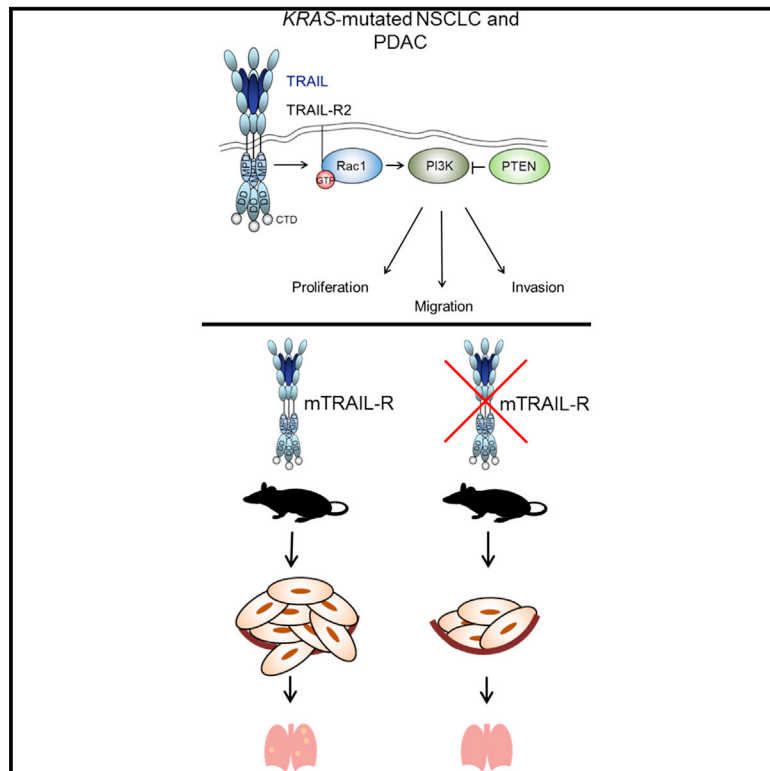


Cancer Cell

Cancer Cell-Autonomous TRAIL-R Signaling Promotes KRAS-Driven Cancer Progression, Invasion, and Metastasis

Graphical Abstract



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In Brief

von Karstedt et al. show that mouse TRAIL-R and human TRAIL-R2, but not TRAIL-R1, are important for the progression, invasion, and metastasis of KRAS-mutant tumors through the regulation of Rac-1.

Highlights

- mTRAIL-R promotes KRAS-driven lung and pancreatic cancer growth and metastasis
- Human TRAIL-R2 promotes tumor growth, migration, invasion, and metastasis
- Endogenous mTRAIL-R constitutively activates Rac1 in vivo in tumors
- TRAIL-R2 expression positively correlates with the onset of metastasis in patients



Cancer Cell-Autonomous TRAIL-R Signaling Promotes KRAS-Driven Cancer Progression, Invasion, and Metastasis

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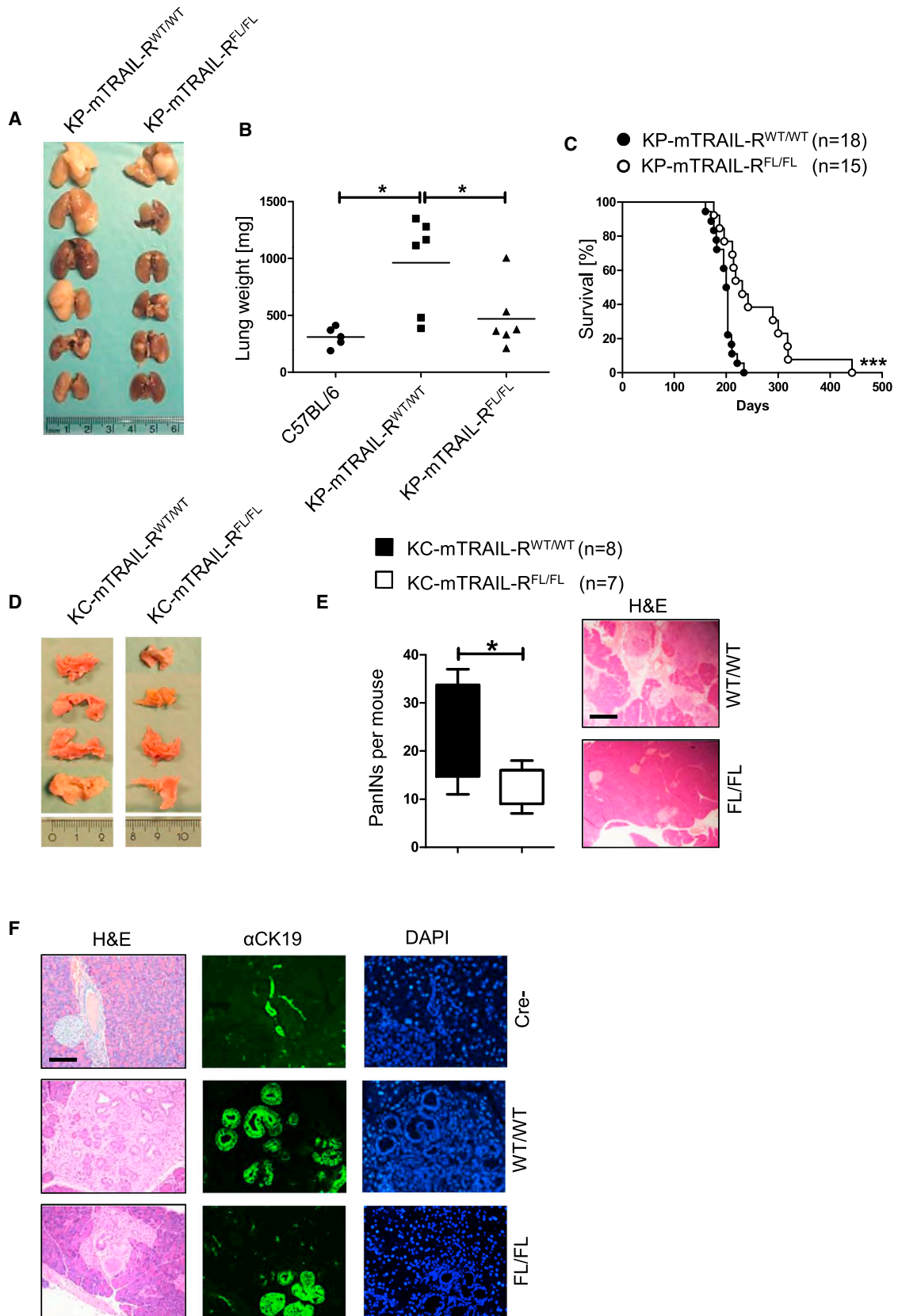
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SUMMARY

Many cancers harbor oncogenic mutations of *KRAS*. Effectors mediating cancer progression, invasion, and metastasis in *KRAS*-mutated cancers are only incompletely understood. Here we identify cancer cell-expressed murine TRAIL-R, whose main function ascribed so far has been the induction of apoptosis as a crucial mediator of *KRAS*-driven cancer progression, invasion, and metastasis and in vivo Rac-1 activation. Cancer cell-restricted genetic ablation of murine TRAIL-R in autochthonous *KRAS*-driven models of non-small-cell lung cancer (NSCLC) and pancreatic ductal adenocarcinoma (PDAC) reduces tumor growth, blunts metastasis, and prolongs survival by inhibiting cancer cell-autonomous migration, proliferation, and invasion. Consistent with this, high TRAIL-R2 expression correlates with invasion of human PDAC into lymph vessels and with shortened metastasis-free survival of *KRAS*-mutated colorectal cancer patients.

Significance

Previous preclinical and clinical studies have explored the pro-apoptotic function of TRAIL and TRAIL-R agonists as anti-cancer drugs exploiting the fact that many cancers highly express TRAIL-Rs. However, the cancer biological function of high TRAIL-R expression is still unknown. Here, we identify cancer cell endogenous TRAIL-R signaling to drive cancer progression, invasion, and metastasis in *KRAS*-mutated cancers, suggesting that this nonapoptotic function of the TRAIL/TRAIL-R system is positively selected for in these cancers. Importantly, this receptor/ligand system is amenable to therapeutic blockade. Therefore, inhibiting, rather than triggering, human TRAIL-R2 should be explored as a therapeutic approach for patients with *KRAS*-mutated cancers.



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INTRODUCTION

Twenty-one percent of all human cancers bear oncogenic mutations in *KRAS* (Bos, 1989), and their frequency is highest in some of the most aggressive cancers, such as 95% of pancreatic ductal adenocarcinoma (PDAC) (Hidalgo, 2010; Jaffee et al., 2002), 50% of colorectal adenocarcinoma (Grady and Markowitz, 2002), and 30% of non-small-cell lung cancers (NSCLCs) (Mitsuuchi and Testa, 2002). Whereas direct therapeutic targeting of mutated *KRAS*^{G12C} has recently been achieved (Ostrem et al., 2013), cancers driven by all other forms of oncogenic *KRAS* remain difficult to treat. Many effector pathways downstream of *KRAS* have been implicated in *KRAS*-driven tumor development and maintenance (Castellano et al., 2013; Downward, 2008; Engelman et al., 2008; Gupta et al., 2007; Ling et al., 2012). Therefore, small molecule inhibitors targeting these pathways have been developed (Baines et al., 2011). Their recent clinical application has, however, highlighted that many of these inhibitors activate resistance-causing feedback mechanisms and give cause for concern over toxicity in the clinic.

TNF-related apoptosis-inducing ligand (TRAIL, also known as Apo2L) can selectively kill tumor cells in vivo (Ashkenazi et al., 1999; Walczak et al., 1999). These findings have initiated the development of TRAIL and other TRAIL-receptor (TRAIL-R) agonists for cancer therapy (reviewed in Lemke et al., 2014). Many tumor cell lines and primary tumors are, however, resistant to TRAIL-induced apoptosis (Koschny et al., 2007; Todaro et al., 2008). In addition, TRAIL also has been shown to activate nonapoptotic signaling pathways in cancer cell lines such as nuclear factor- κ B (NF- κ B) (Degli-Esposti et al., 1997; Ehrhardt et al., 2003; Harper et al., 2001; Ishimura et al., 2006; Varfolomeev et al., 2005), the mitogen-activated kinases (MAPKs) JNK, p38 (Varfolomeev et al., 2005), and ERK (Belyanskaya et al., 2008), as well as the kinase Src (Azijli et al., 2012). These adverse effects were confirmed to be relevant in vivo as therapeutic TRAIL administration promoted liver metastasis of xenotransplanted PDAC (Trauzold et al., 2006) and *KRAS*-mutated colorectal cancer (CRC) cell lines (Hoogwater et al., 2010).

Whereas these findings have highlighted potential adverse effects of TRAIL as a single agent therapy, the basic biological question as to why cancer cells would retain TRAIL-R expression if they were resistant to apoptosis induction by TRAIL remained unresolved.

We hypothesized that the endogenous TRAIL/TRAIL-R system might play a role in promoting apoptosis-resistant cancers, which could provide an explanation for the seemingly counterintuitive high expression of TRAIL-Rs and TRAIL in many types of cancer (Elrod et al., 2010; Ganten et al., 2009; Macher-Goepfinger et al., 2009; Ozawa et al., 2001; Sanioglu et al., 2007),

including NSCLC in which high TRAIL-R2 expression positively correlated with increased risk of death (Spierings et al., 2003). Therefore, we set out to interrogate the role of cancer cell-expressed TRAIL-Rs in *KRAS*-driven cancer.

RESULTS

Cancer Cell-Expressed mTRAIL-R Promotes Autochthonous *KRAS*-Driven Lung Cancer and Formation of Pancreatic Intraepithelial Neoplasia

We started our investigation using a variant of the KP murine NSCLC model (Jackson et al., 2001) in which oncogenic *KRAS*^{G12D} and p53^{R172H} are expressed from their respective endogenous loci upon adenoviral-Cre (Ad-Cre) infection to give rise to lung tumors that closely resemble human NSCLC (Kasinski and Slack, 2012). Unlike humans, mice express only one death-domain (DD)-containing TRAIL-R, mTRAIL-R (MK/mDR5), which is homologous to both human TRAIL-R1 and TRAIL-R2 (Wu et al., 1999). To interrogate the role of mTRAIL-R in NSCLC, we crossed conditional mTRAIL-R knockout (KO) mice (Grosse-Wilde et al., 2008) with KP mice and induced lung tumors by Ad-Cre infection. Genetic ablation of mTRAIL-R in cancer cells substantially reduced lung tumor size (Figure 1A) and weight (Figure 1B) in age-matched groups (mTRAIL-R deletion within the largest tumor nodule per lung was confirmed; data not shown). Importantly, reduced lung tumor burden in mTRAIL-R-ablated lungs translated into significantly prolonged survival after tumor initiation (Figure 1C).

Although the incidence of *KRAS* mutations is relatively high among NSCLC (30%), it is even higher in PDAC (95%) (Hidalgo, 2010; Jaffee et al., 2002), which also overexpresses TRAIL-Rs (Ozawa et al., 2001). Thus, we next determined the role of mTRAIL-R in the initiation of pancreatic cancer. To this end, we made use of *Kras*^{G12D};PDX-1-Cre (KC) mice, in which oncogenic *KRAS*^{G12D} expression is initiated via Cre-recombinase expressed from the PDX-1 promoter leading to mosaic expression of Cre-recombinase and the development of pancreatic intraepithelial neoplasia (PanIN) (Hingorani et al., 2003). Pancreata of age-matched KC mice crossed to conditional mTRAIL-R KO mice were macroscopically normal, albeit slightly smaller than KC-mTRAIL-R WT pancreata (Figure 1D). Importantly, cancer cell-specific absence of mTRAIL-R markedly reduced the number of PanINs that developed in these mice (KC-mTRAIL-R^{FL/FL}) as compared with littermate controls (KC-mTRAIL-R^{WT/WT}) (Figure 1E). The presence of PanINs was confirmed by inspecting sections stained for the epithelial marker CK19, commonly used to distinguish PanINs from normal tissue (Figure 1F). Together, these results demonstrate that cancer cell-expressed mTRAIL-R promotes *KRAS*-driven NSCLC and PanIN formation.

Figure 1. *KRAS*-Driven Lung and Pancreatic Cancers Are Promoted via Endogenous mTRAIL-R

(A) Images of representative lungs of the indicated 6-month-old KP mice.

(B) Lungs from (A) were weighed, and their weight was plotted in comparison to age-matched C57BL/6 mice.

(C) Kaplan-Meier survival curve of mice with indicated genotypes. Statistics were determined by log-rank test, ***p = 0.0008.

(D) Images of representative pancreata of the indicated 4.5-month-old KC mice.

(E) Fixed pancreata were stained with H&E, and the number of PanINs per pancreatic section was determined by histopathological examination. The bottom and top of the box are the 25th and 75th percentile of the data, respectively. The whiskers represent maximum and minimum values, *p < 0.05 (Student's t test). Scale bar represents 100 μ m.

(F) Representative H&E, α CK19 and DAPI stainings of pancreas of mice of indicated genotypes. Scale bar represents 25 μ m.

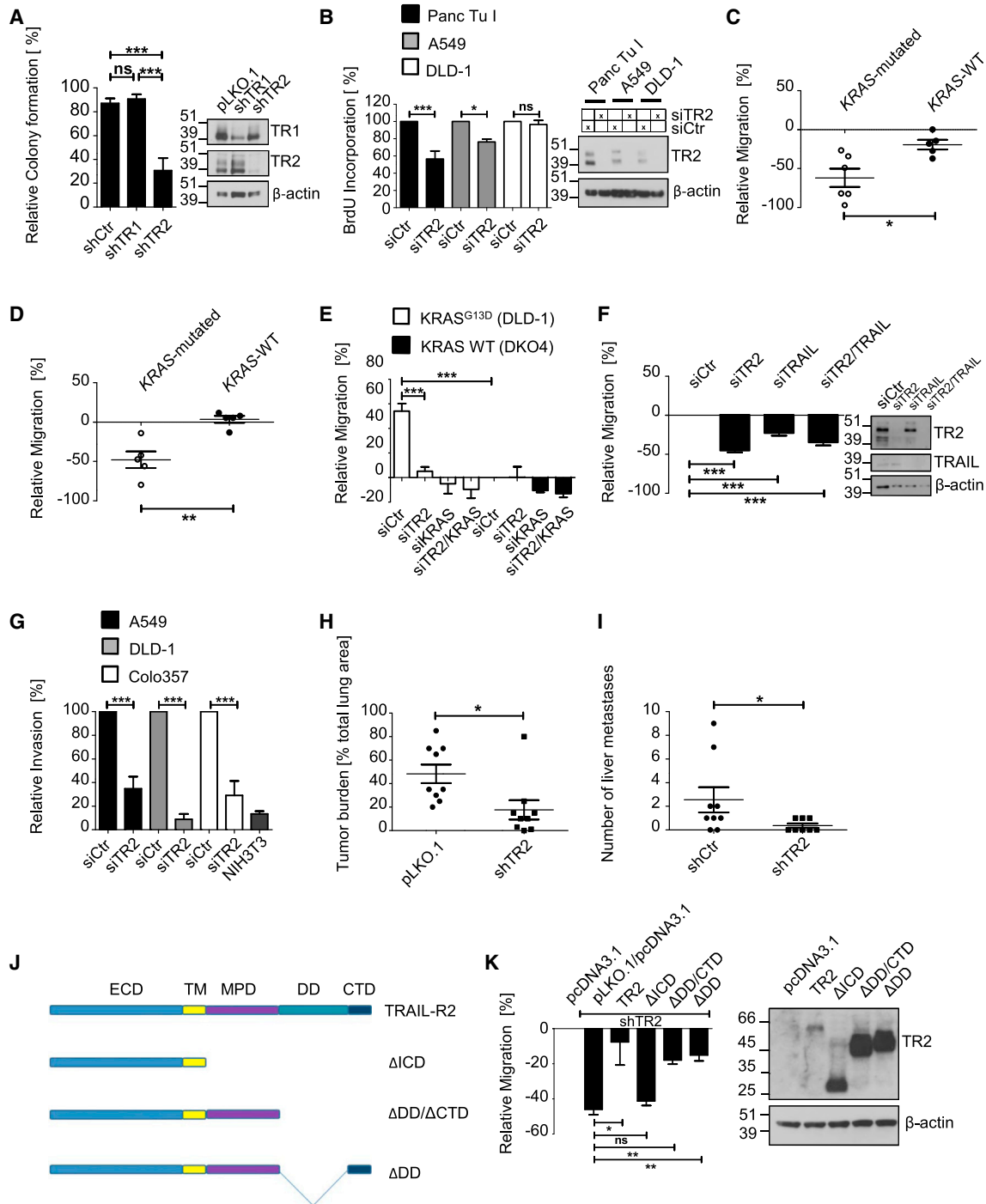


Figure 2. Human TRAIL-R2 Drives Proliferation, Migration, and Invasion

(A) A549 shControl, shTRAIL-R1, and shTRAIL-R2 were subjected to soft agar colony formation assays. Relative colony formation was determined normalized to A549-luc pLKO.1 after 4 weeks.

(B) *KRAS*-mutated Panc Tu I, A549-luc and DLD-1 cells were subjected to control or TRAIL-R2 KD for 72 hr and then BrdU proliferation assays.

(C) Six *KRAS*-mutated and five *KRAS*-WT cell lines were transfected with siRNAs silencing TRAIL-R2 or mTRAIL-R and subsequently subjected to migration assays. Migration was normalized to control transfected cells in each of the cell lines.

(D) *KRAS*-mutated cells as in (C) apart from H460 and *KRAS*-WT cells as in (C) were transfected with siRNAs silencing human or murine *KRAS* and subjected to migration assays.

(E) DLD-1 and DKO4 cells were transfected as indicated and subjected to migration assays. Migration was normalized to control transfected DKO4 cells.

(F) Panc Tu I cells were transfected as indicated and subjected to migration assays.

(G) A549-luc, DLD-1, and Colo357 cells were subjected to invasion assays.

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Human TRAIL-R2 Drives Cancer Cell Proliferation, Migration, and Invasiveness

Humans express two mTRAIL-R homologs, TRAIL-R1 and TRAIL-R2. Therefore, we next tested whether and which of these two receptors could be responsible for the promotion of oncogenic cellular traits. Interestingly, stable RNA interference (RNAi)-mediated suppression of TRAIL-R2, but not TRAIL-R1, in the *KRAS*-mutated NSCLC cell line A549 led to reduced colony formation in soft agar (Figure 2A). In addition, knockdown (KD) of TRAIL-R2 in *KRAS*-mutated Panc Tu 1 (pancreas), A549 (lung), and DLD-1 (colon) cells, representing the three most common cancer types with *KRAS* mutations led to decreased proliferation in two of three cell lines (Figure 2B). Irrespective of this mixed response between cell lines, KD of TRAIL-R2 or mTRAIL-R reduced basal migration of *KRAS*-mutated cell lines further than that of *KRAS*-WT cells, irrespective of tissue origin (Figures 2C, S1A, and S1B; Table S1). Despite detectable expression of both DD-containing TRAIL-Rs (Figure S1C), migration was affected only by KD of TRAIL-R2 (Figure S1D).

Intriguingly, the suppressive effect of KD of TRAIL-R2 on migration was almost identical to that of KD of *KRAS* (Figure 2D). To test whether TRAIL-R2-mediated migration would be responsible for the increased migratory capacity observed in *KRAS*-mutated as compared with *KRAS*-WT cells, we used isogenic colon carcinoma cell lines that contained the oncogenic mutant *KRAS* (*KRAS*^{G13D}) and WT allele (DLD-1) or only the WT allele (DKO4) (Shirasawa et al., 1993). KD of TRAIL-R2 abrogated the enhanced migratory capacity of *KRAS*-mutated DLD-1 cells, whereas basal migration of *KRAS*-WT DKO4 cells remained unaltered (Figure 2E) despite expression of comparable levels of TRAIL-R2 and efficient KD (Figure S1E). Moreover, TRAIL-R2-mediated migration was ligand dependent as KD of endogenous TRAIL reduced migration to a similar extent as TRAIL-R2 KD alone or the combined KD of both proteins (Figure 2F). In addition, TRAIL blockage using TRAIL-R2-Fc similarly inhibited migration (Figure S1F). Interestingly, when subjecting native cell lysates to size-exclusion chromatography (SEC), a portion of TRAIL-R2 was present in high molecular weight fractions along with endogenous TRAIL (Figure S1G). Furthermore, KD of TRAIL led to a shift of TRAIL-R2 from high molecular weight fractions to the size range of trimerized TRAIL-R2 (Figure S1H). Besides affecting migration, TRAIL-R2-KD also blunted invasion to nearly the same low level exhibited by the noninvasive cell line NIH 3T3 (Figure 2G).

Finally, absence of human TRAIL-R2 or systemic inhibition of TRAIL using TRAIL-R2-Fc treatment also suppressed lung tumor growth (Figures 2H, S1I, and S1J) and PDAC liver metastasis in a previously described PDAC metastasis model (Figures 2I and

S1K) (Egberts et al., 2008). Thus, TRAIL-R2 is the human homolog of mTRAIL-R responsible for stimulating tumor growth, migration, and metastasis.

TRAIL triggers apoptosis through formation of the death-inducing signaling complex (DISC) containing FADD, caspase-8 and caspase-10 and cFLIP (Kischkel et al., 2000, 2001; Sprick et al., 2000, 2002). Surprisingly, we found that suppression of caspase-8 did not affect migration (Figure S1L), and TRAIL-R2 KD still inhibited migration of three independent A549 FADD KO clones (Figure S1M). TRAIL-R2-mediated migration therefore does not require the DISC.

To identify the intracellular migration effector domain of TRAIL-R2, we next reconstituted stable TRAIL-R2 KD cells with either full-length TRAIL-R2 or mutants lacking the entire intracellular domain (Δ ICD), the DD (Δ DD), the short C-terminal domain (Δ CTD), or the DD and CTD (Δ DD/CTD) (Figure 2J). Expression of the full-length TRAIL-R2, Δ DD/CTD, or Δ CTD was sufficient to reconstitute migration (Figure 2K). Therefore, the membrane-proximal domain (MPD) of TRAIL-R2, a domain with previously unknown function, is the minimum intracellular domain required to reconstitute migration.

Spontaneous Metastasis in *KRAS*-Driven PDAC Requires Cancer Cell-Expressed mTRAIL-R

Since human TRAIL-R2 was required for migration and invasion besides proliferation (Figure 2), we next investigated the role of mTRAIL-R in the metastatic KPC mouse model of PDAC (Hingorani et al., 2005). Recombination of the floxed mTRAIL-R allele could be detected in the pancreata of KPC-mTRAIL-R^{FL/FL} mice along with remaining floxed alleles due to mosaic expression of Cre (Figure S2A). Absence of mTRAIL-R from KPC pancreatic cancer cells increased survival from a median of 112 days in KPC-mTRAIL-R^{WT/WT} mice to 180 days in KPC-mTRAIL-R^{FL/FL} mice (Figure 3A). As primary tumor size is known to be the main determinant of endpoint in this model, mTRAIL-R^{FL/FL} KPC tumors must have taken longer to reach the same size as WT tumors in order to result in prolonged survival in the absence of mTRAIL-R. Moreover, both groups had equally sized primary tumors at their respective times-of-death (Figure S2B) despite the fact that there was considerably more time for tumors to grow in the mTRAIL-R^{FL/FL} group. These results, in conjunction with the results obtained in the KC model (Figure 1E), imply that delayed primary tumor growth is a consequence of delayed PanIN formation and that this is responsible for increased survival of KPC mice with conditional mTRAIL-R deletion.

When examining animals for presence of distant metastases, we detected liver and lung metastases in a high percentage of KPC-mTRAIL-R^{WT/WT} mice while liver metastases were

(H) 2×10^6 A549-luc pLKO.1 or shTRAIL-R2 were injected intravenously into SCID beige mice ($n = 9$ mice per group). Tumor burden was quantified in paraffin sections of H&E-stained lungs by pathological inspection.

(I) 1×10^6 PancTu-1 shCtr ($n = 9$) or shTRAIL-R2 ($n = 8$) were orthotopically implanted into pancreata of SCID beige mice. After 10 days, primary tumors were resected, and the number of liver macrometastases was determined.

(J) A schematic representation of TRAIL-R2 mutants.

(K) A549 shTRAIL-R2 cells were transiently transfected with either vector (pcDNA3.1) or with the indicated TRAIL-R2 version and subsequently subjected to migration assays. Migration was normalized to A549-luc pLKO.1 cells.

Representative western blots are shown. Figures represent means of three independent experiments \pm SEM; individual dots represent the results of measurements from a single cell line shown as a mean of three independent experiments or sample from a single mouse when applicable. Ctr, control; TR2, TRAIL-R2; ICD, intracellular domain; CTD, C-terminal domain. ns, not significant, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (ANOVA: A, B, E–G, K; Student's *t* test: C, D, H; Mann-Whitney test, I).

See also Figure S1 and Table S1.

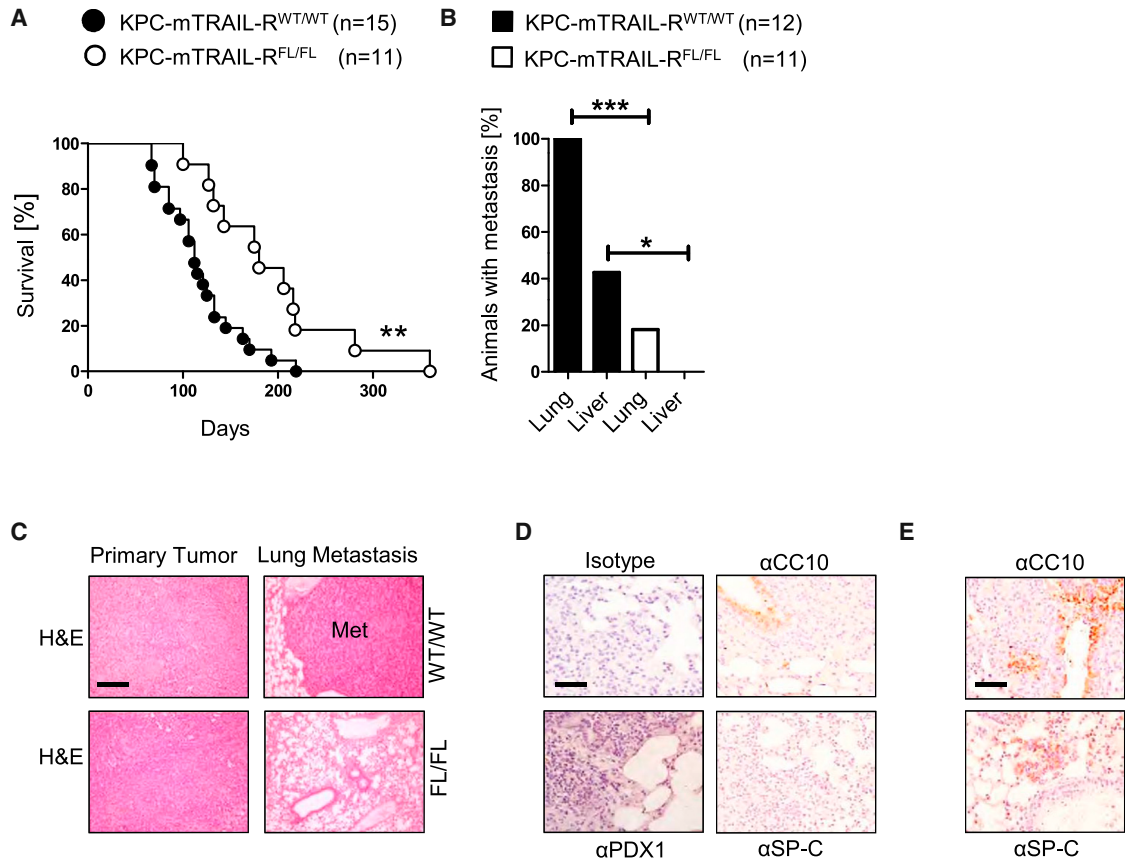


Figure 3. mTRAIL-R Promotes KRAS-Driven Pancreatic Cancer Progression and Metastasis

(A) Kaplan-Meier survival curve, log-rank test, **p = 0.004.

(B) Animals with metastasis in the liver and lungs as determined by H&E staining at time of death were quantified. Three of KPC-TRAIL-R^{WT/WT} could not be determined due to tissue decay. Fisher's exact test ***p > 0.001 and *p > 0.05.

(C) Representative H&E stainings of pancreatic adenocarcinomas and lung tissues. Met, metastasis. Scale bar represents 50 μ m.

(D) Representative images of isotype, α PDX-1, α Sp-C, and α CC10 stainings of KPC lung metastases. Scale bar represents 25 μ m.

(E) Representative images of α CC10 stainings of KP lung tumors. Scale bar represents 25 μ m.

See also Figure S2.

completely absent and lung metastases present in only two out of eleven mTRAIL-R^{FL/FL} KPC mice (Figures 3B and 3C).

To confirm that lung lesions in KPC mice originated from the pancreas, we stained for PDX1, a weakly expressed but specific pancreatic marker, Sp-C, a marker for alveolar type II epithelial cells, and CC10, a specific marker for Clara cells because KRAS-driven lung tumors in mice have previously been shown to be Sp-C positive and CC10 negative (Sotillo et al., 2010). KPC lung lesions stained positive for PDX1 and negative for Sp-C and CC10 and were therefore of pancreatic origin (Figure 3D), whereas comparative stainings of primary murine lung cancers from KP mice were positive for Sp-C and negative for CC10 within tumors (Figure 3E). Of note, the overall occurrence of metastasis did not correlate with primary tumor size (Figure S2C). Together, this demonstrates that failure to efficiently metastasize in the absence of mTRAIL-R is not a consequence of delayed primary tumor formation but specifically disabled by absence of mTRAIL-R from KRAS-driven pancreatic cancer. Thus, mTRAIL-R deletion in KRAS-driven PDAC delays primary tumor growth, prolongs survival, and blunts metastasis.

Cell-Autonomous TRAIL-R2 Stimulation Promotes Activation of Promigratory Rac1/PI3K Signaling

We noted that TRAIL-R2-KD cells had a more rounded morphology than control cells and lacked lamellipodia (Figure 4A). Interestingly, the same morphology was described for cells deficient in the small GTPase Rac1 (Steffen et al., 2013), an important mediator of migration and invasion (Sanz-Moreno and Marshall, 2010). When testing whether this pathway was affected, we found that Rac1 activity was substantially reduced by TRAIL-R2 KD (Figure 4B). Moreover, silencing of Rac1 reduced migration to a similar extent as TRAIL-R2 KD, and no additional reduction was achieved by co-suppression of TRAIL-R2 and Rac1, suggesting that TRAIL-R2 and Rac1 might act sequentially in one pathway (Figure 4C). Further substantiating this notion, while suppression of the small GTPase RhoA, known to negatively regulate Rac1 activity via ROCK activation (Sanz-Moreno et al., 2008), significantly increased migration, this was completely reversed by co-KD of TRAIL-R2 (Figure S3A). Intriguingly, overexpressed Rac1 but not dominant negative (dn) Rac1 bound to TRAIL-R2 (Figure 4D) and GTP-bound but not

GDP-bound Rac1 formed part of a complex with TRAIL-R2 and TRAIL (Figure 4E). Rac1 is known to be able to promote both migration and proliferation/tumor growth (Heasman and Ridley, 2008; Myant et al., 2013). In line with this, pharmacological Rac1 inhibition strongly impaired proliferation only in cell lines in which TRAIL-R2 promoted proliferation (Figure 4F).

Rac1 activation has been described to be either dependent or independent of phosphoinositide 3-kinase (PI3K) (Lambert et al., 2002; Nimnual et al., 1998). Therefore, we first interrogated whether KD of PTEN, which counteracts PI3K activity (Ali et al., 1999), would stimulate PI3K-dependent migration. Indeed, suppression of PTEN strongly induced migration, all of which could be blocked by treatment with the PI3K inhibitor GDC-0941 (Figure 4G). Furthermore, KD of TRAIL-R2 reduced phosphorylation of AKT in both presence and absence of FADD and was induced by expression of TRAIL-R2 Δ DD/CTD but not by Δ ICD (Figure 4H). KRAS requires direct p110 α subunit binding to activate PI3K (Gupta et al., 2007). In order to test whether oncogenic KRAS required TRAIL-R2 for this, we subjected a KRAS-WT cell line with inducible KRAS^{G13D} expression (Figure S3B) to TRAIL-R2 or control KD and treatment with either the p110 α - (A66) or p110 β -specific inhibitor (TGX221). TRAIL-R2-KD did not affect KRAS-mediated pAKT induction that was p110 α dependent (Figure S3C). PI3K-dependent migration instead required p110 β activity (Figure S3D). Interestingly, a recent study demonstrated that GTP-bound Rac1 interacts with p110 β (Fritsch et al., 2013), which was also the case in cells using TRAIL-R2 for migration (Figure S3E). Importantly, PI3K-dependent migration and increased phosphorylation of AKT could be reversed by co-suppression of either TRAIL-R2 or Rac1 (Figure 4I). Together, these results place TRAIL-R2 upstream of Rac1 and Rac1 upstream of PI3K activity in the cell migration pathway triggered by endogenous TRAIL/TRAIL-R2.

The question remained, however, how oncogenic KRAS allowed for TRAIL-R2-mediated migration. Interestingly, KRAS-mutated cells are resistant to TRAIL-induced apoptosis through KRAS-mediated ROCK suppression (Hoogwater et al., 2010). Moreover, Rac1-mediated mesenchymal migration is inhibited by ROCK (Sanz-Moreno et al., 2008). Based on these findings, we interrogated whether pharmacological ROCK inhibition might be sufficient to release a constraint on Rac1 activation in KRAS-WT cells and thus enable TRAIL-R2-mediated migration. KRAS-mutated cells showed constitutive suppression of ROCK activity as compared with isogenic WT cells (Hoogwater et al., 2010) (Figure S3F). Pharmacological ROCK inhibition using the inhibitor Y27632 was indeed sufficient to induce TRAIL-R2-mediated migration in KRAS-WT cells (Figure S3G). Moreover, this same ROCK-inhibitor-induced migration that was dependent on TRAIL-R2 could be reversed by PI3K inhibition (Figure S3H) demonstrating that ROCK inhibition, mediated by oncogenic KRAS, removes a constraint on TRAIL-R2-mediated migration. Thus, we propose a model in which endogenous TRAIL/TRAIL-R2 promote a Rac1/PI3K signaling axis in KRAS-mutated cells, which in turn drives proliferation and migration (Figure 4J).

mTRAIL-R Activates Rac1 in KPC-Derived Tumors In Vivo

Förster resonance energy transfer (FRET) is a highly sensitive method that has been used widely to determine RhoGTPase ac-

tivity in real time (Aoki and Matsuda, 2009; Hodgson et al., 2010; Kraynov et al., 2000). In order to monitor Rac1 activity, the “Raichu” (Ras superfamily and interacting protein chimeric unit) Rac-FRET probe has been developed in which Rac1 coupled to a fluorescent protein is fused to the fluorophore-coupled CRIB domain of the RacGTP-binding protein Pak1 (Itoh et al., 2002) (Figure S4A). To test whether mTRAIL-R would affect Raichu-Rac activity, we generated stable mTRAIL-R KD lines using two different shRNA sequences (23 and 25) from previously generated KPC-derived cell lines (Morton et al., 2010). These cells were subsequently transfected with a Rac-FRET probe suitable for intravital imaging and transplanted into Nude mice (Figure S4B). Subsequently, in vivo Rac1 activity was determined by intravital microscopy using cutaneous optical imaging windows (Ritsma et al., 2013). Strikingly, absence of mTRAIL-R from KPC subcutaneous tumors resulted in a significant increase in average fluorescent lifetime, corresponding to decreased Rac1 activity in both stable KD clones in live KPC tumors in vivo (Figures 5A and 5B). Moreover, blocking mTRAIL by systemic treatment with mTRAIL-R-Fc also strongly inhibited Rac1 activity in subcutaneous KPC tumors, providing evidence for endogenous TRAIL as stimulus for mTRAIL-R-mediated Rac1 activation in vivo (Figure 5C). Since systemic blockade of TRAIL can equally block host and cancer cell-derived TRAIL, we tested whether KPC-derived Rac-FRET cells were in principle self-sufficient in producing TRAIL to stimulate mTRAIL-R in vitro. Indeed, in vitro treatment of KPC-Rac-FRET cells with mTRAIL-R-Fc was sufficient to inhibit Rac1 activity (Figure 5D). Together, these data show that KRAS-mutated KPC-derived tumors use the cancer cell-expressed mTRAIL/mTRAIL-R system to promote Rac1 activity in vivo.

High TRAIL-R2 Expression Correlates with Parameters of Malignancy in Patients with KRAS-Mutated Cancers

To evaluate the relevance of TRAIL-R2 in human cancers with KRAS mutations, we analyzed TRAIL-R1 and TRAIL-R2 expression in primary PDAC resected from patients. In a cohort of 95 tumors, 84% of PDAC highly expressed TRAIL-R2, whereas expression of TRAIL-R1 was high in only 9% of PDAC with all other tumors being either negative or low for TRAIL-R1 expression (Figure 6A). Of note, it was previously shown that only TRAIL-R1 is used for apoptosis induction in PDAC cell lines (Lemke et al., 2010), suggesting that human PDAC might select for expression of TRAIL-R2 and against that of TRAIL-R1. Importantly, when analyzing another PDAC cohort (n = 106) with additional clinical information, high TRAIL-R2 expression correlated with lymph vessel invasion of tumors (Figure 6B).

Metastases from PDAC patients are rarely resected and thus seldom available for histological analysis. We therefore analyzed TRAIL-R1 and TRAIL-R2 expression in lung metastases of a cohort of 36 CRC patients of which 18 carried oncogenic KRAS mutations and 18 were KRAS-WT. In contrast to the PDAC cohorts, TRAIL-R1 and TRAIL-R2 were both expressed at detectable levels in lung metastases of KRAS-mutated and KRAS-WT CRC patients (Figure 6C), indicating that a selection for expression of one versus the other receptor had not taken place in CRC. Strikingly, however, low expression of TRAIL-R2, but not TRAIL-R1, correlated with prolonged metastasis-free survival of patients with KRAS mutant CRC (Figure 6D) but not

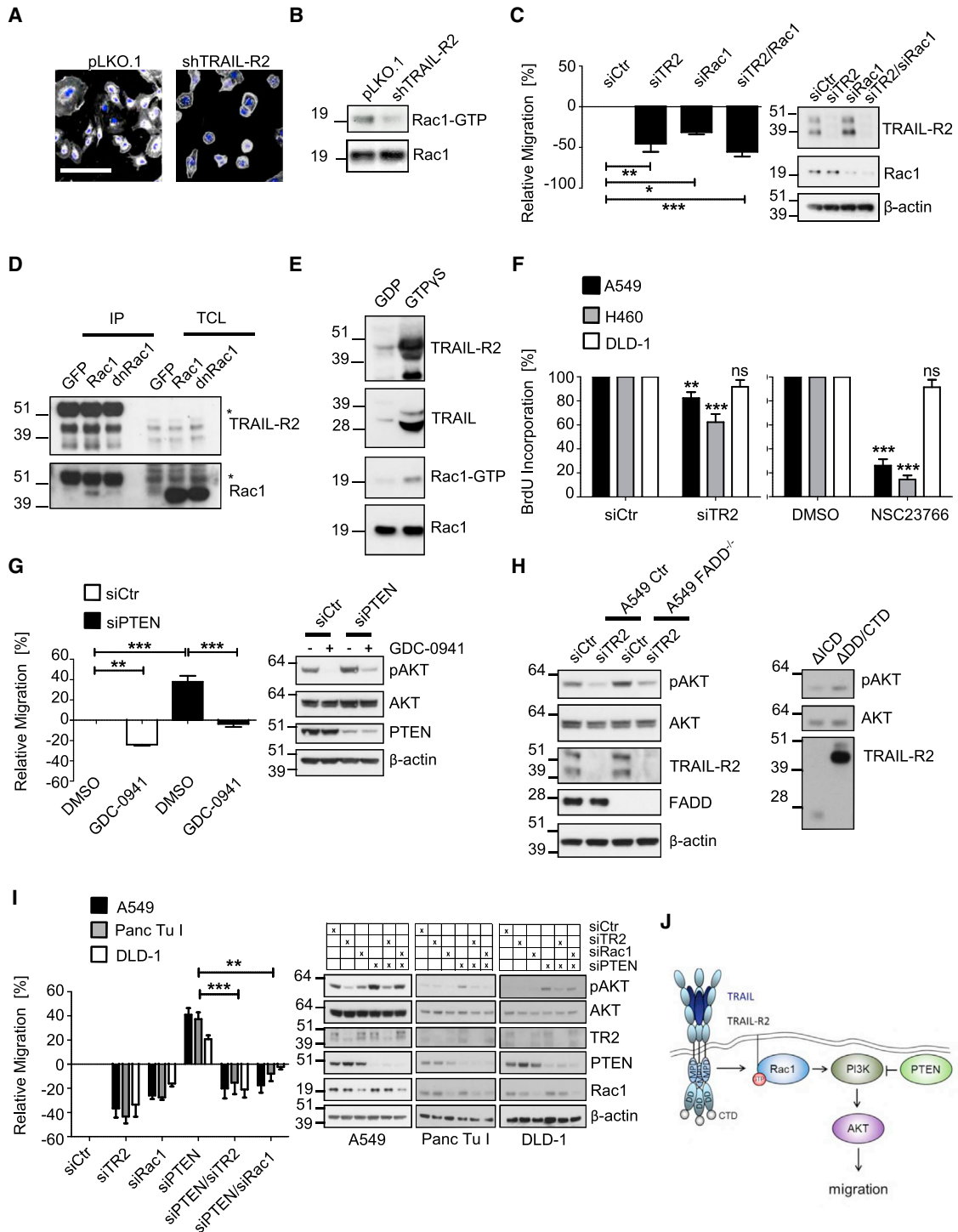


Figure 4. TRAIL-R2 Promotes Activation of a Promigratory Rac1/PI3K Signaling Axis

(A) A549-luc pLKO.1 and shTRAIL-R2 cells were fixed and stained using Texas red-coupled phalloidin and DAPI. Representative confocal images are shown; scale bar represents 25 μ m.

(B) A549-luc pLKO.1 and shTRAIL-R2 were subjected to pulldown of GTP-bound Rac1 using PAK1 beads and western blotting.

(C) A549-luc cells were transfected as shown and subjected to migration assays.

(D) A549-luc cells were transfected with GFP, Rac1, or dnRac1 (Rac1T17N) and subjected to immunoprecipitation via addition of Flag-tagged TRAIL after lysis and western blot analysis. Asterisks indicate the immunoprecipitation (IP) antibody heavy chain.

(E) Endogenous Rac1 in A549-luc cell lysates was loaded with GDP/GTP γ S in vitro, and GTP-bound Rac1 was isolated using PAK1-RBD beads and analyzed by western blot.

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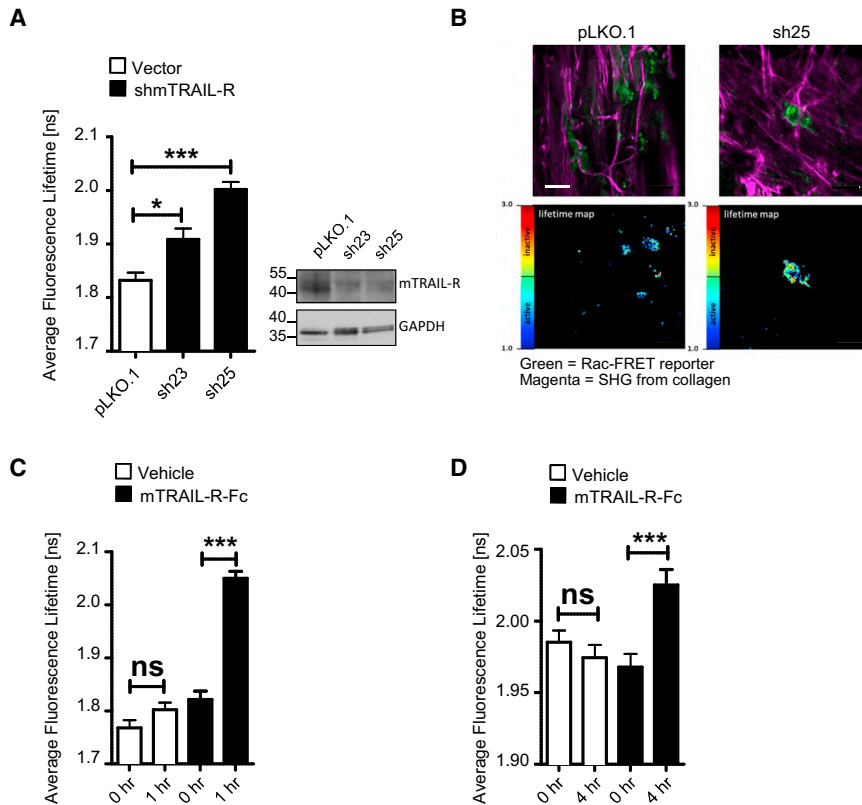


Figure 5. mTRAIL-R and mTRAIL Promote Rac1 Activity in KPC Tumors In Vivo

(A) Intravital imaging was performed on mice bearing either KPC-pLKO ($n = 3$) or -shmTRAIL-R (23, 25, $n = 3$ each) tumors, and average lifetime fluorescence was quantified. Representative western blots are shown.

(B) Lifetime maps of representative single KPC-pLKO.1 or -shmTRAIL-R (sh25) cells in vivo. SHG, second harmonic generation. Scale bar represents 50 μm .

(C) Intravital imaging was performed on mice bearing KPC tumors before and 1 hr after treatment with either vehicle or mTRAIL-R-Fc. Average lifetime fluorescence was quantified.

(D) Parental KPC cells were transiently transfected with a Rac-FRET reporter and treated with mTRAIL-R-Fc for the indicated times in vitro. Single cells were analyzed as in (A).

Values are means \pm SEM of three independent experiments. ns, not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$ (Mann-Whitney test); [ns], nano seconds. See also Figure S4.

been shown to be required for early metaplastic changes in autochthonous KRAS-driven NSCLC (Kissil et al., 2007) and PanIN formation (Heid et al., 2011) in line with our finding that mTRAIL-R promotes Rac1 activity in both cancers.

in patients with WT KRAS CRC (Figure 6E). Together, these data support a proinvasive and prometastatic role of TRAIL-R2 in KRAS-mutated human cancers.

DISCUSSION

Treatment with recombinant TRAIL was selectively able to kill tumor cells in vivo without toxicity (Ashkenazi et al., 1999; Walczak et al., 1999). It is therefore counterintuitive that apoptosis-inducing TRAIL-Rs are highly expressed in many cancers (Daniels et al., 2005; Elrod et al., 2010; Ganten et al., 2009; Macher-Goeppinger et al., 2009; Ozawa et al., 2001; Sanlioglu et al., 2007; Spierings et al., 2003).

We show here that cancer cell-expressed mTRAIL-R promotes cancer progression, invasion, and metastasis in KRAS-driven autochthonous mouse models of NSCLC and PDAC. Constitutive signaling from TRAIL-R2 promotes activation of a Rac1/PI3K signaling axis that increases migration and invasion in a cancer cell-autonomous manner. Moreover, we provide in vivo evidence that endogenous mTRAIL and mTRAIL-R activate Rac1 in KPC tumor cells. Interestingly, Rac1 has

Moreover, elevated Rac1 activity has been measured in KPC tumors as compared with normal pancreatic tissue (Johnsson et al., 2014). Our results now show that blocking the endogenous TRAIL/mTRAIL-R interaction reduces Rac1 activation, delays tumor growth, and blunts metastasis. Since TRAIL- and TRAIL-R-deficient mice do not show a gross phenotype (Cretney et al., 2002; Finnberg et al., 2008; Grosse-Wilde et al., 2008), therapeutic inhibition of TRAIL or TRAIL-R2 is unlikely to cause toxicity and might therefore be advantageous over other therapeutic strategies targeting KRAS-effector pathways.

Mechanistically, we identify that TRAIL-R2 mediates migration via the MPD of TRAIL-R2. Intriguingly, human TRAIL-R2 and mTRAIL-R share a part of the MPD that is not present in the TRAIL-R1 MPD. It will therefore be interesting to delineate the precise molecular pathway that is triggered from this domain. This will, however, require more sensitive experimental approaches, as the endogenous TRAIL/TRAIL-R complex is not abundant enough for proteomic analysis (data not shown).

Recently, endogenous TRAIL-R2 has been shown to promote proliferation of pancreatic cancer cell lines via inhibition of let-7 maturation in the nucleus in a ligand-independent manner

(F) The indicated cell lines were subjected to KDs or NSC23766 treatment (100 μM) for 72 hr and then BrdU proliferation assays.

(G) A549-luc cells were subjected to the indicated KDs and migration assays or western blot analysis in the presence or absence of GDC-0941 (1 μM).

(H) FADD-deficient and proficient A549-luc cells were transfected with TRAIL-R2-targeting siRNAs and A549 shTRAIL-R2 cells were reconstituted with either ΔICD or $\Delta\text{DDD/CTD}$. All cells were subjected to western blotting.

(I) A549-luc, PancTu-1, and DLD-1 cells were transfected as indicated and subjected to migration assays or western blot analysis.

(J) Proposed model of migration signaling mediated by endogenous TRAIL/TRAIL-R2 in KRAS-mutated cells.

Representative western blots are shown. Values are means \pm SEM of three independent experiments. ICD, intracellular domain; CTD, C-terminal domain; Ctr, Control; TR2, TRAIL-R2; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$ (ANOVA). See also Figure S3.

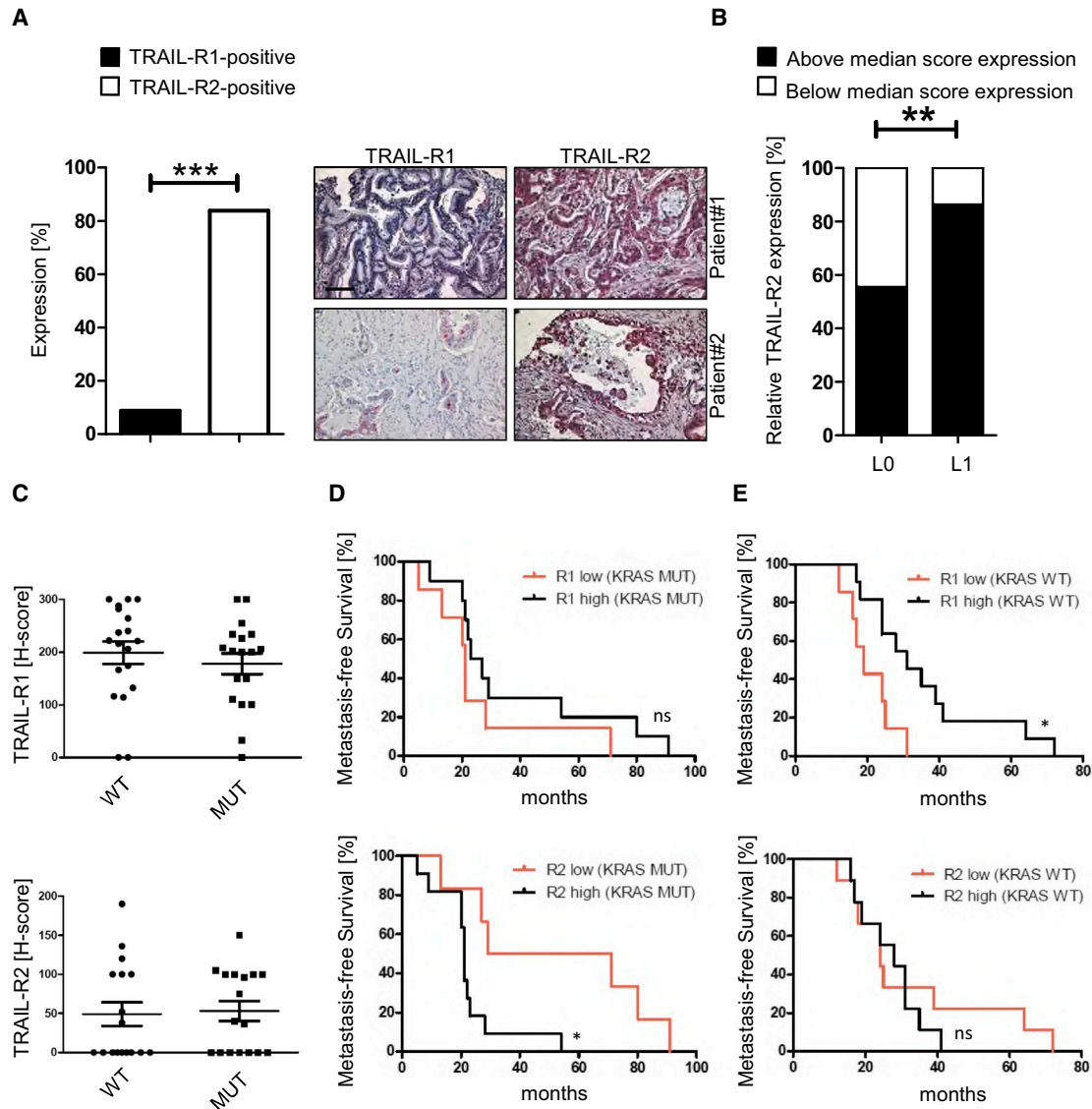


Figure 6. High TRAIL-R2 Expression Correlates with Markers of Malignancy in Patients with *KRAS*-Mutated Cancers

(A) TRAIL-R1 and TRAIL-R2 expression in human PDAC samples of 95 patients was quantified. More than 20% of positive (intensity of a minimum of 1 on a scale of 1 to 3; Ganten et al., 2009) cancer cells within one patient sample were considered high expression and included into the figure, which depicts percentage of high TRAIL-R expression within the patient population. Representative stainings from two patients are shown. Scale bar represents 25 μ m. Fisher's exact test *** $p < 0.0001$.

(B) TRAIL-R2 expression was determined in a human PDAC cohort ($n = 106$). All tumors were positive ranging from score 3 to 7 (median = 5). Data were dichotomized by the median 5, and the percentage of cases above (black) and below (white) median was calculated separately for cases with lymph vessel invasion (L1, $n = 71$) and cases without lymph vessel invasion (L0, $n = 30$). In five patients, lymph vessel invasion had not been determined, although tumors also stained positive. Fisher's exact test ** $p = 0.001$.

(C) TRAIL-R1 and TRAIL-R2 expression levels (H score) in lung metastases of *KRAS*-mutated and *KRAS*-WT CRC. The central line represents the mean. Error bars are \pm SEM.

(D) Metastasis-free (Kaplan-Meier) survival according to high or low TRAIL-R1/2 expression (i.e., above or below respective H-score median) in patients with *KRAS*-mutated CRC. Log-rank test, ns = not significant; * $p < 0.02$.

(E) As in (D), in patients with *KRAS*-WT CRC. Log-rank test, ns = not significant; * $p < 0.02$.

(Haselmann et al., 2014). We find endogenous TRAIL bound to TRAIL-R2 associated with the active form of Rac1, which is mainly found in association with the plasma membrane, suggesting that the TRAIL/TRAIL-R2-mediated Rac1 activation occurs at the plasma membrane.

We previously showed in an autochthonous model of skin cancer formation, driven by oncogenic mutation of *Hras* (Balmain et al., 1984), that constitutive mTRAIL-R deficiency suppressed metastasis (Grosse-Wilde et al., 2008). This antimetastatic role of the mTRAIL-R seems to contradict the findings reported

here. However, we could confirm an antimetastatic role for TRAIL-R1 in patients with *KRAS*-WT CRC as high expression of this receptor correlated with prolonged metastasis-free survival. In contrast to this, high TRAIL-R2 expression in *KRAS*-mutated CRC correlated with shortened metastasis-free survival, suggesting that the role of TRAIL-R2 and TRAIL-R1 as well as mTRAIL-R is dependent on the oncogenic context. Accordingly, TRAIL-R2 suppression does not affect migration in *KRAS*-WT or *HRAS*-mutated cells (data not shown). Furthermore, we identify ROCK inhibition to be the prerequisite for TRAIL/TRAIL-R2-mediated migration. In addition, induction of oncogenic *HRAS* expression, which did not allow for TRAIL-R2-mediated migration, also did not inhibit ROCK activity (data not shown). It will therefore be interesting to investigate whether other pathways that inhibit ROCK might utilize TRAIL/TRAIL-R2 similarly.

In conclusion, we identify cancer cell endogenous TRAIL-R2/mTRAIL-R stimulation as a promoter of cancer progression, invasion, and metastasis of *KRAS*-mutated cancers offering an explanation as to why these cancers not only retain TRAIL-R expression but also frequently upregulate it. On the basis of these results, it is tempting to speculate whether patients with *KRAS*-mutated cancers that are resistant to apoptosis induction might benefit from inhibition of the constitutive TRAIL/TRAIL-R2 interaction.

EXPERIMENTAL PROCEDURES

Animal Models

A549 Lung Tumors

Twelve-week-old female Fox Chase SCID Beige mice (Charles River) were injected with 2×10^6 A549-luc vector control or shTRAIL-R2 cells via the lateral tail vein. Starting on day 1 after cell injection, all mice were imaged weekly for bioluminescence using the IVIS Spectrum at 1-min exposure time (Caliper Life Science). Photons per second (Photon Flux) were quantified using IVIS Spectrum software.

Orthotopic PDAC Metastasis

Experiments were carried out in female SCID beige mice (Charles River). 1×10^6 PancTu-1 shCtr or shTRAIL-R2 were orthotopically transplanted into the pancreas, and tumors were resected 10 days later as previously described (Egberts et al., 2008). Animals were sacrificed and organs preserved at day 26 postresection.

K(P)C-mTRAIL-R Mice

Mixed-gender *Kras*^{G12D}, *p53*^{R172H}, and PDX-1-Cre mice on C57BL/6 background were kindly provided by D. Tuveson, and mTRAIL-R^{FL/FL} mice on C57BL/6 background were previously generated by our laboratory (Grosse-Wilde et al., 2008).

KP-mTRAIL-R Mice

Mixed-gender *Kras*^{G12D}, *p53*^{R172H}, mTRAIL-R^{WT/WT} or ^{FL/FL} mice were intranasally infected with a single dose of 2×10^7 pfu Ad-Cre to induce tumors.

Rac-FRET Tumors

After transfection of KPC shCtr and shTRAIL-R2 cell lines with a GFP and RFP version of the Rac-FRET reporter (Mack et al., 2012) and selection for stable expression with neomycin, 2×10^6 cells were subcutaneously injected into the flanks of nude mice. Following primary tumor development of 7 to 8 days, a cutaneous version of the abdominal imaging window described previously (Ritsma et al., 2013) was surgically implanted on top of the tumors under isoflurane. Mice were subsequently imaged for up to 2 hr on a 37°C heated stage under isoflurane, using the described multiphoton TCSPC system. For treatment with mTRAIL-R-Fc, mice were imaged prior to and 1 hr after intraperitoneal injection of either PBS or 0.5 mg per mouse of mTRAIL-R-Fc.

The orthotopic PDAC metastasis experiment was conducted in accordance with the Office of Laboratory Animal Welfare and the German Federal Animal Protection Laws and approved by the Institutional Animal Care and Use Com-

mittees of the University of Kiel. The Rac-FRET experiments were conducted under an appropriate animal project license approved by the UK home office and in accordance with the Animal Welfare and Experimental Ethics Committee at the Beatson Institute of Cancer Research. All other animal experiments were conducted under an appropriate animal project license approved by the UK home office, in accordance with the revised (2013) Animals (Scientific Procedures) Act (ASPA) and the institutional guidelines of the UCL Cancer Institute.

siRNA-Mediated KD

KD experiments were performed by transient transfection of siRNA smartpools or single siRNA sequences to silence the indicated proteins or RISC-free control using Dharmafect I (Dharmacon) following the instruction manual. All cell lines were incubated for 48 hr to achieve sufficient KD efficiency. See [Supplemental Experimental Procedures](#) for detailed description.

Migration/Invasion Assays

Migration/Invasion assays were performed using the xCELLigence System (Cambridge Bioscience). See [Supplemental Experimental Procedures](#) for detailed description.

Patient Cohorts

Informed consent was obtained from all individuals, and use of samples for histology was approved by the ethics committees of the University of Heidelberg (PDAC cohort 1), Kiel (PDAC cohort 2), and of the Medical University of Vienna (CRC lung metastasis cohort). See [Supplemental Experimental Procedures](#) for detailed description.

Statistical Analysis

Data were analyzed using GraphPad Prism 5 software (GraphPad Software). Results are expressed as means \pm SEM. Statistical significance between groups was determined using Student's *t* test and/or one-way ANOVA, followed by the Bonferroni post-test. A *p* value of < 0.05 was considered significant: ***p* < 0.01 and ****p* < 0.001 (ns = nonsignificant).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.ccell.2015.02.014>.

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