sorting to the basolateral surface of proximal tubular cells of the kidney (2).

In polarized epithelial cells, transforming growth factor-α (TGFα) is delivered preferentially to the basolateral cell surface where it is rapidly cleaved by TNF-α-converting enzyme/a disintegrin and metalloprotease-17 (TACE/ADAM-17) (3, 4). Soluble TGFα is then avidly captured by basolateral EGFRs (4). The rapid cleavage and avid capture of TGFα suggest that its cell surface delivery may be rate-limiting in the spatial and temporal regulation of endogenous TGFα activity (1). We have identified two basolateral sorting determinants in the cytoplasmic tail of TGFα (LL and HCCQVRKH) (5), both of which contribute to its interaction with Naked2 (6), a previously identified negative regulator of Wnt signaling (7–9). Naked2 binds to the cytoplasmic tail of a Golgi-processed form of TGFα through its TGFα tail-binding (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 (10, 11). 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, signal transduction, and protein transport (12–14). Ubiquitylation is a multistep posttranslational protein modification catalyzed by E1s (ubiquitin-activating enzymes), E2s (ubiquitin-conjugating enzymes), and E3s (ubiquitin ligases). Cycling of these reactions can conjugate polyubiquitin 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 >500.

Here, we demonstrate that Naked2 is a short-lived protein with a half-life of 60 min caused by rapid ubiquitin-mediated proteasomal degradation. AO7, a RING finger protein that exhibits ubiquitin ligase activity (15), 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 proteasomal degradation. This effect of TGFα is EGFR-independent; a physical interaction between the cytosolic tail of TGFα and Naked2 is necessary and sufficient to attenuate Naked2 ubiquitylation. This work identifies an E3 for Naked2 and an EGFR-independent action of TGFα.

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, Madin–Darby canine kidney (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 Western blotting. As shown in Fig. 1*A*, Naked2 protein was rapidly degraded with a half-life of 60 min. When MDCK cells were pretreated with a proteasome inhibitor, MG132 (or lactacystin; data not shown), for 2 h before the addition of CHX, Naked2 degradation was significantly retarded. By contrast, pretreatment with a lysosomal inhibitor, ammonium chloride (NH_4Cl), did not retard Naked2 degra-

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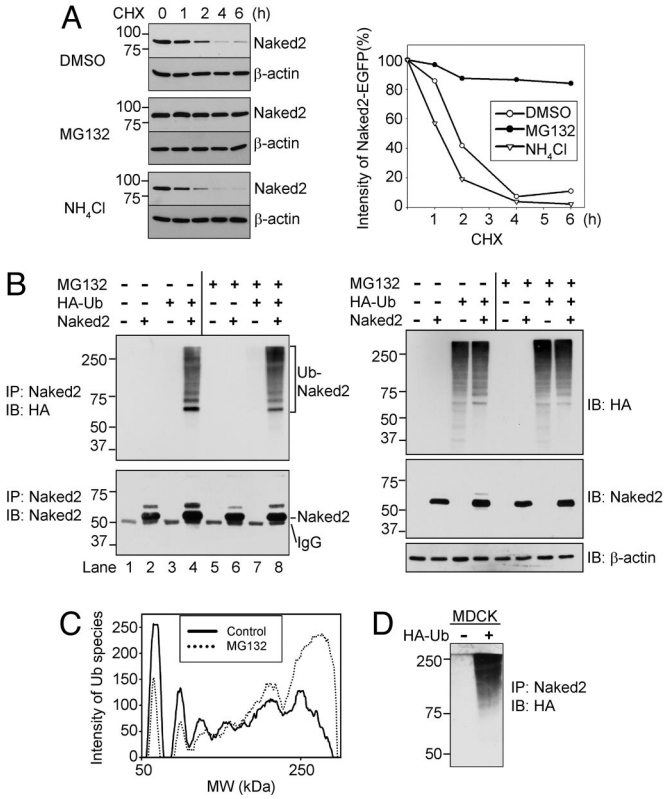

Fig. 1. Ubiquitylation and proteasomal degradation of Naked2. (*A*) (*Left*) Proteasomal degradation of Naked2. MDCK cells expressing Naked2-EGFP were treated with 1 μg/ml CHX. Cell lysates were collected at the indicated time points and then subjected to Naked2 Western blotting. The majority of Naked2-EGFP was degraded within 4 h. Naked2 degradation was blocked by 2-h exposure to a proteasome inhibitor (5 μM MG132) before CHX treatment but not by preexposure to a lysosomal inhibitor (20 mM NH_4Cl). All blots were reprobed for β-actin as a loading control. (*Right*) Band densities quantified. (*B*) Ubiquitylation of Naked2. (*Left*) HEK293 cells transiently expressing Naked2 and HA-ubiquitin were exposed to DMSO or 5 μM MG132 for 5 h. Cells were lysed and immunoprecipitated (IP) by using Naked2 antibody VU308 (see *Materials and Methods*) followed by anti-HA Western blotting (IB). A ladder of ubiquitylated Naked2 (Ub-Naked2) was detected. (*Lower*) The blot was stripped and reprobed with VU308 to demonstrate levels of Naked2 protein. (*Right*) Aliquots of whole-cell lysates (WCL) were blotted with HA, Naked2, and β-actin antibodies to show their respective expression levels. (*C*) Density plotting of Ub-Naked2 species in *B* showed that MG132 treatment resulted in a compositional shift toward higher-molecular-mass species. (*D*) Ubiquitylation of endogenous Naked2 in MDCK cells by using an *in vivo* ubiquitylation assay (see *Materials and Methods*).

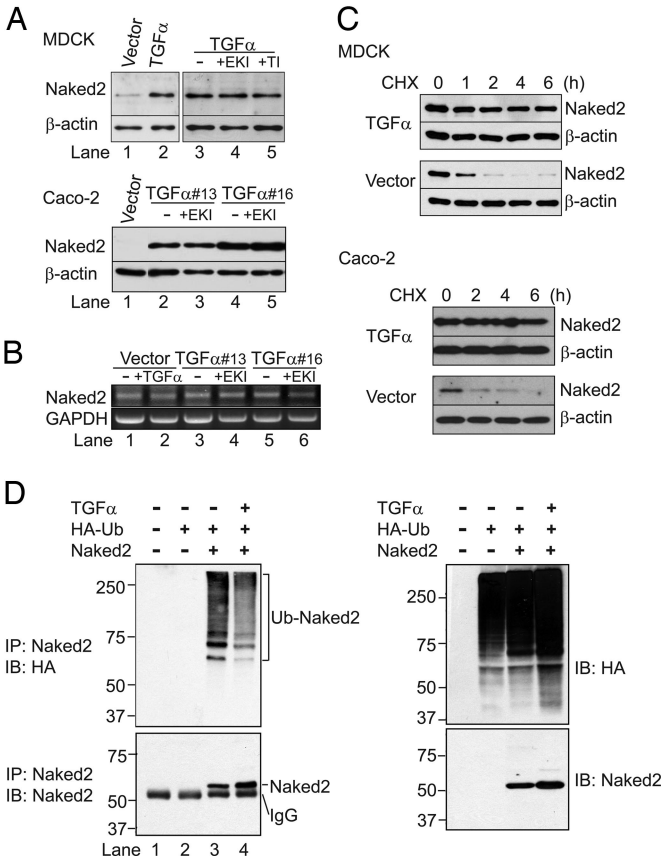

Fig. 2. Overexpression of TGFα delays Naked2 degradation and up-regulates endogenous Naked2 levels. (*A*) Up-regulation of Naked2 protein by TGFα. Compared with vector control cells, MDCK cells stably expressing TGFα exhibited increased Naked2 levels. Pharmacological blockade of EGFR activation by using an irreversible EGFR tyrosine kinase inhibitor (EKI-785, 1 μM) or TGFα cell surface shedding with a selective TACE inhibitor (WAY022, 1 μM; designated TI) did not affect Naked2 levels in TGFα-transfected MDCK cells. In similar experiments in Caco-2 cells, Naked2 was dramatically up-regulated in two independent clones 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. (*C*) Delayed Naked2 degradation by overexpressed TGFα. MDCK cells stably expressing Naked2-EGFP and TGFα or Naked2-EGFP and blank pCB7 vector were incubated with 1 μg/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 monitored by Western blotting with VU308 antibody. (*D*) Reduced Naked2 ubiquitylation by TGFα overexpression. (*Left*) Cell lysates from HEK293 cells transiently expressing Naked2, TGFα, and HA-ubiquitin, as indicated, were subjected to immunoprecipitation (IP) by using VU308 antibody followed by HA (*Upper*) or Naked2 (*Lower*) Western blotting (IB). The overall ubiquitylation of Naked2 was reduced in the presence of TGFα. (*Right*) WCL were subjected to Western blotting by using anti-HA and Naked2 VU308 antibodies to show expression levels.

dation (Fig. 1*A*). Thus, Naked2 is a short-lived protein that undergoes rapid proteasomal degradation.

Because polyubiquitylation is a signature for delivering substrates to the proteasome for degradation (16, 17), we next performed an *in vivo* ubiquitylation assay (18) to examine directly the ubiquitylation of Naked2. We transiently transfected human embryonic kidney (HEK293) cells with plasmids encoding nontagged Naked2 and HA-tagged ubiquitin (HA-Ub) (18); 48 h later, cell lysates were subjected to immunoprecipitation by using a Naked2 antibody VU308 (see *Materials and Methods*) and blotted with an HA antibody. Clear laddering of polyubiquitylated Naked2 was detected when HA-Ub was coexpressed with Naked2 (Fig. 1*B Left*, lane 4). The lowest band detected by the HA antibody migrates at $\approx$9 kDa (the molecular mass of a single HA-Ub molecule) above the Naked2 band and likely represents monoubiquitylated Naked2, which might be an intermediate product of polyubiquitylation. The presence of immunoreactive forms migrating at higher molecular mass represents polyubiquitylated Naked2, which was confirmed by reprobing with Naked2 antibody (data not shown). The identification of polyubiquitylated Naked2 is consistent with it being a short-lived protein as demonstrated in the previous degradation assays (Fig. 1*A*). We have performed density plots of ubiquitylated Naked2 in the presence and absence of MG132 (Fig. 1*B*, lanes 4 and 8). This analysis demonstrated a clear accumulation of higher-molecular-mass species of ubiquitylated Naked2 in the presence of MG132 (Fig. 1*C*). In the absence of MG132, we speculate that these higher-molecular-mass species undergo proteasomal degradation.

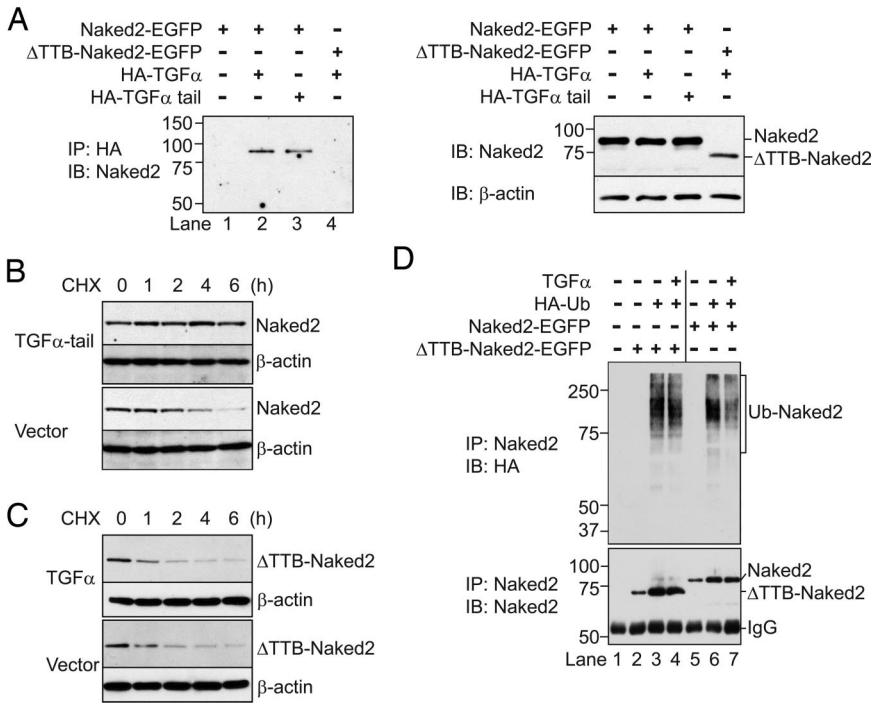

Fig. 3. A physical interaction between Naked2 and TGFα is required to stabilize Naked2 protein. (*A*) Validation of binding activities of HA-TGFα tail and ΔTTB-Naked2-EGFP. (*Left*) HEK293 cells were transiently cotransfected with Naked2-EGFP, ΔTTB-Naked2-EGFP, HA-tagged TGFα, and a TGFα cytoplasmic tail construct (HA-TGFα tail), as indicated. Cell lysates were prepared 48 h after transfection; HA-tagged TGFα was immunoprecipitated (IP) by using HA antibody and then blotted (IB) for Naked2. The cytoplasmic tail of TGFα was sufficient to immunoprecipitate Naked2, but deletion of the TTB domain within Naked2 abolished the interaction between Naked2 and TGFα. (*Right*) WCL were immunoblotted for Naked2 and β-actin to show equivalent expression and loading. (*B*) TGFα tail is sufficient to retard Naked2 degradation. MDCK cells stably expressing Naked2-EGFP and HA-tagged TGFα cytoplasmic tail (or pCB7 vector control) were exposed to CHX for the times indicated, and the stability of Naked2 protein was monitored by Western blotting. Expression of the TGFα cytoplasmic tail was sufficient to stabilize Naked2 protein. (*C*) TTB domain of Naked2 is required for Naked2 stabilization. An experiment similar to *B* was performed in MDCK cells stably expressing TGFα (or pCB7 vector control) and ΔTTB-Naked2-EGFP. TGFα expression did not prevent degradation of this mutant Naked2 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 cDNA. Cell lysates were prepared 48 h later and subjected to Naked2 immunoprecipitation followed by HA and Naked2 Western blotting. In contrast to full-length Naked2, ubiquitylation of the ΔTTB-Naked2 mutant was not inhibited by TGFα expression.

We also observed polyubiquitylation of endogenous Naked2 in parental MDCK cells (Fig. 1*D*).

TGFα Up-Regulation of Naked2 Is EGFR-Independent. Because Naked2 acts as a CaRT protein for the proper delivery of TGFα-containing exocytic vesicles to the basolateral surface of polarized epithelial cells (10, 11), we considered whether the cargo (TGFα) might regulate its CaRT (Naked2). We found a marked increase in endogenous Naked2 protein levels in MDCK cells stably overexpressing TGFα (Fig. 2*A Upper*, lanes 2 and 3). An even more dramatic up-regulation of Naked2 protein was observed when TGFα was stably overexpressed in Caco-2 cells, a human colorectal cancer cell line (Fig. 2*A Lower*, lanes 2 and 4). Because TGFα is a major EGFR ligand, we presumed that this effect was caused by EGFR activation. However, levels of Naked2 in TGFα-expressing MDCK cells were not altered by either an irreversible EGFR tyrosine kinase inhibitor (EKI-785) or a selective TACE inhibitor (WAY-022) (Fig. 2*A*). Nor did administration of 1 nM recombinant human (rh) TGFα 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*A* and data not shown). To determine whether Naked2 is up-regulated 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 with vector control cells with or without the EGFR tyrosine kinase inhibitor EKI-785 (Fig. 2*B*). Taken together, these findings indicate that Naked2 is up-regulated by TGFα in an EGFR-independent manner, and this effect occurs at a posttranscriptional level.

Overexpression of TGFα Stabilizes Naked2 by Inhibiting Its Ubiquitylation. We next asked whether overexpressing TGFα would up-regulate Naked2 protein by retarding its proteasomal degradation. CHX was added to MDCK cells stably expressing Naked2-EGFP with or without coexpressed TGFα. We found that coexpression of TGFα significantly delayed Naked2 turnover compared with vector control cells (Fig. 2*C*). We also observed stabilization of endogenous Naked2 in TGFα-expressing Caco-2 cells (Fig. 2*C*). Consistent with these findings, the intensity of total ubiquitylated Naked2 was significantly reduced by TGFα in an *in vivo* ubiquitylation assay in transiently cotransfected HEK293 cells (Fig. 2*D Left*, lanes 3 and 4). Collectively, these results with both transfected and endogenous Naked2 support our contention that TGFα up-regulates Naked2 by inhibiting its polyubiquitylation 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 (11) and that up-regulation of Naked2 by TGFα persists despite pharmacological blockade of TGFα cell surface cleavage and EGFR tyrosine kinase activity (Fig. 2), we predicted that (*i*) the TGFα tail alone would be sufficient to stabilize Naked2 and (*ii*) internal deletion of the TTB domain within Naked2 would abolish this effect (Fig. 3).

To test the first prediction (Fig. 3*C*), 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; an HA tag was inserted between the signal peptide and transmembrane sequences for immunoprecipitation studies. Coimmunoprecipitation experiments in transiently transfected HEK293 cells showed that Naked2-EGFP was pulled down as efficiently by the HA-tagged TGFα tail (Fig. 3*A*, lane 2) as by full-length TGFα (lane 3). As we predicted, the TGFα tail alone delayed the degradation of Naked2 when stably coexpressed in MDCK cells (Fig. 3*B*).

To examine the second prediction (Fig. 3*D*), an internal deletion of the TTB motif (ΔTTB-Naked2-EGFP) was generated (see *Materials and Methods*). As expected, the ΔTTB-Naked2-EGFP mutant no longer interacted with HA-tagged TGFα (Fig. 3*A*, lane 4). Stably overexpressing full-length TGFα did not protect the ΔTTB-Naked2-EGFP mutant from rapid degradation in MDCK

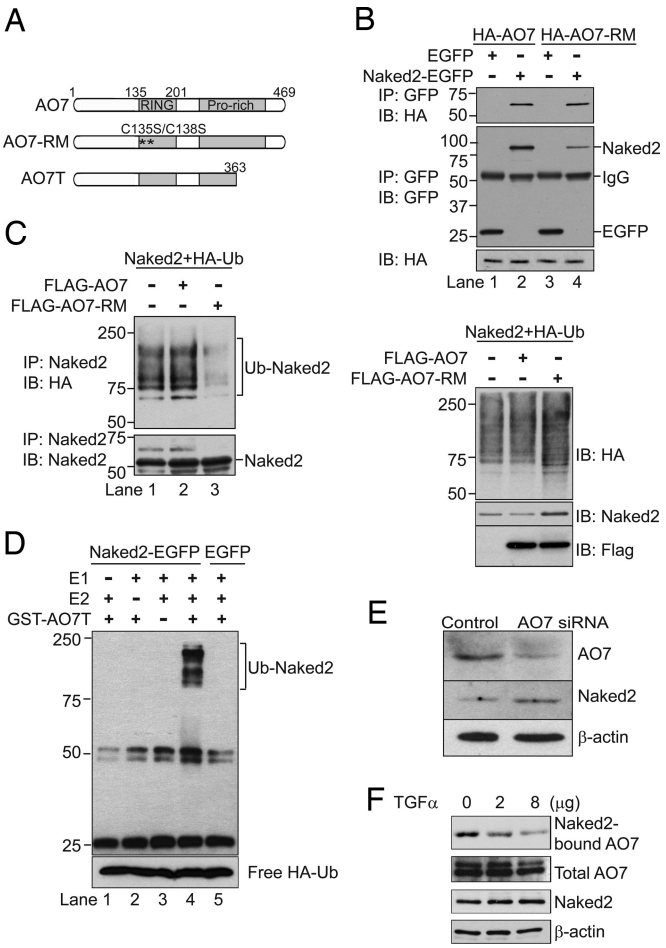

Fig. 4. Identification of a ubiquitin ligase for Naked2. (*A*) Schematic illustration of AO7 and its mutants. AO7 contains a RING finger motif and a proline (Pro)-rich region. AO7-RM contains two point mutations at Cys-135 and Cys-138 and lacks ubiquitin ligase activity. AO7T is a C-terminal truncation mutant that retains E3 activity and is soluble when expressed in *E. coli*. (*B*) Interaction of Naked2 with wild-type AO7 and its RING mutant. Naked2-EGFP and HA-tagged AO7/AO7-RM were coexpressed in HEK293 cells followed by coimmunoprecipitation (IP) with a GFP antibody. Both forms of AO7 were coimmunoprecipitated by using GFP antibody in the presence of Naked2-EGFP but not EGFP alone. AO7 Western blotting (IB) demonstrated levels of AO7/AO7-RM in the cell lysates. (*C*) Attenuation of Naked2 ubiquitylation by AO7-RM. The expression of FLAG-AO7 slightly enhanced Naked2 ubiquitylation. In contrast, expression of FLAG-AO7-RM dramatically reduced ubiquitylation of Naked2. (*D*) *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. Ubiquitylated Naked2 was detected by HA Western blotting only when E1, E2, and GST-AO7T were added together. (*E*) AO7 siRNA in MDCK cells. Transient transfection of AO7 siRNA oligonucleotides reduced AO7 protein and resulted in increased levels of Naked2 protein by Western blotting. (*F*) TGFα transfection dose-dependently displaced Naked2-bound AO7. Increasing concentrations of TGFα expression plasmid were transfected into HEK293 cells that express HA-AO7 and Naked2. Coimmunoprecipitation by using VU308 antibody followed by HA Western blotting showed the levels of Naked2 bound to AO7.

cells (Fig. 3*C*). As we predicted, ubiquitylation of ΔTTB-Naked2-EGFP was not reduced when TGFα was coexpressed (Fig. 3*D*, lane 4) compared with the markedly attenuated ubiquitylation of wild-type Naked2 upon TGFα coexpression (Figs. 2*D* and 3*D*). 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 degradation of Naked2.

Identification of AO7 as a Ubiquitin Ligase for Naked2 Ubiquitylation. 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 with the N-terminal half of human AO7 (data not shown). AO7, also known as RING finger protein 25 (RNF25), exhibits RING finger-dependent ubiquitin ligase activity (15) (Fig. 4*A*). We confirmed this interaction by coimmunoprecipitation in HEK293 cells. HA-tagged AO7 was pulled down by GFP antibody when EGFP-tagged Naked2, but not EGFP alone, was coexpressed (Fig. 4*B*, lanes 1 and 2). Two-point mutations at Cys-135 and Cys-138 in the conserved RING finger domain of AO7 abolished the E3 activity of AO7 (Fig. 4*A*) (15), but this AO7 RING mutant (AO7-RM) retained the ability to interact with Naked2 (Fig. 4*B*, lane 4). Therefore, we used FLAG-tagged AO7-RM as a dominant-negative to examine whether AO7 participates in Naked2 ubiquitylation. Overexpression of AO7-RM dramatically reduced the levels of ubiquitylated Naked2 in an *in vivo* ubiquitylation assay in HEK293 cells (Fig. 4*C*, lane 3). We also conducted an *in vitro* ubiquitylation assay, which used 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 (15). Because of insolubility of full-length Naked2 when expressed in *Escherichia coli* (19), Naked2-EGFP fusion protein was expressed in HEK293 cells and immunoprecipitated by using anti-GFP to serve as ubiquitylation substrate. Western blotting with HA antibody revealed ubiquitylation of Naked2-EGFP when E1, E2, and GST-AO7T were all present in the reaction (Fig. 4*D*, 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 copurified from HEK293 cells. The specificity of ubiquitylation is demonstrated by the lack of ubiquitylation of EGFP that was not fused to Naked2. In addition, AO7 siRNA reduced AO7 protein in MDCK cells, and this resulted in increased levels of Naked2 protein (Fig. 4*E*). Thus, we conclude 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 were transfected with Naked2 and HA-AO7 and then split into 3 wells, followed by a second transfection with different concentrations of a TGFα expression plasmid. Naked2-bound AO7 was coimmunoprecipitated by using Naked2 antibody and examined by HA Western blotting (Fig. 4*F*). 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 (Fig. 5).

Discussion

We determined that Naked2 acts as a multifunctional protein to ensure the efficient delivery of TGFα to the basolateral surface of polarized epithelial cells. Based on the multiple functions carried out by Naked2, we have designated it a CaRT protein for the delivery of TGFα to the basolateral surface of polarized epithelial cells (10). 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 (20). It is generally thought that adaptors regulate recruitment and stability of cargo but not vice versa. However, we have found that Naked2 is polyubiquitylated and undergoes rapid proteasomal degradation (Figs. 1 and 2). Moreover, its stability appears to be regulated by its cargo TGFα (Fig. 2). This is similar to the recent report of Hirst *et al*. (21) showing that the degradation of a relatively short-lived

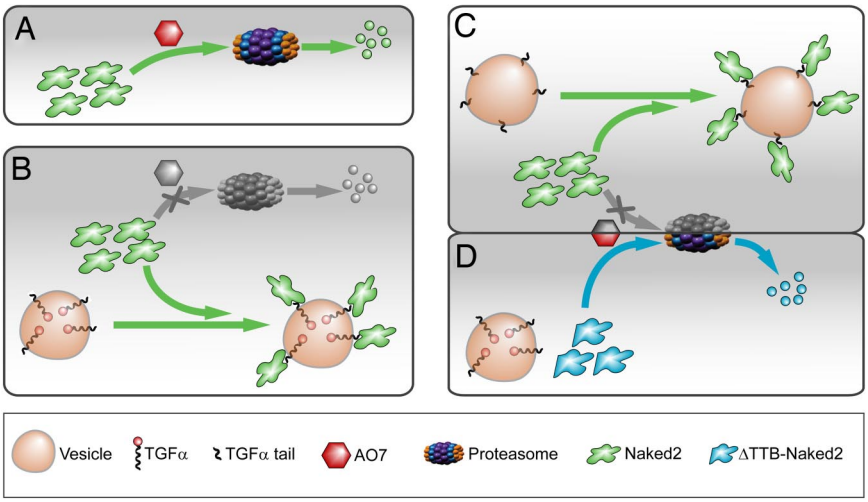

Fig. 5. Model for TGFα-induced Naked2 stabilization. (*A*) In the absence of TGFα, Naked2 is ubiquitylated by AO7 and undergoes rapid proteasomal degradation. (*B*) Naked2 binds to the cytoplasmic tail of TGFα on the surface of TGFα-containing vesicles and/or at the plasma membrane. In the presence of 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.

clathrin-dependent adaptor, Golgi-localized, γ-ear-containing, ADP ribosylation factor (ARF)-binding protein 2 (GGA2), was delayed when one of its cargos, cation-independent mannose 6-phosphate receptor (CIMPR), was overexpressed in HeLa cells. 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. In this work, we not only identify a similar phenomenon in the context of Naked2-coated TGFα vesicles, but we also elucidate the biochemical basis for this phenomenon.

Given the multiple tasks orchestrated by Naked2 (cargo selection, basolateral targeting, and vesicle fusion), it is perhaps not surprising that it is a highly regulated protein. We show that the up-regulation of Naked2 by TGFα is posttranscriptional (Fig. 2 *A* and *B*). Naked2 is a short-lived protein with a half-life of 60 min caused by ubiquitin-mediated proteasomal degradation (Fig. 1). We have identified AO7 as a ubiquitin ligase for Naked2. AO7 mediates RING finger-dependent autoubiquitylation *in vitro* (15). A subsequent study implicated AO7 in NF-κB-mediated transcriptional activity (22) 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. We show that AO7 interacts with and ubiquitylates Naked2. Thus, we have identified a heterologous substrate for AO7 and an E3 ligase for Naked2. However, we cannot exclude that additional E3s may participate in degradation of Naked2.

We demonstrated that TGFα binds to Naked2 residues 300–385 (11). Because 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. 4*F*). This result favors the first possibility but does not exclude the second possibility. Thus, studies are under way to map the Naked2–AO7 interaction domains and to identify the ubiquitylation site(s) within Naked2.

The discrepancy between the marked efficiency of TGFα in Naked2 stabilization and only partial inhibition of Naked2 ubiquitylation and AO7 binding by TGFα raises the possibility that additional mechanisms may be involved in TGFα-mediated Naked2 up-regulation. 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 that lack protein degradative 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 needed 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 coworkers (23). 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 (10, 11). 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 hypomagnesemia is caused by 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 (2). The 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 (11), another basolaterally targeted EGFR ligand that is also cleaved by cell surface TACE/ADAM17. Thus, TGFα appears to use a unique Naked2-mediated trafficking machinery; it ensures its basolateral cell surface delivery by stabilizing its CaRT Naked2. Taken together, these studies highlight the complexity of basolateral trafficking and underscore the need for considering ligand trafficking and delivery in a systems biology approach to EGFR signaling.

Materials and Methods

Plasmid Construction, Antibodies, and Chemicals. Generation of full-length human Naked2 and TGFα expression plasmids has been described in ref. 10. Truncated Naked2 sequences were obtained by PCR amplification from full-length human Naked2 cDNA. All 5′ primers contained EcoRI restriction sites, and all 3′ primers contained BamHI sites. The PCR products were cloned into pEGFP-N2 vector (Clontech) 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-PCRs 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 (18), was a generous gift from Michael Freeman (Vanderbilt University, Nashville, TN). Murine AO7, AO7-RM ,and GST-AO7T expression plasmids have been described in

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ref. 15. FLAG-AO7 and FLAG-AO7-RM mammalian expression plasmids were generated by PCR amplification from mouse HA-AO7 and HA-AO7-RM plasmids by using a 5′ primer encoding an EcoRI site and a FLAG epitope tag plus a 3′ primer encoding a NotI site. The PCR products were cloned into pcDNA3.1(+) (Invitrogen) between the EcoRI and NotI sites. We combined two AO7 siRNA oligonucleotides: no. 1 sense (GGACCAGGAUUCACAGUAUUU) and antisense (5′-AUACUGUGAAUCCUGGUCCUU); and no. 4 sense (GAGCGAAACCGAUACUUCAUU) and antisense (5′-UGAAGUAUCGGUUUCGCUCUU).

The monoclonal HA and β-actin antibodies were obtained from Sigma. 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. Horseradish peroxidase-donkey anti-mouse and anti-rabbit IgG were obtained from Jackson ImmunoResearch. Protein G–agarose beads were purchased from Invitrogen. rhTGFα was purchased from R&D Systems. MG-132 and CHX were purchased from Calbiochem. The selective TACE/ADAM-17 inhibitor WAY-022 was provided by Jay Gibbons (Wyeth Ayerst Laboratories). 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 Lines. Parental HEK293 cells, MDCK cells, Caco-2 cells, and all transiently or stably transfected derivatives were grown in DMEM supplemented with 10% FBS, glutamine, nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin (HyClone) with or without 500 μg/ml geneticin and/or 200 μg/ml hygromycin (Roche).

Transient transfections were performed in HEK293 cells by using FuGENE 6 (Roche Applied Science) transfection reagent according to the manufacturer's instructions. Twenty-four hours after transfections, cells were harvested or processed for further experiments. Stable transfections were performed by using Lipofectamine 2000 (Invitrogen). Transfected cells were split and diluted at least 10-fold into selection medium 24 h after transfections. A dual-tagged rat TGFα cDNA (a generous gift from David Lee at University of North Carolina, Chapel Hill) was stably transfected into Caco-2 cells.

RT-PCR. Total RNA was isolated from Caco-2 cells by using the RNeasy mini kit (Qiagen). RT-PCR was performed to determine the relative gene expression by using the SuperScript one-step RT-PCR kit (Invitrogen). 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 the same reaction tubes to serve as an internal control for an equivalent starting amount of RNA.

Immunoprecipitation and Western Blotting. For immunoprecipitations, cells were lysed in 1× lysis buffer (25 mM Tris·HCl, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM DTT). Supernatants were incubated with HA antibody or Naked2 antibody VU308 and then with protein G beads. Beads were washed and then boiled in sample buffer for 5 min, and proteins were resolved in 8% SDS/PAGE for ubiquitylation analysis or 10% SDS/PAGE otherwise. Western blotting of whole cell lysates was performed as described in ref. 11.

Expression and Purification of GST-AO7T. A GST fusion of a C-terminal truncation of AO7 at amino acid 363 (GST-AO7T) was used in the *in vitro* ubiquitylation assay because the full-length GST fusion is poorly expressed (15). GST-AO7T plasmid was transformed into BL-21 strain and grown overnight at 37°C. The expression of GST-AO7T was induced by adding 0.2 mM IPTG for 18 h at 30°C. Cells then were harvested and lysed as described in ref. 19. The clear supernatant of bacterial lysate was used to extract GST-AO7T protein with a B-PER GST fusion protein purification kit (Pierce). Finally, a 12-ml volume was eluted from the glutathione column, and 4 ml of GST-AO7T-enriched fractions was dialyzed three times against 4 liters of 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 described in ref. 15, unless indicated. One hundred nanograms of rhE1 and UbcH5b (E2), 1 μg of HA-ubiquitin, and 400 ng of purified GST-AO7T were used in each 100-μl reaction. Naked2-EGFP expressed in HEK293 cells was immunoprecipitated by using GFP antibody and protein G–agarose beads followed by five successive washes in RIPA buffer. Finally, beads were resuspended in 125 μl of 50 mM Tris (pH 7.4). Twenty microliters of agarose-bound Naked2-EGFP was 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.

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