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TGF- β receptor I/II trafficking and signaling at primary cilia are inhibited by ceramide to attenuate cell migration and tumor metastasis

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Abstract

Signaling by the transforming growth factor- β (TGF- β) receptors I and II (T β RI/II) and the primary cilia-localized sonic hedgehog (Shh) pathway promote cell migration and, consequently, tumor metastasis. In contrast, the sphingolipid ceramide inhibits cell proliferation and tumor metastasis. We investigated whether ceramide metabolism inhibited T β RI/II trafficking to primary cilia to attenuate cross-talk between T β RI/II and the Shh pathway. We found that ceramide synthase 4 (CerS4)-generated ceramide stabilized the association between T β RI and the inhibitory factor Smad7, which limited the trafficking of T β RI/II to primary cilia. Expression of a mutant T β RI that signals but does not interact with Smad7 prevented the CerS4-mediated inhibition of migration in various cancer cells. Genetic deletion or knockdown of CerS4 prevented the formation of the Smad7-T β RI inhibitory complex and increased the association between T β RI and the transporter Arl6 through a previously unknown cilia-targeting signal (Ala³¹Thr³²Ala³³Leu³⁴Gln³⁵) in T β RI. Mutating the cilia-targeting signal abolished the trafficking of T β RI to the primary cilia. Localization of T β RI to primary cilia activated a key mediator of Shh signaling, Smoothened (Smo), which stimulated cellular migration and invasion. T β RI-Smo cross-talk at the cilia in CerS4-deficient 4T1 mammary cancer cells induced liver metastasis from orthotopic allografts in both wild-type and CerS4-deficient mice, which was prevented by overexpression of Smad7 or knockdown of intraflagellar transport protein 88 (IFT88). Overall, these data reveal a ceramide-dependent mechanism that suppresses cell migration and invasion by restricting T β RI/II-Shh signaling selectively at the plasma membrane of the primary cilium.

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SUPPLEMENTARY MATERIALS

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INTRODUCTION

Transforming growth factor- β (TGF- β) signaling is involved in the regulation of various cellular signaling processes, including apoptosis, cell proliferation, differentiation, and migration (1–4). TGF- β signaling is activated by the binding of the ligand to its specific serine-threonine kinase TGF- β type I and type II receptors (T β RI/II) on the plasma membrane (PM) (1–4). The ligand binding initiates the formation of the T β RI/II heteromeric complex, in which T β RII phosphorylates and activates T β RI (1–4). Activation of the T β RI leads to the recruitment and formation of Smad protein complexes, which are translocated to the nucleus for the regulation of target genes (5–8). Inhibitory Smad7 negatively regulates TGF- β signaling by binding T β RI, leading to the recruitment of Smurf2, an E3 ubiquitin ligase that labels the T β RI-Smad7 complex for degradation (9–13).

The primary cilium is an organelle with a distinct membrane composition of lipids and proteins, which controls various signaling functions, such as enhanced cell-to-cell communication, autophagy, and/or cell migration (14–16). Intraflagellar transport (IFT) is a cargo-trafficking pathway, involved in cilium genesis, which maintains the microtubule axoneme (16–18). IFT machinery along with several proteins encoded by genes mutated in Bardet-Biedl syndrome (BBS) provides specificity for ciliary cargo transport (16–18). This includes targeting several receptors, including G protein-coupled receptors, to cilia via binding of BBS, such as BBS3 (Bardet-Biedl syndrome 3 protein) [Arl6 (adenosine diphosphate-ribosylation factor-like protein 6)], to their cilia transport signal (CTS) comprising AX(S/A)XQ sequence (X is any amino acid) (17, 18). Sonic hedgehog (Shh) signaling is localized to primary cilia with a complex inhibitory (Patched) and activating [Smoothered (Smo)] pathways (19–21), leading to increased cell migration and metastasis. T β RI/II signaling has been observed at the base of primary cilia (22), and ciliary TGF- β signaling is linked to enhanced cell migration (23, 24).

Ceramide, a bioactive signaling sphingolipid, is involved in the regulation of stress-related antiproliferative responses in cancer cells, such as apoptosis, mitophagy, and/or necroptosis (25). Endogenous ceramides are synthesized de novo by six distinct ceramide synthases, CerS1 to CerS6 (26–29), which are specialized for the synthesis of ceramides with different fatty acyl chain lengths. For example, CerS5/CerS6 induces medium-chain C₁₂- to C₁₆-ceramides, CerS1/CerS4 induces long-chain C₁₈- to C₂₀-ceramides, and CerS2 induces very-long-chain C₂₂- to C₂₄-ceramides (26–29). CerS3, which is expressed selectively in testes and skin tissues, generates ultralong-chain ceramides (30, 31). Ceramides with different fatty acyl chain lengths play distinct physiological roles in various biological processes, including providing skin barrier, liver homeostasis, insulin resistance, induction of apoptosis, and regulation of cancer pathogenesis (32–39). However, the roles of ceramides generated by CerS enzymes in the regulation of cancer cell migration and/or metastasis through regulation of T β RI/II trafficking and/or signaling have not been described previously. Here, we investigated the mechanistic cross-talk between ceramide and T β RI/II signaling to control cell migration, invasion, and/or metastasis. Our data revealed that CerS4-generated long-chain ceramides play key roles in targeting T β RI/II selectively to the primary cilia to limit Shh/Smo-mediated cell migration and tumor metastasis.

RESULTS

CerS4/ceramide metabolism plays a key role in the regulation of cancer cell migration and invasion

To define the clinical significance of de novo ceramide synthesis in tumor metastasis, we performed unbiased screens to examine mRNAs encoding CerS1 to CerS6 using published microarray data sets (40–42), obtained from tumor tissues isolated from patients with local (early stage) versus metastatic (late stage) head and neck squamous cell carcinoma (HNSCC), renal cell carcinoma, and melanoma. The abundance of the mRNA encoding CerS4 but not CerS1, CerS2, or CerS6 was significantly decreased in metastatic HNSCCs ($n = 27$, $P < 0.05$), melanomas ($n = 83$, $P < 0.05$), and renal cell carcinomas ($n = 48$, $P < 0.05$) compared to nonmetastatic primary tumors (fig. S1A). No information was available for CerS3 or CerS5 in these microarrays. These data suggest that decreased CerS4 might be associated with metastasis in patients with various advanced solid tumors.

To examine whether down-regulation of CerS4 plays any role in the regulation of cancer cell migration or invasion in general with a common mechanism (regardless of the initial tumor site), we determined the effects of small interfering RNA (siRNA)-mediated knockdown of CerS4 compared with that of CerS1 on these processes in various cancer cell lines. Knockdown of CerS4 (~75% decreased mRNA and protein abundance; fig. S1B, middle and right) but not of CerS1 (50% decreased mRNA abundance; fig. S1B, left) enhanced the invasion of A549 human lung cancer cells in collagen-coated Boyden chambers by ~5-fold (Fig. 1A) compared to scramble (Scr) siRNA-transfected controls without affecting cell proliferation (fig. S1C). Similarly, siRNA-mediated knockdown of CerS4 increased the migration of A549 cells ~7-fold compared to controls, measured using the single-cell fluorescence track assay (Fig. 1B). Stable down-regulation of CerS4 using two distinct short hairpin RNAs (shRNAs) (#1 and #2) resulted in ~90% reduction in CerS4 mRNA compared to controls (fig. S1D) and increased the invasion and single-cell migration (~5-fold) of UM-SCC-22A human HNSCC cells compared to controls (Fig. 1, C and D). Thus, these data suggest that CerS4 knockdown increases invasion and/or migration capacity in various human cancer cell lines.

CerS4 knockdown results in T β RI/II activation on the PM to induce migration and invasion

Because TGF- β signaling is a master regulator of cancer cell invasion/migration (43–45), and genetic loss of CerS4 resulted in alopecia in CerS4^{-/-} mice (46, 47), which is a similar phenotype observed in response to enhanced epidermal T β RI signaling in mice (48), we hypothesized that alterations of CerS4 might induce cancer cell migration as a result of increased T β RI/II signaling. To test this hypothesis, we measured the effects of CerS4 down-regulation on the global abundance versus PM localization of T β RI/II in A549 cells by Western blotting and flow cytometry, respectively. Using two sets of distinct antibodies that recognize these receptors in the presence or absence of TGF- β , CerS4 knockdown using shRNA (fig. S2A) had no effect on total receptor abundance relative to controls (fig. S2B), whereas it increased the amount of T β RI/II (~2.0-fold) and decreased the amount of Smad7 (~60%) that was localized at the PM (Fig. 2A, left and right, and fig. S2C). As an additional control, CerS4 knockdown had no detectable effect on the cell surface abundance of tumor

necrosis factor- α receptor I or sphingosine 1-phosphate receptor 1 compared to Scr shRNA-transfected control cells (fig. S2C). Inhibition of T β RI/II signaling using SB431542 completely prevented A549 cell migration in response to CerS4 knockdown compared to controls (Fig. 2B). In addition, ectopic expression of a dominant-negative (D/N) mutant of T β RII (K277A) (1) prevented CerS4 knockdown-mediated cell migration and invasion ~70% compared to controls in the absence or presence of exogenous TGF- β exposure (Fig. 2C and fig. S2D). Protein abundance of wild-type-T β RII and K277A-T β RII was confirmed by Western blotting compared to vector-transfected controls (Fig. 2C, bottom). Increased cell surface localization of T β RI/II in response to CerS4 knockdown was also detected in biotin-labeled A549 cells (Fig. 2D). In biotin-labeling assays (49), the extracellular portion of receptors is labeled by exogenous biotin, and labeled receptors are then detected by immunoprecipitation followed by Western blotting. As a positive control, the abundance of Na⁺- and K⁺-dependent adenosine triphosphatase (Na⁺/K⁺-ATPase) on cell surface was not affected in response to CerS4 knockdown compared to Scr shRNA-transfected controls (Fig. 2D, bottom). Biotin labeling also revealed that ectopic expression of FLAG-Smad7, an inhibitor of T β RI/II signaling, prevented both T β RI/II localization at the PM (Fig. 2E) and decreased cell migration and invasion by ~75% (Fig. 2F and fig. S2, E and F) in response to siRNA-mediated CerS4 knockdown. Notably, CerS4 knockdown had no significant effect on endogenous Smad7 protein abundance (Fig. 2E, bottom) or canonical TGF- β signaling compared to Scr shRNA-transfected A549 controls, measured by Smad3-dependent luciferase reporter assay (Fig. 2G), and phosphorylation of Smad3 using Western blotting with/without TGF- β exposure (Fig. 2H). Moreover, CerS4 knockdown had no additional effect on TGF- β -mediated phosphorylation of Smad2 compared to controls (Fig. 2I). Together, these data suggest that knockdown of CerS4 mediates the localization (potentially via trafficking) of T β RI/II to the PM, inducing T β RI/II-dependent cell migration and invasion without substantially affecting the phosphorylation of Smad2/3. This process is prevented by inhibition of receptor signaling through ectopic expression of D/N-T β RII or Smad7.

Genetic loss of CerS4 induces migration or invasion by T β RI/II activation in skin keratinocytes and mouse embryonic fibroblasts

To investigate the physiological roles of CerS4 in the regulation of cell migration or invasion, we have generated a CerS4^{-/-} knockout mouse model (fig. S3, A and B), which decreased the generation of long-chain C₁₈- and C₂₀-ceramides in all tissues and the serum (fig. S3C). The genetic loss of CerS4 resulted in irreversible alopecia, which was concomitant with alterations in sebaceous glands and sebum contents in adult CerS4^{-/-} compared to wild-type CerS4^{+/+} mice (fig. S3D), as previously reported (46, 47).

We isolated skin keratinocytes and mouse embryonic fibroblasts (MEFs) from age-matched CerS4^{+/+} and CerS4^{-/-} mice and examined the abundance of T β RII at the PM and cell migration or invasion after immortalization by ectopic expression of the SV40 large T antigen. The loss of CerS4 protein was confirmed by Western blotting in CerS4^{-/-} compared to CerS4^{+/+} keratinocytes and MEFs (fig. S3E, top and bottom). Abundance of T β RII at the PM was elevated ~3-fold in CerS4^{-/-} compared to CerS4^{+/+} MEFs and keratinocytes (Fig. 3A). In addition, single-cell migration and Boyden chamber invasion assays showed that

migration or invasion of CerS4^{-/-} skin keratinocytes was increased ~6-fold compared to wild-type keratinocytes, and ectopic expression of inhibitory Smad7 prevented the effects of CerS4 loss on migration or invasion (Fig. 3, B and C). In addition, reconstitution of wild-type CerS4 protein attenuated (~70%) migration or invasion of CerS4^{-/-} keratinocytes compared to vector-transfected controls in Boyden chambers and single-cell migration assays (Fig. 3, D and E). Together, these data suggest that genetic loss of CerS4 increases TβRI/II PM abundance/localization, leading to increased migration or invasion of skin keratinocytes and MEFs, which is prevented by ectopic expression of inhibitory Smad7 or reconstitution of wild-type CerS4 protein.

Restoring CerS4 and subsequent ceramide generation inhibit cancer cell migration or invasion

We then measured the effects of expressing wild-type CerS4 (with the CerS4^{WT}-V5 vector) versus catalytically inactive CerS4 (with the “mutant-CerS4-V5” vector expressing CerS4^{H212A/H213A}) on the invasive behaviour of A549 cells that were stably transfected with CerS4-targeted or control (Scr) shRNA. Knockdown of CerS4 increased cell migration or invasion and reconstitution of wild-type but not catalytically inactive CerS4 restored the generation of C₁₈- and C₂₀-ceramides (measured by lipidomics; fig. S4, A and B) and substantially inhibited cell migration in CerS4-depleted cells (Fig. 3F). We also examined the effects of ectopic co-transfection of TβRI/II on A549 cell migration or invasion in response to tetracycline (+tet)-induced CerS4 (CerS4^{WT}-V5) expression, confirmed by Western blotting or lipidomics for ceramide measurements after 24-, 48-, or 72-hour tet induction (Fig. 3G and fig. S4, C and D). Cotransfection of TβRI/II increased A549 cell migration or invasion by ~2-fold compared to vector-transfected control cells, and tet-induced CerS4^{WT}-V5 expression attenuated this effect (~75% at 72 hours) compared to controls (Fig. 3H). Overall, these data suggest that restoring CerS4 abundance and, consequently, long-chain C₁₈- and C₂₀-ceramide generation inhibit TβRI/II-mediated A549 cell migration and invasion.

TβRI/II localization at the PM is regulated by a ceramide-Smad7 inhibitory complex

We then determined the mechanisms by which CerS4/ceramide controls TβRI/II signaling within the PM. Interaction between ceramide (green in Fig. 4A), TβRI (cyan), and Smad7 (red) was detectable in ScrshRNA-transfected 4T1-luciferase (4T1-Luc) murine mammary carcinoma cells (white in the merged images), whereas their interaction was attenuated in response to CerS4 knockdown (Fig. 4A, right and left). Decreased abundance of long-chain C₁₈- and C₂₀-ceramides after CerS4 knockdown compared to Scr shRNA-transfected control 4T1-Luc cells was detected using lipidomics (Fig. 4B and fig. S5, A and C). Inhibitory effects of shRNA-mediated knockdown of CerS4 on the ceramide-Smad7 interaction were also detected in UM-SCC-22A and A549 cells (~85%) using proximity ligation assay (PLA) (50), using antibodies that recognize ceramide and Smad7 (Fig. 4C, left and right). CerS4 knockdown and decreased generation of long-chain ceramides in these cells were confirmed by quantitative polymerase chain reaction (qPCR) and lipidomics (fig. S5, A and C). We then assessed the effects of expressing wild-type (CerS4^{WT}-FLAG vector) or catalytically inactive (mutant-CerS4-FLAG vector; CerS4^{H212A/H213A} as in the V5 vector above) CerS4 on the association between TβRI and inhibitory protein Smad7, which were

measured in A549 cells that were stably transfected with CerS4-shRNA versus Scr shRNA by immunoprecipitation and Western blotting. CerS4 knockdown reduced Smad7 protein abundance, without affecting its mRNA abundance (fig. S5, A and B), decreasing the association (~40%) between T β RI and Smad7 compared to Scr/vector-transfected control cells (Fig. 4D, lanes 2 and 8). Reconstitution of CerS4^{WT}-FLAG, but not the inactive mutant-CerS4-FLAG, increased T β RI-Smad7 association (~2.3-fold) compared to that in Scr/vector-transfected controls (Fig. 4D, lanes 6, 4, and 2). CerS4^{WT}-FLAG, but not mutant-CerS4-FLAG, restored T β RI-Smad7 association in response to CerS4 knockdown (Fig. 4D, lanes 12, 10, and 8). CerS4 knockdown or ectopic expression of CerS4 had no detectable effect on the association of Smad7 and Smurf2 (Fig. 4D, bottom). Moreover, inhibition of T β RI-Smad7 interaction was also observed in CerS4^{-/-} MEFs compared to CerS4^{+/+} cells by immunoprecipitation/Western blotting (~40%) and PLA (~80%) (Fig. 4, E and F). Reconstitution of CerS4^{WT}-FLAG restored T β RI-Smad7 association in CerS4^{-/-} MEFs (Fig. 4E) compared to CerS4^{+/+}/vector-transfected controls. Genetic loss of CerS4 had no visible effects on the localization of T β RI to the caveolin-containing membrane microdomains (51) or clathrin-containing late endosomes (3) in CerS4^{-/-} compared to CerS4^{+/+} skin keratinocytes (fig. S5D). Moreover, CerS4 knockdown had no detectable effect on the colocalization of caveolin or clathrin with acetylated tubulin (Ac-tubulin) compared to Scr shRNA-transfected controls (fig. S5D). Overall, these data suggest that reconstitution of CerS4 induces, whereas knockdown or genetic loss of CerS4 inhibits, the association between T β RI and inhibitory Smad7, which regulates the localization/trafficking of the T β RI/II at the PM without affecting general receptor protein abundance.

To examine the effects of Smad7-T β RI interaction on the regulation of cell migration and invasion by CerS4/ceramide signaling, we first identified amino acids of T β RI, which might be involved in Smad7 association using molecular docking and simulation studies. These data suggested that Asn²⁷¹ and Thr²⁰⁸ might be key for T β RI to interact with Smad7 (fig. S5E). Thus, we generated a double mutant of T β RI with N271A and T208A conversion and measured its effects on Smad7 association in response to CerS4 induction in UM-SCC-22A cells by PLA using anti-Smad7 and anti-FLAG antibodies. Ectopic expression of CerS4 by tet induction increased the association between Smad7 and wild-type T β RI, whereas we detected no interaction between Smad7 and T β RI^{N271A/T208A} regardless of CerS4 expression (Fig. 4G). We then measured the effects of T β RI^{N271A/T208A} on cell migration and invasion in Boyden chambers as compared to wild-type T β RI with or without CerS4 expression. Ectopic CerS4 expression (+tet) inhibited wild-type T β RI-mediated invasion compared to controls (-tet); however, T β RI^{N271A/T208A} (the mutant receptor with decreased interaction with Smad7) restored invasion in response to CerS4/ceramide induction compared to controls (Fig. 4H). Protein abundance of wild-type [WT-T β RI-influenza hemagglutinin peptide (HA)] and mutant (T β RI^{N271A/T208A}-HA) T β RI expression was confirmed by Western blotting using anti-HA antibody in the absence or presence of TGF- β (fig. S5F). Moreover, mutant T β RI^{N271A/T208A} had no detectable effect on the phosphorylation of Smad2 or Smad3, regardless of the presence of TGF- β (fig. S5G). Thus, these data suggest that CerS4 and ceramide modulate cell migration and invasion by inducing T β RI-Smad7 association, which appears to involve the Asn²⁷¹/Thr²⁰⁸ residues of T β RI.

T β RI/II activation at the PM mediates Shh/Smo signaling for induction of cell migration and invasion in response to CerS4/ceramide knockdown

To determine the downstream mechanism by which CerS4 and ceramide regulate cell migration and invasion via T β RI/II signaling, we measured the expression of genes that are regulated by TGF- β /T β RI/II signaling using qPCR-based microarrays (52). Several mRNAs were altered in response to CerS4 knockdown compared to controls. For example, the abundance of mRNAs encoding serpine1 and Shh was increased 7.6- and 5.3-fold, whereas that of brain-derived neurotrophic factor and Fos (proto-oncogene, activator protein-1 subunit) was decreased 5.7- and 3.79-fold, respectively, compared to controls (fig. S6A). Among these, we validated only increased Shh (53) by measuring its mRNA or release to the media in response to CerS4 knockdown compared to vector-transfected controls (Fig. 5, A and B). Inhibition of Shh signaling using robotnikinin or shRNA-mediated knockdown of Shh inhibited cell migration or invasion in response to siRNA/shRNA-mediated knockdown of CerS4 compared to controls in A549 cells (Fig. 5, C to E). Activation of Shh signaling was also consistent with increased abundance of mRNA encoding Gli1 (54) (~2-fold) in response to CerS4 knockdown compared to controls (Fig. 5, F and G), which was prevented by cyclopamine, another pharmacologic inhibitor of Shh signaling, consistent with the inhibition of cell migration or invasion (Fig. 5H). Similarly, shRNA-mediated knockdown of Gli1 (confirmed by Western blotting and qPCR; Fig. 5, I and J) substantially prevented cell migration or invasion in response to CerS4 knockdown in 4T1-Luc cells (Fig. 5K). Inhibition of Smad3 (5–8), using a pharmacologic inhibitor (SIS3), had no effect on increased migration in response to CerS4 knockdown in A549 cells compared to controls (fig. S6B). Shh inhibition had no effect on the abundance of phosphorylated Smad3 or cell migration in response to CerS4 knockdown regardless of TGF- β exposure (fig. S6, C and D). Thus, these data suggest that CerS4 knockdown induces cell migration or invasion via mediating “non-canonical” (Smad-independent) T β RI/II-Shh signaling at the PM.

To determine the mechanism of how CerS4 and ceramide regulate the cross-talk between T β RI/II and Shh signaling in cell migration, we examined the effects of CerS4 knockdown on the interaction between T β RI and Smo, the key activator of Shh signaling, by immunoprecipitation and Western blotting. Stable knockdown of CerS4 in A549 cells using shRNA resulted in ~7-fold increase in the association between T β RI and Smo compared to Scr shRNA controls (Fig. 6A). CerS4 transfections and equal pulldown of Smo by immunoprecipitation were confirmed by Western blotting (Fig. 6A). Actin was measured by Western blotting as loading controls in total cell lysates (Fig. 6A). Equal immunoprecipitation of Smo or T β RI was confirmed by Western blotting (Fig. 6A). Increased association between T β RI and Smo (~8-fold) and between T β RII and Smo (~7-fold) in response to shRNA-mediated CerS4 knockdown was also determined by PLA in 4T1-Luc cells (Fig. 6B). Immunoglobulin G (IgG) was used as a negative control in PLA (Fig. 6B). Inhibition of T β RI/II signaling by expression of wild-type Smad7 prevented the association between T β RI and Smo (as detected by immunoprecipitation and Western blotting) in response to CerS4 knockdown in 4T1 and A549 cells (Fig. 6, C and D). These data suggest that down-regulation of CerS4/ceramide signaling enhances Shh-dependent cell migration by inducing T β RI/II-mediated activation of Smo.

TβRI/II trafficking to primary cilia membrane mediates cell migration through Shh/Smo signalling

Because Shh, specifically Smo, signaling is localized mainly to primary cilia within the PM, we then explored whether TβRI/II and Smo crosstalk and consequent cell migration are regulated by inhibitory signaling by ceramide-Smad7 complexes at the primary cilia membrane. To determine whether CerS4 knockdown plays a role in the regulation of TβRI/II localization to primary cilia, we performed electron microscopy (EM) or immunofluorescence and confocal microscopy using antibodies that recognize TβRI and Ac-tubulin, a marker of primary cilia (55). CerS4 knockdown induced the localization of TβRI to primary cilia membranes, detected by transmission electron microscopy (TEM) using gold-labeled anti-TβRI and Ac-tubulin antibodies in isolated primary cilia from 4T1-Luc-CerS4-shRNA compared to controls (Fig. 6E). There was no detectable Glu-tubulin, a deetyrosinated form of tubulin and a marker of mid bodies (as a negative control), in isolated primary cilia in Scr shRNA- or CerS4-shRNA-transfected cells (Fig. 6E). These data were also confirmed in A549 and UM-SCC-22A (U22A) cells transfected with control versus CerS4-targeted shRNA and in wild-type and CerS4^{-/-} MEFs and keratinocytes by immunofluorescence (Fig. 6F, left and right). There was no detectable association between TβRI and Glu-tubulin or γ-tubulin, a marker of basal bodies and TβRI (fig. S7, A to C), whereas TβRI was highly colocalized with Ac-tubulin (fig. S7D). There was no detectable association between γ-tubulin and Ac-tubulin in these cells (fig. S7E). CerS4 knockdown did not affect number of primary cilia in 4T1 cells compared to controls (fig. S7F). CerS4 knockdown resulted in almost complete loss of ceramide-Ac-tubulin interaction (Fig. 6G, top and bottom) while inducing TβRI-Ac-tubulin (~2.5-fold) and Smo-Ac-tubulin (~3-fold) colocalization, which is also confirmed by enhanced TβRI-Smo-Ac-tubulin colocalization (~80%) (Fig. 6H, top and bottom) when compared to shScr-transfected cells detected by immunofluorescence (Fig. 6H). Immunoprecipitation and Western blotting revealed an enhanced interaction between Smo and TβRI in primary cilia-enriched lysates isolated from CerS4 knockdown 4T1-Luc cells compared to those isolated from control cells (fig. S7, G and H).

To determine whether increased cross-talk between TβRI and Smo at the primary cilia membrane plays any biological roles in enhancing 4T1 cell migration in response to CerS4 knockdown, we measured the effects of cilia inhibition by shRNA-mediated down-regulation of intraflagellar transport protein 88 (IFT88), which is a key mediator of cilia formation (54, 55). IFT88 knockdown (~75% efficiency; fig. S7I, top) almost completely prevented cell migration or invasion in response to stable CerS4 knockdown (fig. S7I, bottom), consistent with the abrogation of TβRI abundance at the cilia membrane in response to IFT88 knockdown compared to control shRNA-transfected cells (fig. S7J). Decreased primary cilia formation after IFT88 knockdown was confirmed by immunofluorescence for Ac-tubulin (fig. S7K). Thus, these data suggest that CerS4 knockdown mediates the cross-talk between TβRI/II and Smo in primary cilia membranes, which enhances the migratory and invasive behaviors of cells.

The CerS4/ceramide-Smad7 axis attenuates T β RI trafficking to the primary cilia membrane via Arl6-dependent transport

To determine the mechanism of how T β RI is transported to primary cilia membrane and how CerS4/C₁₈-ceramide-Smad7 axis regulates this process, we first examined whether the complex composed of Arl6 and BBS4 (the “Arl6/BBSome”) (56–58) is involved in the trafficking of T β RI/II to primary cilia in response to CerS4 knockdown. shRNA-mediated knockdown of Arl6 decreased T β RI protein abundance at the primary cilia membrane (Fig. 7A), and Arl6 or BBS4 knockdown inhibited migration and invasion in cells that are stably transfected with shRNA against endogenous CerS4 compared to those transfected with control shRNA (Fig. 7B). In fact, down-regulation of CerS4 resulted in ~6.5-fold increase in the association between Arl6 and T β RI in isolated cilia in A549 cells (Fig. 7C, top and bottom).

We next assessed whether 31-ATLQ-35 residues present at the N terminus of T β RI, which match the canonical CTS (56–58), play a role in the trafficking of T β RI/II complex to the primary cilia membrane when CerS4/ceramide is down-regulated. Notably, we discovered that a mutant T β RI-HA with altered CTS (Ala³³Phe conversion mutant) prevented the transport of the receptor to the primary cilia in response to CerS4 knockdown (Fig. 7D, left and right). Moreover, Ala³³Phe-T β RI, which has an altered CTS, prevented A549 cell invasion in response to CerS4 knockdown compared to controls in the absence/presence of exogenous TGF- β exposure (Fig. 7E). Overall, these novel data suggest that CerS4/ceramide signaling controls the targeting of T β RI/II complex to the primary cilia membrane, which is regulated by the Arl6/BBSome via the CTS present at the N terminus of T β RI (31-ATLQ-35), inducing T β RI-Smo-dependent cell migration and/or invasion.

T β RI/II signaling in primary cilia mediates tumor metastasis

To determine the effects of CerS4-dependent ceramide signaling on lung colonization/metastasis, we used an orthotopic tumor engraftment assay (59) in which we injected the mammary pads of female wild-type and CerS4^{-/-} mice (C57/black) with 4T1-Luc murine breast cancer cells that also stably coexpressed Scr shRNA or CerS4-shRNA. Twenty-one days later, we performed a luciferase activity assay *ex vivo* to assess for metastasis of 4T1-Luc cells to the lungs or liver of wild-type and CerS4^{-/-} mice. We detected ~4-fold increase in metastasis of 4T1-CerS4-shRNA-Luc cells to the liver compared to 4T1-shScr-Luc cells (fig. S8, A and B). No metastatic lesions were observed in the lungs in these mice (fig. S8A). Notably, the systemic loss of CerS4 in the mouse hosts did not appear to promote the metastasis of 4T1-Luc cells (fig. S8, A and B). Because 4T1 cells are Balb/c syngeneic, we repeated these studies in wild-type Balb/c mice. Knockdown of CerS4 resulted in increased metastasis of 4T1-CerS4-shRNA-Luc cells to the lungs and liver of wild-type mice compared to 4T1-shScr-Luc cells after mammary fat pad injections (Fig. 7, F and G). CerS4 knockdown in cells before injection also increased the number of liver lesions in wild-type Balb/c mice (Fig. 7H, left and right). Thus, these data suggest that knockdown of tumor CerS4 but not the systemic loss of CerS4 in the host tumor microenvironment plays key roles in enhancing metastasis of 4T1-Luc cells mainly to the liver, increasing tumor development.

To determine whether inhibitory Smad7 plays any roles in the regulation of liver metastasis in response to CerS4 knockdown, we examined the effects of IFT88 knockdown or stable Smad7 expression in the liver and lung metastasis of 4T1-shScr-Luc or 4T1-CerS4-shRNA-Luc cells in wild-type C57/black and Balb/c mice. Inhibiting cilia formation by knocking down of IFT88 or overexpressing Smad7 in the 4T1-CerS4-shRNA-Luc cells (fig. S8, B, E, and F) substantially decreased the incidence of liver and lung metastasis in C57/black (fig. S8, C and D) and Balb/c mice (Fig. 7, G and H). These data were also consistent with the inhibition of the T β RI-Smo association by ectopically expressed Smad7 or by knockdown of IFT88, as measured by immunoprecipitation and Western blotting ex vivo in the liver and lung homogenates of these mice (Fig. 7I, left and right). Overall, these data suggest that T β RI-Smo cross-talk within primary cilia in 4T1 graft-derived tumors plays key roles in the induction of liver and/or lung metastasis in response to loss of CerS4 and ceramide and can be attenuated by restoring Smad7 abundance or inhibiting IFT88.

DISCUSSION

Here, we have found that CerS4-generated long-chain ceramides inhibit the localization of T β RI/II to the primary cilia membrane by forming an inhibitory complex with Smad7. Molecular and genetic alterations of CerS4 or ceramide abundance attenuated the Smad7-T β RI interaction, inducing T β RI/II targeting to the PMs of primary cilia. This process was mediated by the Arl6/BBSome transporter complex, involving a previously unknown cilia-targeting signal (31-Ala-Thr-Ala-Leu-Gln-35; “CTS”) within T β RI. Activated T β RI/II signaling in the primary cilium by CerS4 and/or Smad7 knockdown led to the induction of Shh signaling through enhanced T β RI-mediated activation of Smo, resulting in increased cell migration in culture and liver metastasis in orthotopic mouse models of cancer (Fig. 7A).

The activity of several signaling pathways, including those mediated by receptor tyrosine kinases (like T β RI/II), Wnt, Notch, mTOR, and Shh (60, 61), is coordinated within cell surface projections called primary cilia. Thus, various biological and physiological functions depend on the formation of functional primary cilia. Alterations related to primary cilia or the signaling therein have been associated with various diseases, such as cardiac anomalies, obesity, and diabetes (18, 62) as well as cancer progression and alopecia. As lipids, which comprise PMs of cells and cellular vesicles, ceramides are critical to primary cilium formation. Our findings with CerS4-deficient mice and cells indicate that some ceramides directly regulate signaling within cilia as well, namely, by Smad7-dependent inhibition of TGF- β receptors and their cross-talk with the Hedgehog pathway. Furthermore, the data suggest that “non-canonical” (Smad3-independent) as well as the previously reported “canonical” (Smad-dependent) TGF- β signaling in the primary cilium promotes cell migration (22, 23). In the context of cancer, we observed that this increased ciliary TGF- β signaling and Shh activation enabled by the loss of CerS4 and long-chain ceramides in tumor cells promoted metastatic progression in mice. Our observations of alopecia and skin abnormalities in CerS4^{-/-} mice are consistent with other studies (46, 47), which link increased epidermal T β RI abundance with alopecia (48). Elsewhere, loss of Patched2 (Ptch2), a receptor protein that inhibits Shh signaling, has been associated with alopecia in Ptch2-deficient mice (63). It should also be noted that loss of CerS4 and C₁₈- and C₂₀-

ceramides can also be compensated by up-regulation of other ceramide synthase enzymes and/or ceramide species (64). Thus, whether the CerS4^{-/-}-associated ciliary ceramide-Smad7 alterations or activation of the TβRI/II-Shh-Smo axis specifically identified here and tested in tumor cells also contributes to CerS4^{-/-}-associated alopecia requires further investigation.

On a biochemical level, our data suggest that CerS4-generated long-chain ceramide promoted the association between Smad7 and TβRI, at least in part through Asn²⁷¹ and Thr²⁰⁸ in TβRI, which compromised ciliary trafficking of the TβRI by Arl6/BBSome, in which we identified a previously unknown functional motif, the CTS (31-ATALQ-35) in TβRI. Suppression of TβRI trafficking prevented its interaction with Smo and, consequently, the activation of Shh signaling. Mutational analysis revealed that Smad7's interaction with TβRI was critical to the antitumorigenic roles of CerS4/ceramide. Curiously, loss of CerS4 resulted in decreased abundance of Smad7; further work is needed to understand how that happens, but increasing Smad7 abundance was able to restore suppression of the TβRI-Smo axis. The antimetastatic effects of CerS4/ceramide appear to be tumor-intrinsic because only disruption of CerS4-dependent ceramide metabolism within the tumor cells but not systemically in the tumor hosts caused an increase in metastatic incidence. A recent study also showed that genetic loss of CerS4 had no effect on T cell function and phenotype, a critical factor in tumor progression (65). However, the same study showed that mice deficient in another ceramide synthase, CerS6, showed global impairment in their T cell responses to allogeneic hematopoietic stem cell transplantation. Moreover, the role of other ceramides, such as neutral sphingomyelinase-generated ceramide, in the formation of cilia via induced acetylation of tubulin in Madin-Darby canine kidney cells, neuronal progenitors, and stem cells has been reported (66, 67). In contrast, our data suggest that decreased generation of long-chain ceramides (C₁₈- and C₂₀-ceramides) due to loss of CerS4 had no major effect on the formation of cilia in MEFs, skin keratinocytes, and cancer cells. Thus, together, these data suggest that ceramide metabolism and signaling might have distinct functions for the regulation of cilia formation and the signaling therein, and that even among similar ceramide species, roles may be context- and cell type-dependent. For cancer patients, these points are likely to be critical to clinical and therapeutic considerations because tumor-versus-host/microenvironment-specific changes as well as the abundance (and signaling effects) of distinct ceramide species are increasingly being recognized for discrete contributions to tumor development and progression. Overall, these data suggest that activation of the CerS4/ceramide-Smad7 inhibitory complex may selectively target TβRI/II signaling in primary cilia, without affecting general primary cilia generation or perhaps even compromising canonical TGF-β signaling, and thus may be an effective strategy to inhibit cell migration and invasion and tumor metastasis.

MATERIALS AND METHODS

Cell lines and culture conditions

A549 and UMSCC-22A cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Cellgro) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin and streptomycin (Cellgro). Primary MEFs or skin keratinocytes were derived

from age-matched wild-type and CerS4-knockout C57/black mice, as previously described (68). MEFs (immortalized using SV40 large T antigen) and 4T1 murine mammary carcinoma cells were cultured in DMEM. Primary mouse skin keratinocytes (nonimmortalized or immortalized by SV40 large T antigen) were cultured in keratinocyte serum-free basal medium with growth supplement (Gibco) at 37°C/5% CO₂. Inhibition of TβRI/II was performed with SB431542 {4-4-(1,3-benzodioxol-5-yl)-5(2-pyridinyl)-1*H*-imidazol-2-yl]benzamide}, which inhibits TGF-β-superfamily receptors ALK5, ALK4, and ALK7 (Cayman Chemical). Inhibition of Shh signaling and primary cilia signaling was performed with robotnikinin (a small molecule that binds the extracellular Shh protein and inhibits Shh signaling; Calbiochem) and CA2 (which abrogates ciliogenesis; ChemDiv), respectively.

siRNAs and shRNAs

Molecular knockdown studies were performed using the following siRNA or shRNAs:

CerS4 siRNA: 5'-GUGCCAACCUGCUGCGCAU-3'

CerS1 siRNA: 5'-AACCAGTAGAGGTTTCATAAGG-3'

CerS4 shRNA (#1): 5'-ATGGACTCGTAGTATGTGGTG-3'

CerS4 shRNA (#2): 5'-ATGAATCTCTCAAAGGCAAGG-3'

Shh shRNA: 5'-ATATGTGCCTTGGACTCGTAG-3'

Smo shRNA: 5'-AACTGAGATGTGAATGTAGGG-3'

IFT88 shRNA: 5'-ATAGGTCTAGTAACTGCCGTG-3'

Cells were transfected with siRNAs (obtained from Dharmacon) or shRNAs (obtained from Sigma-Aldrich) (100 nM for 48 hours) using DharmaFECT transfection kit (Dharmacon, Thermo Fisher Scientific). Scrambled siRNAs and shRNAs were obtained from Dharmacon and Sigma-Aldrich using their nontargeting control siRNA and shRNAs, respectively.

Plasmids and antibodies

Expression of wild-type and catalytically inactive mutant (H212A/H213A) CerS4 was performed using the tet-inducible vector system as described previously (51, 67). Expression vectors pBABE-puro (#1764), pMD2.G (#12259), psPAX2 (#12260), TβRI (#19161), and TβRII (#11766) were purchased from Addgene. In TGF-β-responsive luciferase reporter plasmids, six copies of the Smad binding element or its mutant form were cloned upstream of the SV40 promoter in the pGL3 vector.

Anti-CerS1 and anti-CerS4 antibodies were purchased from Exalphi Biologicals. Anti-p-Smad3-Ser^{423/425} (C255A9) and anti-HA (3724) antibodies were purchased from Cell Signaling Technology. Anti-V5 tag (ab9116) and anti-TβRI (for flow cytometry; ab31013) antibodies were purchased from Abcam. Anti-Smad6/7 (N19, sc-7004), anti-Shh (H-160, sc-9024), anti-Smo (H-300, sc-13943), anti-TβRI (V-22, sc-398), anti-TβR2 (L-21, sc-400), anti-Smad7, anti-IFT88, anti-Ac-tubulin, anti-caveolin-1 (7C8, sc-53564), anti-clathrin heavy chain (TD.1, sc-12734), anti-calnexin (sc-6465), and anti-Smurf2 (H-50, sc-25511)

antibodies were purchased from Santa Cruz Biotechnology. Anti-ceramide antibody (MID 15B4) was purchased from Enzo Life Sciences.

Measurement of cell migration and invasion

For the Boyden chamber assay, the inserts for 24-well plates (transparent polyethylene terephthalate membrane, 8.0- μ m pore size, Falcon) were coated with Matrigel or fibronectin (Invitrogen). Cells (50×10^5) were placed in the upper chamber in 1% serum, and media with 10% serum were placed in the lower chamber. After 24-hour incubation at 37°C in a humidified atmosphere with 5% CO₂, cells were fixed in cold methanol and stained with crystal violet. Bright-field images were taken with 10 $\times$ and 4 $\times$ objectives in an Olympus CKX41 phase-contrast microscope. Images were analyzed using ImageJ.

For the scratch “wound healing” assay, cells were plated onto a 60-mm dish to create a confluent monolayer. Dishes were incubated for about 24 hours at 37°C, allowing cells to adhere and spread on the substrate completely. A straight line was then scraped through the cell monolayer with a p200 pipet tip. Cell debris was removed, and the edge of the scratch was smoothened by washing the cells once with 1 ml of the growth medium. To obtain the same field during the image acquisition, we created markings to be used as reference points close to the scratch. After the reference points were made, we placed the dish under a phase-contrast microscope (Olympus CKX41) and left the reference mark outside the capture image field but within the eyepiece field of view. Images were analyzed by Wimasis image analysis software.

In the single-cell tracking assay, 100 μ l [phosphate-buffered saline (PBS), 5 μ g/ml] of fibronectin was added to 24-well plates and left unperturbed for a minimum of 4 hours at room temperature (or overnight at 4°C). Next, 1 ml of 1 $\times$ PBS and 1 μ l of fluorescent microspheres (vortexed well) were added to the fibronectin-coated wells and left to settle at 4°C for at least 2 hours. Excess beads were then gently aspirated from the wells, and wells were washed gently twice in 1 $\times$ PBS. About 4 cells/mm² were seeded in 1 ml of media into each well, incubated at 37°C (humidified) in 5% CO₂ for 24 hours, and then examined under a Nikon Eclipse TE2000-S microscope.

Measurement of PM abundance of T β RI/II

PM abundance of murine or human T β RI/II was measured by flow cytometry using antibodies that recognize these receptors. Biotin labeling was also performed to measure the PM abundance of T β RI/II in various cells as described (Thermo Fisher Scientific). Briefly, the thiol-cleavable amine-reactive biotinylation reagent EZ-Link Sulfo-NHS-SS-Biotin (Pierce) was used for protein labeling. After washing cells in PBS, cells were treated with biotin (1 mg/ml) in PBS at 4°C for 2 hours. Then, we removed the biotin-containing PBS and quenched the reaction using tris-buffered saline containing EGTA and EDTA. After immunoprecipitation using avidin-coated agarose beads, we treated the beads with 1 $\times$ immunoprecipitation urea sample buffer containing 50 mM dithiothreitol for 2 hours (to cleave the biotinylation reagent) with vigorous shaking at 800 rpm and performed Western blotting.

Isolation of primary cilia

Primary cilia were isolated as described previously (69) using the peel-off technique. Briefly, 4T1 or A549 cells were grown in a 150-mm culture dish in 10% DMEM and 1% penicillin/streptomycin in a humidified incubator at 37°C in 5% CO₂ for a maximum of 10 days, and they were starved for differentiation before cilia isolation for 24 to 48 hours. The cells were washed with culture medium without serum three times, and 15 ml of fresh medium with protease inhibitors (0.01% soybean trypsin inhibitor and 0.1 mM phenylmethylsulfonyl fluoride) was added. The poly-L-lysine-coated coverslips were placed face down on top of coverslips with a cell monolayer. A filter paper was placed on top of the poly-L-lysine-coated coverslips, and light pressure was applied by placing a rubber cork (20 mm in diameter) or by slightly pushing down with a finger for 20 s. After the coverslips were lifted off from the cell monolayer, samples were scraped off, centrifuged, and then frozen for biochemical analysis. To assess the efficiency of cilia isolation, both samples (that is, poly-L-lysine-coated coverslip with attached isolated cilia and remaining cilia on coverslips after isolation) were measured with Ac-tubulin by Western blotting.

Isolation of primary cilia using differential centrifugation

The primary cilia were isolated using shear force. In brief, 4T1-Luc-shScr or CerS4-shRNA cells were grown in a 150-mm dish for up to 7 days, and they were starved for 24 to 48 hours before cilia isolation. After cells were rinsed with PBS, 10 ml of PBS was added, and dishes were placed on a rotary shaker for 4 min at 350 rpm. This was followed by two centrifugation steps: (i) 10 min at 1000g at 4°C (pellet was discarded) and (ii) 30 min at 40,000g at 4°C. Primary cilia (pellet) was resuspended in radioimmunoprecipitation assay buffer for Western blotting or used for TEM.

Transmission electron microscopy

Isolated cilia pellets were directly immersed in 4% paraformaldehyde for 45 min, rinsed with PBS, and incubated with anti-TβRI (1:50) or Ac-tubulin (1:50) antibodies for 14 hours at 4°C. After washing with PBS, cilia were incubated with Nanogold-labeled anti-rabbit and mouse (Nanoprobes) and secondary antibody conjugate (1:1000) for 1 hour. After postfixing with 2% glutaraldehyde in PBS for 30 min, silver enhancement (HQ Silver enhancement kit; Nanoprobes) was performed. This was followed by treatment with 0.2% of OSO₄ in PBS and staining with uranyl acetate and embedding. These sections were visualized using a JEOL 1010 transmission electron microscope.

Measurement of ceramide by lipidomics

Ceramides with various fatty acyl chain lengths were measured by lipidomics using liquid chromatography and mass spectrometry, as described previously (50, 68). Inorganic phosphate in lipid extracts or total protein amounts in total cell lysates (measured before lipid extractions) were used for normalization.

Generation of CerS4^{-/-} mice

The CerS4^{+/-} heterozygous mice (C57/black) were obtained from the Texas A&M Institute for Genomic Medicine, and CerS4^{-/-} mice were generated after crossbreeding of CerS4^{+/-} parental mice.

Microarray analysis and immunohistochemistry

CerS1 to CerS6 mRNAs were analyzed in already existing microarray data using GDS1062, GDS3966, and GSE22541 arrays for metastatic (advanced stage) compared to nonmetastatic (stage 1) head and neck cancer, melanoma, or renal cell carcinoma, respectively. CerS4 protein abundance was detected by immunohistochemistry using anti-CerS4 antibody in commercially available tumor microarrays (US Biomax) containing metastatic versus nonmetastatic tumor tissues obtained from patients with breast (BR100106-DO36), lung (LC817-F093), and head and neck cancers (HN803a-M215) or melanoma (ME1004a-I224), as we described previously (50, 52, 68). The expression of genes regulated by TGF- β signaling was measured by qRT-PCR using the Super Array (PAHS-235ZA; Qiagen).

Immunofluorescence

Cells (50×10^5 cells per well) were plated on glass coverslips in a six-well plate for 18 hours, fixed, and permeabilized using 4% paraformaldehyde (20 min) and 0.1% Triton X-100 in 1 $\times$ PBS (pH 7.4) for 10 min. The cells were then blocked with 1% bovine serum albumin and dissolved in PBS (pH 7.4) for 1 hour. Cells were incubated for 18 hours at 4°C with primary antibodies in blocking solution followed by Alexa Fluor 488-, Alexa Fluor 594-, or Cy5-conjugated secondary antibodies (1:500) for 60 min. Immunofluorescence was performed using a Leica TSC SP2 AOBs TCS confocal or Olympus FV10i microscope with 543-nm and 488-nm channels for visualizing red and green fluorescence (50). Cilia were imaged using anti-Ac-tubulin antibody. Images were taken at 63 $\times$ magnification. At least three random fields were selected for images. Images and overlays were analyzed in Leica LAS AF software (52, 68).

Proximity ligation assay

Cells were fixed using 4% paraformaldehyde for 15 min and permeabilized using 0.2% glycine (2 min). Cells were then incubated with various antibodies (10 to 20 μ g/ml) at 4°C for 18 hours. PLA (50) was performed using the Duolink in situ hybridization kit as described by the manufacturer (Olink Bioscience).

Immunoprecipitation

Cells were lysed with 1 $\times$ lysis buffer (Thermo Fisher Scientific) including a protease inhibitor cocktail (Sigma-Aldrich) for 20 min on ice. Cell lysates were centrifuged at 12,000g for 15 min at 4°C, and supernatants were used for immunoprecipitation and Western blotting (50).

Molecular modeling and docking

Interactive docking prediction of protein-protein complexes and modeling between T β RI and Smo were performed using ZDOCK and Phyre2 as described (68, 70). In summary,

Phyre2 was used to predict secondary structures of human T β RI (ID: 7046) and Smad7 (ID: 4092) based on their amino acid sequences in GenBank. The generated Protein Data Bank files were analyzed using the ZDOCK Server (<http://zdock.umassmed.edu/>). The top model was then used to predict the sites of association between these two proteins.

Measurement of metastasis in mice

To measure metastasis, 4T1-Luc cells (with/without shRNA and/or ectopic expression vectors) were injected to the mammary fat pads (1×10^5 cells) of wild-type (C57/black and Balb/c) or CerS4^{-/-} mice (C57/black). Seeding of the cells into the mammary fat pads was visualized using a bioluminescence imaging system (50, 52). After 21 days, lungs, bones, brain, and liver were isolated from these recipient mice, and 4T1-Luc metastasis to these organs was detected using luciferase assay ex vivo. Animal protocols used in this study were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

Statistical analysis

All data are presented as means $\pm$ SD of at least three independent studies ($n > 3$). Group comparisons were performed with either a two-tailed Student's *t* test or a one-way ANOVA with Tukey's posttest, as appropriate. $P < 0.05$ was considered significant. Statistical analysis was professionally reviewed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. CerS4 knockdown mediates cell migration

(A) Fibronectin-coated Boyden chamber assays measuring migration of A549 cells after being transfected with control (Scr) or CerS1- or CerS4-targeted siRNA. Scale bar, 100 μm . (B) Effect of siRNA-mediated CerS4 knockdown on A549 cell migration measured by the clearance of fluorescent micro-spheres on fibronectin-coated wells. The ability of cells to create phagokinetic nonfluorescent tracks was evaluated by fluorescence microscopy and quantified using National Institutes of Health Image software. (C) As described in (A), using two shRNAs against CerS4. Scale bar, 100 μm . (D) As in (B), using UM-SCC-22A cells transfected with CerS4-targeted or control shRNA. Data are means $\pm$ SD of $n = 3$ independent experiments. * $P < 0.05$ by Student's t tests.

Fig. 2. CerS4 knockdown induces cell migration through increased TβRI/II membrane trafficking and signaling

(A) Cell surface abundance of TβRI/II measured by flow cytometry in A549 cells transfected with CerS4-shRNAs or Scr shRNAs using two different sets of anti-TβRI or anti-TβRII antibodies. (B) Effects of TβRI inhibition by SB431542 on migration of A549 cells transfected with CerS4-targeted compared to Scr shRNAs, as measured in fibronectin-coated Boyden chambers. (C) Boyden chamber assays using fibronectin-coated chambers assessing migration of 4T1 cells after expression of wild-type (WT) or D/N-mutant TβRI (confirmed with Western blot, below the graph) and CerS4-targeted or control siRNA in the presence of vehicle or TGF-β (5 ng/ml). OD, optical density. (D) Effects of shRNA-mediated knockdown of CerS4 on total and cell surface protein abundance of Smad7, TβRI, and TβRII measured by biotin-labeling followed by Western blotting. Actin and Na⁺/K⁺-ATPase were used as controls for total or cell surface, respectively. Graph shows the amount of each protein at the cell surface (quantified relative to total protein in the whole lysate) in CerS4-shRNA-transfected cells (gray bars) relative to that in control, Scr shRNA-transfected cells (black bars). (E) Western blotting for Smad7 in whole-cell lysates from 4T1 cells cotransfected with a control (vector) or FLAG-tagged Smad7 expression vector and Scr- or CerS4-targeting shRNA. (F) Effects of WT-Smad7 on migration in Boyden chambers in A549 cells expressing Scr shRNA or CerS4-shRNAs were measured. (G to I) Luciferase

reporter assay (G) and Western blotting (H and I) to assess the effects of CerS4 knockdown by shRNA in 4T1 cells on Smad-dependent promoter activation (G) and the phosphorylation of Smad3 (H) and Smad2 (I). Cells were cultured in the presence or absence of exogenous TGF- β exposure (5 ng/ml), as indicated. Data are means $\pm$ SD ($n = 3$ independent experiments). Blots are representative of $n = 3$. * $P < 0.05$ by a Student's t test on log-transformed data. AU, arbitrary units; MBP, myelin basic protein; BE, binding element.

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Fig. 3. Genetic CerS4 inhibition results in increased outer membrane localization of TβRII and signaling to induce migration in CerS4^{-/-} MEFs and keratinocytes

(A) Outer membrane abundance of TβRII in MEFs and skin keratinocytes isolated from age-matched WT and CerS4^{-/-} mice was measured by flow cytometry using the anti-TβRII antibody. Data are means ± SD ($n = 3$ independent experiments; $*P < 0.05$). P values were calculated using t test on log-transformed data. (B and C) Effects of WT-Smad7 on the migration of WT and CerS4^{-/-} skin keratinocytes were measured using fibronectin-coated Boyden chambers (B) or single-cell migration track assay (C) compared to vector-transfected controls. Data are means ± SD ($n = 3$ independent experiments). KO, knockout. (D and E) Effects of reconstitution of CerS4 on the migration of WT and CerS4^{-/-} skin keratinocytes were measured using fibronectin-coated Boyden chambers (D) or single-cell tracking assay (E) compared to vector-transfected controls. Data are means ± SD ($n = 3$ independent experiments) ($*P < 0.05$). (F) Effects of reconstitution of CerS4^{WT} versus catalytically inactive CerS4^{H212A/H213A}, unable to generate ceramide, on migration in A549 cells, stably expressing CerS4-shRNA or Scr shRNA, were measured in fibronectin-coated Boyden chambers. Vector-transfected cells were used as controls. Data are means ± SD ($n = 3$ independent experiments). (G) Tet-induced CerS4-V5 at 24 to 72 hours in A549 cell extracts was detected by Western blotting using anti-CerS4 or anti-V5 antibodies. Actin was used as a loading control. Data represent at least three independent experiments. (H) Effects of TβRI/II cotransfections on A549 cell migration in the absence/presence (–/+tet) of CerS4 induction were measured in fibronectin-coated Boyden chambers. Vector-transfected A549 cells were used as controls. Data are means ± SD ($n = 3$ independent experiments). Student's t test was performed.

Fig. 4. Association of TβRI with inhibitory Smad7 regulates receptor signaling

(A) Colocalization (white) of ceramide (Cer) (green), Smad7 (red), and TβRI (cyan) was detected by immunofluorescence in 4T1 cells stably expressing Scr shRNA or CerS4-shRNAs. Images represent three independent studies, which are quantified using ImageJ, and $*P < 0.05$ was considered significant. Scale bars, 100 μ m. (B) Ceramides were measured using lipidomics and normalized to milligrams of protein. Data are means $\pm$ SD ($n = 4$ independent studies). (C) Ceramide-Smad7 interaction in immortalized MEFs isolated from WT or CerS4^{-/-} mice (left) and in Scr shRNA- or CerS4-shRNA-transfected A549 cells (right) was measured by PLA using antibodies against ceramide and Smad7. PLA signals were quantified using the Duolink Image Tool. Data are means $\pm$ SD ($n = 3$ independent experiments; $*P < 0.05$). (D and E) TβRI-Smad7 (D) or Smad7-Smurf2 (E) association was determined by immunoprecipitation (IP) and Western blotting [immunoblotting (IB)] using antibodies that recognize TβRI (R1), Smad7, or Smurf2 in extracts isolated from A549 cells stably transfected with Scr shRNA or CerS4-shRNAs in the absence or presence of vector, CerS4^{H212A/H213A}, or CerS4^{WT}. Anti-IgG (IgG) antibody was used to detect nonspecific immunoprecipitation of proteins in these extracts. Actin was used as a loading control in Western blots. Blots shown represent at least three independent studies. (F) Association between TβRI (R1) and Smad7 was measured by PLA using antibodies that recognize TβRI or Smad7 in immortalized WT or CerS4^{-/-} MEFs. Anti-IgG antibody was used as a negative control. PLA signals were quantified using the Duolink Image Tool. Data are means $\pm$ SD (n

= 3 independent experiments; * $P < 0.05$). Scale bars, 100 μm . (**G** and **H**) Effects of Asn²⁷¹Ala/Thr²⁷²Ala conversions in T β RI on Smad7 recognition (**G**) or on A549 cell migration or invasion (**H**) with/without CerS4 induction were measured using PLA or Matrigel-coated Boyden chamber invasion assay compared to vector- and WT-T β RI-transfected controls. Data are means $\pm$ SD ($n = 3$ independent experiments; * $P < 0.05$). Student's t test was performed. EV, empty vector; RI, T β RI.

Fig. 5. Activation of T β RI/II signaling at the PM increases cell migration by Shh/Smo signaling in response to CerS4/ceramide knockdown

(A and B) Effects of shRNA-mediated CerS4 knockdown on Shh mRNA (A) or secreted protein abundance (B) were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) or enzyme-linked immunosorbent assay using Shh detection kit (Abcam). Scr shRNA-transfected A549 cells were used as controls. Data are means $\pm$ SD ($n = 3$ independent experiments; $*P < 0.05$). (C) Effects of Shh inhibition by robonikinin (+Rob) on A549 cell migration in response to siRNA-mediated CerS4 knockdown were measured using single-cell migration tracking assay compared to Scr-siRNA-transfected controls. Data are means $\pm$ SD ($n = 3$ independent experiments; $*P < 0.05$). (D and E) Effects of shRNA-mediated knockdown of Shh on the migration of A549 cells stably expressing CerS4-shRNA compared to Scr shRNA were measured in fibronectin-coated Boyden chambers (D). Quantification was performed using ImageJ (E). Data are means $\pm$ SD ($n = 3$ independent experiments; $*P < 0.05$). Scale bars, 100 μm . (F to H) Effects of Shh signaling inhibition by cyclopamine (10 μM) on Gli protein abundance (F), *Gli* mRNA (G), and cell migration (H) were measured in response to CerS4 knockdown compared to Scr shRNA-transfected controls. Quantification was performed using ImageJ (H, right). Data are means $\pm$ SD ($n = 3$ independent experiments; $*P < 0.05$). Scale bar, 100 μm . (I to K) Effects of Gli1 knockdown using siRNAs on Gli1 abundance (I), Gli1 mRNA (J), and cell migration (K) were measured in response to CerS4 knockdown compared to controls. Quantification was performed using ImageJ. Data are means $\pm$ SD ($n = 3$ independent

experiments; $*P < 0.05$). Student's t test was performed, and P values were calculated using t test on log-transformed data (for A, G, and J). Scale bar, 100 μm .

Fig. 6. Trafficking of TβRI/II to primary cilia membrane mediates the cross-talk between TβRI and Smo

(A) Effects of shRNA-mediated CerS4 knockdown on Smo-TβRI interaction were measured by immunoprecipitation using anti-TβRI antibody followed by Western blotting using anti-Smo or anti-TβRI antibodies compared to Scr-transfected 4T1 cells (bottom). Data are means ± SD ($n = 3$ independent experiments; * $P < 0.05$). Total protein abundance of Smo in cell extracts before immunoprecipitation studies was measured by Western blotting (top).

(B) Interaction between TβRI-Smo and TβRII-Smo was measured by PLA using anti-TβRI, anti-TβRII, and anti-Smo antibodies in A549 cells expressing Scr shRNA or CerS4-shRNAs. PLA signals were quantified using the Duolink Image Tool. Data are means ± SD ($n = 3$ independent experiments; * $P < 0.05$). Scale bars, 100 μm. (C and D) TβRI and Smo interaction was measured by immunoprecipitation and Western blotting using anti-TβRI and anti-Smo antibodies in 4T1 (C) or A549 (D) cells stably expressing Scr shRNA or CerS4-shRNAs in response to vector or WT-Smad7 (top). Anti-IgG antibody was used as a negative control. Equal immunoprecipitation of TβRI was confirmed by Western blotting (middle). Actin was measured for equal input of proteins in ceramide binding studies. Data represent at least three independent studies. o/e, overexpression. (E) Effects of shRNA-mediated knockdown of CerS4 on the localization of TβRI in primary cilia membrane (black arrows)

were measured in primary cilia-enriched fractions using EM with gold-labeled antibodies that recognize Ac-tubulin or T β RI compared to controls (Scr shRNA) (left and middle). EM images obtained using a gold-labeled anti-Glu-tubulin antibody in primary cilia-enriched fractions obtained from Scr shRNA- or CerS4-shRNA-transfected 4T1 cells were used as negative controls (right). Data represent at least three independent studies. Scale bars, 100 nm. **(F)** Primary cilia localization of T β RI was detected by colocalization of Ac-tubulin using immunofluorescence in response to CerS4 knockdown or genetic loss in A549, UM-SCC-22A (U22A), MEFs, and keratinocytes. Data represent at least three independent studies ($n = 3$). Scale bars, 100 μ m. **(G)** Ceramide, T β RI, and Smo localization in primary cilium (merged yellow) was detected by immunofluorescence in 4T1 cells stably expressing Scr shRNAs or CerS4-shRNAs. Quantification was performed using ImageJ (bottom). Data are means $\pm$ SD ($n = 3$ independent experiments; $*P < 0.05$). Scale bar, 100 μ m. **(H)** Colocalization of Ac-tubulin (Ac-Tub) (green), T β RI (purple), and Smo (red) was measured by immunofluorescence using anti-Ac-Tub, anti-T β RI, and anti-Smo antibodies in 4T1 stably expressing Scr shRNAs or CerS4-shRNAs. Images represent three independent experiments, which are quantified using ImageJ. Data are means $\pm$ SD ($n = 3$ independent experiments; $*P < 0.05$) (bottom). Student's t test was performed. Scale bars, 100 μ m.

Fig. 7. TβRI/II trafficking to primary cilium by CTS and Arl6 in response to CerS4 knockdown in mammary cancer cells mediates liver metastasis

(A and B) Effects of shRNA-mediated knockdown of Arl6 and/or BBS4 on primary cilia localization of TβRI in PM/primary cilia by flow cytometry (A) and on cell migration or invasion in Boyden chambers (B) were measured in 4T1-Luc cells stably expressing shRNA against CerS4 (left). Data are means ± SD ($n = 3$ independent experiments; $*P < 0.05$). Knockdowns of Arl6 and BBS4 were confirmed by Western blotting (B, middle and right). Blots represent three independent studies. (C) Association between Arl6 and TβRI in 4T1 cells stably expressing Scr shRNAs or CerS4 shRNAs was measured by immunoprecipitation and Western blotting using anti-Arl6 and anti-TβRI antibodies in primary cilia-enriched fractions. Images were quantified using ImageJ. Data are means ± SD ($n = 3$ independent experiments; $*P < 0.05$). (D and E) Effects of WT-TβRI or mutant of TβRI (TβRI^{mut}) (31-Ala-Thr-Ala-Leu-Gln-35) on its primary cilia localization or cell migration were measured by immunofluorescence (D) or fibronectin-coated Boyden chamber migration assay in the absence/presence of TGF-β (5 ng/ml) (E) compared to vector-transfected controls. Equal abundance of WT- and mutant-TβRI was confirmed by Western blotting (D, right). Data are means ± SD ($n = 3$ independent experiments; $*P <$

0.05). **(F to H)** Liver (F) or lung (G) metastasis obtained from Balb/c mice after mammary fat pad injections ($n = 6$ mice per group) with 4T1-Luc cells stably expressing Scr shRNAs or CerS4-shRNAs in response to transfections using vector and Smad7 or Scr shRNA and IFT88-shRNAs for inhibition of cilia formation was measured ex vivo using chemiluminescence. Data are means $\pm$ SD. Liver tumor nodules were detected and measured via hematoxylin and eosin staining by an independent pathologist (H). Data are means $\pm$ SD ($n = 6$; $*P < 0.05$). **(I)** Association of T β RI and Smo was detected by immunoprecipitation and Western blotting in liver tissues obtained from mice, as in (F). Data are means $\pm$ SD ($*P < 0.05$). Student's t test and analysis of variance (ANOVA) with Tukey's posttest for pairwise comparisons in animal/allograft studies in (F) and (G) were performed. P values were calculated using t test on log-transformed data (for A, C, and E). **(J)** Graphical summary. Our data suggest that CerS4-generated C₁₈-/C₂₀-ceramide forms an inhibitory ceramide-Smad7 complex, which inhibits the primary cilia membrane localization of T β RI/II and signaling, preventing the cross-talk between T β RI and Smo, attenuating Shh/Smo-dependent cell migration. Molecular and genetic inhibition of CerS4/ceramide prevents ceramide-Smad7 complex formation, inducing Arl6-dependent T β RI/II trafficking and signaling in primary cilia through novel CTS of the T β RI. Activated T β RI/II signaling in primary cilium by CerS4 and/or Smad7 knockdown induces T β RI-Smo interaction and Shh/Smo signaling, increasing cell migration and liver metastasis.