Europe PMC Funders Group
Author Manuscript
Cancer Cell. Author manuscript; available in PMC 2019 June 24.

Published in final edited form as:
Cancer Cell. 2015 April 13; 27(4): 561–573. doi:10.1016/j.ccell.2015.02.014.

Cancer cell-autonomous TRAIL-R signaling promotes KRAS-driven cancer progression, invasion and metastasis

Silvia von Karstedt[1], **Annalisa Conti**[1,2], **Max Nobis**[3], **Antonella Montinaro**[1], **Torsten Hartwig**[1], **Johannes Lemke**[1], **Karen Legler**[4], **Franka Annewanter**[4], **Andrew D. Campbell**[3], **Lucia Taraborrelli**[1], **Anne Grosse-Wilde**[5,6], **Johannes F. Coy**[5,7], **Mona A. El-Bahrawy**[8], **Frank Bergmann**[9], **Ronald Koschny**[10], **Jens Werner**[11], **Tom M. Ganten**[10], **Thomas Schweiger**[12,13], **Konrad Hoetzenecker**[13], **Istvan Kenessey**[14], **Balazs Hegedüs**[12,14], **Michael Bergmann**[12], **Charlotte Hauser**[15], **Jan-Hendrik Egberts**[15], **Thomas Becker**[15], **Christoph Röcken**[16], **Holger Kalthoff**[4], **Anna Trauzold**[4,15], **Kurt I. Anderson**[3], **Owen J. Sansom**[3], and **Henning Walczak**[1,*]

[1]Centre for Cell Death, Cancer and Inflammation, UCL, 72 Huntley Street, London WC1E 6DD, UK

[2]Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, 20133 Milan, Italy

[3]Beatson Institute for Cancer Research, Switchback Road, Bearsden, Glasgow G61 1BD, UK

[4]Division of Molecular Oncology, Institute for Experimental Cancer Research, University of Kiel, Kiel D-24105, Germany

[5]German Cancer Research Centre (DKFZ), Im Neuenheimer Feld 580, 69120 Heidelberg, Germany

[6]Institute for Systems Biology, 401 Terry Ave N, Seattle WA 98109, USA

[7]TAVARLIN AG, Biotechpark Pfungstadt, Reißstraße 1a, 64319 Pfungstadt, Germany

[8]Department of Histopathology, Imperial College London, Du Cane Road, London W12 0NN, UK

[9]Institute of Pathology, University Hospital Heidelberg, Im Neuenheimer Feld 224, 69120 Heidelberg, Germany

[10]Department of Gastroenterology, University Hospital Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany

[11]Department of Surgery, University Hospital Heidelberg, Im Neuenheimer Feld 110, 69120 Heidelberg, Germany

[12]Department of Thoracic Surgery, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

[13]Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

[*]Correspondence: Henning Walczak, h.walczak@ucl.ac.uk, phone: +44 207 67946471; FAX: +44 207 679 6925.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

[14]2nd Department of Pathology, Semmelweis University Budapest, Ulloi ut 93, H-1091 Budapest, Hungary

[15]Department of General Surgery, Visceral, Thoracic, Transplantation and Pediatric Surgery, University Hospital Schleswig-Holstein, Kiel, Germany

[16]Department of Pathology, Christian-Albrechts-University, Kiel, Germany

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.

Introduction

21% 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 KRASG12C 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 non-apoptotic signaling pathways in cancer cell lines such as NF-κB (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

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

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 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-Goeppinger et al., 2009; Ozawa et al., 2001; Sanlioglu 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-R in KRAS-driven cancer.

Results

Cancer cell-expressed mTRAIL-R promotes autochthonous KRAS-driven lung cancer and formation of pancreatic intraepithelial neoplasia (PanIN)

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 amongst 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 (PanINs) (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 to littermate controls (KC-mTRAIL-R$^{WT/WT}$) (Figure 1E). Presence of PanINs was confirmed by

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

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.

Human TRAIL-R2 drives cancer cell proliferation, migration and invasiveness

Humans express two mTRAIL-R homologues, 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 I (pancreas), A549 (lung) and DLD-1 (colon) cells, representing the three most common cancer types with *KRAS* mutations, led to decreased proliferation in two out 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, S1B and Table S1). Despite detectable expression of both DD-containing TRAIL-Rs (Figure S1C), migration was only affected 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 to -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 non-invasive cell line NIH3T3 (Figure 2G).

Finally, absence of human TRAIL-R2 or systemic inhibition of TRAIL using TRAIL-R2-Fc treatment also suppressed lung tumor growth (Figure 2H, S1I, S1J) and PDAC liver metastasis in a previously described PDAC metastasis model (Figure 2I, S1K) (Egberts et al., 2008). Thus, TRAIL-R2 is the human homologue 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 -10 and cFLIP (Kischkel et al., 2000; Kischkel et al., 2001; Sprick et al., 2002; Sprick et al., 2000). Surprisingly, we found that suppression of

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

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 (ICD), the DD (DD), the short C-terminal domain (CTD), or the DD and CTD (DD/CTD) (Figure 2J). Expression of the full length TRAIL-R2, DD/CTD or 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 whilst liver metastases were completely absent and lung metastases present in only two out of eleven mTRAIL-R$^{FL/FL}$ KPC mice (Figure 3B, C).

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

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

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 pro-migratory Rac1/PI3K signalling

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, whilst 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 DD/CTD but not by ICD (Figure 4H). KRAS requires direct p110α 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 p110α- (A66) or p110β-specific inhibitor (TGX221). TRAIL-R2-KD did not affect KRAS-mediated pAKT induction that was p110α-dependent (Figure S3C). PI3K-dependent migration instead required p110β activity (Figure S3D). Interestingly, a recent study demonstrated that GTP-bound Rac1 interacts with p110β (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.

Europe PMC Funders Author Manuscripts

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 to 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 activity 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 fluorophor-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 (Figure 5A, B). 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 colorectal cancer (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 –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 in patients with WT *KRAS* CRC (Figure 6E). Together, these data support a pro-invasive 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 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. Moreover, elevated Rac1 activity has been measured in KPC tumors as compared to 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.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

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 (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 anti-metastatic role of the mTRAIL-R, seems to contradict the findings reported here. However, we could confirm an anti-metastatic 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 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—12-week-old female Fox Chase® SCID Beige Mice (Charles River, Germany) were injected with 2 x 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 minute 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, Germany). 1 x 10^6 PancTu-I 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 post resection.

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, 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 x 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 x 10^6 cells were subcutaneously injected into the flanks of nude mice. Following primary tumor development of 7-8 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 post i.p. injection of either PBS or 0.5 mg/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 Committees of the University of Kiel, Germany. 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, UK. 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, UK.

siRNA-mediated knockdown

Knockdown 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 knockdown efficiency. See Supplemental Experimental Procedures for detailed description.

Migration/Invasion assays

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

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

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, Germany (PDAC cohort 1), Kiel, Germany (PDAC cohort 2), and of the Medical University of Vienna, Austria (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 ± SEM. Statistical significance between groups was determined using Student's t-test and/or one-way analysis of variance (ANOVA), followed by the Bonferroni post-test. A p-value of <0.05 was considered significant and indicated with *, $p<0.01$=** and $p<0.001$=***. ns= non-significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank O. Kranenburg for helpful discussions which inspired our investigation of the endogenous TRAIL-R system and D. Murphy for helpful discussions regarding the autochthonous mouse models. We would also like to thank T. Jacks and D. Tuveson for the KP and KPC mouse models and J. Downward and D. Hancock for many cell lines; A. Malliri and M. Daugaard for providing Rac1 plasmids, A. Daboh, H. Draberova, M. Reichert, L. Hamann, B. García and M. Abd El Hay for practical assistance. This work was supported by a Cancer Research UK programme grant (A17341), a Wellcome Trust Senior Investigator Award (096831/Z/11/Z) and an ERC Advanced grant (294880) held by H.W.. A.C. was supported by the Italian Foundation for Cancer Research (FIRC), J.L. was supported by the Dr. Mildred-Scheel Stiftung/Deutsche Krebshilfe. The contribution of A.T., K.L. and F.A. was funded by the German Research Foundation (DFG TR1063/2-1).

References

Ali IU, Schriml LM, Dean M. Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity. Journal of the National Cancer Institute. 1999; 91:1922–1932. [PubMed: 10564676]

Aoki K, Matsuda M. Visualization of small GTPase activity with fluorescence resonance energy transfer-based biosensors. Nature protocols. 2009; 4:1623–1631. [PubMed: 19834477]

Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest. 1999; 104:155–162. [PubMed: 10411544]

Azijli K, Yuvaraj S, Peppelenbosch MP, Wurdinger T, Dekker H, Joore J, van Dijk E, Quax WJ, Peters GJ, de Jong S, Kruyt FA. Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3 dependent invasion in resistant non-small cell lung cancer cells. Journal of cell science. 2012

Baines AT, Xu D, Der CJ. Inhibition of Ras for cancer treatment: the search continues. Future medicinal chemistry. 2011; 3:1787–1808. [PubMed: 22004085]

Balmain A, Ramsden M, Bowden GT, Smith J. Activation of the mouse cellular Harvey-ras gene in chemically induced benign skin papillomas. Nature. 1984; 307:658–660. [PubMed: 6694757]

Belyanskaya LL, Ziogas A, Hopkins-Donaldson S, Kurtz S, Simon HU, Stahel R, Zangemeister-Wittke U. TRAIL-induced survival and proliferation of SCLC cells is mediated by ERK and dependent on TRAIL-R2/DR5 expression in the absence of caspase-8. Lung Cancer. 2008; 60:355–365. [PubMed: 18093694]

Bos JL. ras oncogenes in human cancer: a review. Cancer research. 1989; 49:4682–4689. [PubMed: 2547513]

Castellano E, Sheridan C, Thin MZ, Nye E, Spencer-Dene B, Diefenbacher ME, Moore C, Kumar MS, Murillo MM, Gronroos E, et al. Requirement for interaction of PI3-kinase p110alpha with RAS in lung tumor maintenance. Cancer cell. 2013; 24:617–630. [PubMed: 24229709]

Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ, Smyth MJ. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. J Immunol. 2002; 168:1356–1361. [PubMed: 11801676]

Daniels RA, Turley H, Kimberley FC, Liu XS, Mongkolsapaya J, Ch'En P, Xu XN, Jin BQ, Pezzella F, Screaton GR. Expression of TRAIL and TRAIL receptors in normal and malignant tissues. Cell Res. 2005; 15:430–438. [PubMed: 15987601]

Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity. 1997; 7:813–820. [PubMed: 9430226]

Downward J. Targeting RAS and PI3K in lung cancer. Nature medicine. 2008; 14:1315–1316.

Egberts JH, Cloosters V, Noack A, Schniewind B, Thon L, Klose S, Kettler B, von Forstner C, Kneitz C, Tepel J, et al. Anti-tumor necrosis factor therapy inhibits pancreatic tumor growth and metastasis. Cancer research. 2008; 68:1443–1450. [PubMed: 18316608]

Ehrhardt H, Fulda S, Schmid I, Hiscott J, Debatin KM, Jeremias I. TRAIL induced survival and proliferation in cancer cells resistant towards TRAIL-induced apoptosis mediated by NF-kappaB. Oncogene. 2003; 22:3842–3852. [PubMed: 12813457]

Elrod HA, Fan S, Muller S, Chen GZ, Pan L, Tighiouart M, Shin DM, Khuri FR, Sun SY. Analysis of death receptor 5 and caspase-8 expression in primary and metastatic head and neck squamous cell carcinoma and their prognostic impact. PloS one. 2010; 5:e12178. [PubMed: 20808443]

Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R, Maira M, McNamara K, Perera SA, Song Y, et al. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nature medicine. 2008; 14:1351–1356.

Finnberg N, Klein-Szanto AJ, El-Deiry WS. TRAIL-R deficiency in mice promotes susceptibility to chronic inflammation and tumorigenesis. J Clin Invest. 2008; 118:111–123. [PubMed: 18079962]

Fritsch R, de Krijger I, Fritsch K, George R, Reason B, Kumar MS, Diefenbacher M, Stamp G, Downward J. RAS and RHO families of GTPases directly regulate distinct phosphoinositide 3-kinase isoforms. Cell. 2013; 153:1050–1063. [PubMed: 23706742]

Ganten TM, Sykora J, Koschny R, Batke E, Aulmann S, Mansmann U, Stremmel W, Sinn HP, Walczak H. Prognostic significance of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor expression in patients with breast cancer. J Mol Med (Berl). 2009; 87:995–1007. [PubMed: 19680616]

Grady WM, Markowitz SD. Genetic and epigenetic alterations in colon cancer. Annu Rev Genomics Hum Genet. 2002; 3:101–128. [PubMed: 12142355]

Grosse-Wilde A, Voloshanenko O, Bailey SL, Longton GM, Schaefer U, Csernok AI, Schutz G, Greiner EF, Kemp CJ, Walczak H. TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. J Clin Invest. 2008; 118:100–110. [PubMed: 18079967]

Gupta S, Ramjaun AR, Haiko P, Wang Y, Warne PH, Nicke B, Nye E, Stamp G, Alitalo K, Downward J. Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. Cell. 2007; 129:957–968. [PubMed: 17540175]

Harper N, Farrow SN, Kaptein A, Cohen GM, MacFarlane M. Modulation of tumor necrosis factor apoptosis-inducing ligand- induced NF-kappa B activation by inhibition of apical caspases. The Journal of biological chemistry. 2001; 276:34743–34752. [PubMed: 11461927]

Haselmann V, Kurz A, Bertsch U, Hubner S, Olempska-Muller M, Fritsch J, Hasler R, Pickl A, Fritsche H, Annewanter F, et al. Nuclear Death Receptor TRAIL-R2 Inhibits Maturation of Let-7 and Promotes Proliferation of Pancreatic and Other Tumor Cells. Gastroenterology. 2014; 146:278–290. [PubMed: 24120475]

Heasman SJ, Ridley AJ. Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nature reviews Molecular cell biology. 2008; 9:690–701. [PubMed: 18719708]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Heid I, Lubeseder-Martellato C, Sipos B, Mazur PK, Lesina M, Schmid RM, Siveke JT. Early requirement of Rac1 in a mouse model of pancreatic cancer. Gastroenterology. 2011; 141:719–730. [PubMed: 21684285]

Hidalgo M. Pancreatic cancer. The New England journal of medicine. 2010; 362:1605–1617. [PubMed: 20427809]

Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer cell. 2003; 4:437–450. [PubMed: 14706336]

Hingorani SR, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH, Rustgi AK, Chang S, Tuveson DA. $Trp53^{R172H}$ and $KRAS^{G12D}$ cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. Cancer cell. 2005; 7:469–483. [PubMed: 15894267]

Hodgson, L, Shen, F, Hahn, K. Biosensors for characterizing the dynamics of rho family GTPases in living cellsCurrent protocols in cell biology. editorial board; 2010. Chapter 14 Unit 14 11 11–26. Juan S Bonifacino [et al]

Hoogwater FJ, Nijkamp MW, Smakman N, Steller EJ, Emmink BL, Westendorp BF, Raats DA, Sprick MR, Schaefer U, Van Houdt WJ, et al. Oncogenic K-Ras turns death receptors into metastasis-promoting receptors in human and mouse colorectal cancer cells. Gastroenterology. 2010; 138:2357–2367. [PubMed: 20188103]

Ishimura N, Isomoto H, Bronk SF, Gores GJ. Trail induces cell migration and invasion in apoptosis-resistant cholangiocarcinoma cells. Am J Physiol Gastrointest Liver Physiol. 2006; 290:G129–136. [PubMed: 16166346]

Itoh RE, Kurokawa K, Ohba Y, Yoshizaki H, Mochizuki N, Matsuda M. Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. Molecular and cellular biology. 2002; 22:6582–6591. [PubMed: 12192056]

Jackson EL, Willis N, Mercer K, Bronson RT, Crowley D, Montoya R, Jacks T, Tuveson DA. Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. Genes & development. 2001; 15:3243–3248. [PubMed: 11751630]

Jaffee EM, Hruban RH, Canto M, Kern SE. Focus on pancreas cancer. Cancer cell. 2002; 2:25–28. [PubMed: 12150822]

Johnsson AK, Dai Y, Nobis M, Baker MJ, McGhee EJ, Walker S, Schwarz JP, Kadir S, Morton JP, Myant KB, et al. The Rac-FRET mouse reveals tight spatiotemporal control of Rac activity in primary cells and tissues. Cell reports. 2014; 6:1153–1164. [PubMed: 24630994]

Kasinski AL, Slack FJ. miRNA-34 prevents cancer initiation and progression in a therapeutically resistant K-ras and p53-induced mouse model of lung adenocarcinoma. Cancer research. 2012; 72:5576–5587. [PubMed: 22964582]

Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity. 2000; 12:611–620. [PubMed: 10894161]

Kischkel FC, Lawrence DA, Tinel A, LeBlanc H, Virmani A, Schow P, Gazdar A, Blenis J, Arnott D, Ashkenazi A. Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. The Journal of biological chemistry. 2001; 276:46639–46646. [PubMed: 11583996]

Kissil JL, Walmsley MJ, Hanlon L, Haigis KM, Bender Kim CF, Sweet-Cordero A, Eckman MS, Tuveson DA, Capobianco AJ, Tybulewicz VL, Jacks T. Requirement for Rac1 in a K-ras induced lung cancer in the mouse. Cancer research. 2007; 67:8089–8094. [PubMed: 17804720]

Koschny R, Holland H, Sykora J, Haas TL, Sprick MR, Ganten TM, Krupp W, Bauer M, Ahnert P, Meixensberger J, Walczak H. Bortezomib sensitizes primary human astrocytoma cells of WHO grades I to IV for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. Clin Cancer Res. 2007; 13:3403–3412. [PubMed: 17545549]

Kraynov VS, Chamberlain C, Bokoch GM, Schwartz MA, Slabaugh S, Hahn KM. Localized Rac activation dynamics visualized in living cells. Science. 2000; 290:333–337. [PubMed: 11030651]

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Lambert JM, Lambert QT, Reuther GW, Malliri A, Siderovski DP, Sondek J, Collard JG, Der CJ. Tiam1 mediates Ras activation of Rac by a PI(3)K-independent mechanism. Nature cell biology. 2002; 4:621–625. [PubMed: 12134164]

Lemke J, Noack A, Adam D, Tchikov V, Bertsch U, Roder C, Schutze S, Wajant H, Kalthoff H, Trauzold A. TRAIL signaling is mediated by DR4 in pancreatic tumor cells despite the expression of functional DR5. J Mol Med (Berl). 2010; 88:729–740. [PubMed: 20354842]

Lemke J, von Karstedt S, Zinngrebe J, Walczak H. Getting TRAIL back on track for cancer therapy. Cell death and differentiation. 2014; 21:1350–1364. [PubMed: 24948009]

Ling J, Kang Y, Zhao R, Xia Q, Lee DF, Chang Z, Li J, Peng B, Fleming JB, Wang H, et al. KRASG12D-induced IKK2/beta/NF-kappaB activation by IL-1alpha and p62 feedforward loops is required for development of pancreatic ductal adenocarcinoma. Cancer cell. 2012; 21:105–120. [PubMed: 22264792]

Macher-Goeppinger S, Aulmann S, Tagscherer KE, Wagener N, Haferkamp A, Penzel R, Brauckhoff A, Hohenfellner M, Sykora J, Walczak H, et al. Prognostic value of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors in renal cell cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009; 15:650–659. [PubMed: 19147771]

Mack NA, Porter AP, Whalley HJ, Schwarz JP, Jones RC, Khaja AS, Bjartell A, Anderson KI, Malliri A. beta2-syntrophin and Par-3 promote an apicobasal Rac activity gradient at cell-cell junctions by differentially regulating Tiam1 activity. Nature cell biology. 2012; 14:1169–1180. [PubMed: 23103911]

Mitsuuchi Y, Testa JR. Cytogenetics and molecular genetics of lung cancer. Am J Med Genet. 2002; 115:183–188. [PubMed: 12407699]

Morton JP, Timpson P, Karim SA, Ridgway RA, Athineos D, Doyle B, Jamieson NB, Oien KA, Lowy AM, Brunton VG, et al. Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107:246–251. [PubMed: 20018721]

Myant KB, Cammareri P, McGhee EJ, Ridgway RA, Huels DJ, Cordero JB, Schwitalla S, Kalna G, Ogg EL, Athineos D, et al. ROS production and NF-kappaB activation triggered by RAC1 facilitate WNT-driven intestinal stem cell proliferation and colorectal cancer initiation. Cell stem cell. 2013; 12:761–773. [PubMed: 23665120]

Nimnual AS, Yatsula BA, Bar-Sagi D. Coupling of Ras and Rac guanosine triphosphatases through the Ras exchanger Sos. Science. 1998; 279:560–563. [PubMed: 9438849]

Ostrem JM, Peters U, Sos ML, Wells JA, Shokat KM. K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions. Nature. 2013; 503:548–551. [PubMed: 24256730]

Ozawa F, Friess H, Kleeff J, Xu ZW, Zimmermann A, Sheikh MS, Buchler MW. Effects and expression of TRAIL and its apoptosis-promoting receptors in human pancreatic cancer. Cancer letters. 2001; 163:71–81. [PubMed: 11163110]

Ritsma L, Steller EJA, Ellenbroek SIJ, Kranenburg O, Rinkes I, van Rheenen J. Surgical implantation of an abdominal imaging window for intravital microscopy. Nature Protocols. 2013; 8:583–594. [PubMed: 23429719]

Sanlioglu AD, Koksal IT, Ciftcioglu A, Baykara M, Luleci G, Sanlioglu S. Differential expression of TRAIL and its receptors in benign and malignant prostate tissues. The Journal of urology. 2007; 177:359–364. [PubMed: 17162091]

Sanz-Moreno V, Gadea G, Ahn J, Paterson H, Marra P, Pinner S, Sahai E, Marshall CJ. Rac activation and inactivation control plasticity of tumor cell movement. Cell. 2008; 135:510–523. [PubMed: 18984162]

Sanz-Moreno V, Marshall CJ. The plasticity of cytoskeletal dynamics underlying neoplastic cell migration. Current opinion in cell biology. 2010; 22:690–696. [PubMed: 20829016]

Shirasawa S, Furuse M, Yokoyama N, Sasazuki T. Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. Science. 1993; 260:85–88. [PubMed: 8465203]

Sotillo R, Schvartzman JM, Socci ND, Benezra R. Mad2-induced chromosome instability leads to lung tumour relapse after oncogene withdrawal. Nature. 2010; 464:436–440. [PubMed: 20173739]

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Spierings DC, de Vries EG, Timens W, Groen HJ, Boezen HM, de Jong S. Expression of TRAIL and TRAIL death receptors in stage III non-small cell lung cancer tumors. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003; 9:3397–3405. [PubMed: 12960128]

Sprick MR, Rieser E, Stahl H, Grosse-Wilde A, Weigand MA, Walczak H. Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. The EMBO journal. 2002; 21:4520–4530. [PubMed: 12198154]

Sprick MR, Weigand MA, Rieser E, Rauch CT, Juo P, Blenis J, Krammer PH, Walczak H. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity. 2000; 12:599–609. [PubMed: 10894160]

Steffen A, Ladwein M, Dimchev GA, Hein A, Schwenkmezger L, Arens S, Ladwein KI, Margit Holleboom J, Schur F, Victor Small J, et al. Rac function is crucial for cell migration but is not required for spreading and focal adhesion formation. Journal of cell science. 2013; 126:4572–4588. [PubMed: 23902686]

Todaro M, Lombardo Y, Francipane MG, Alea MP, Cammareri P, Iovino F, Di Stefano AB, Di Bernardo C, Agrusa A, Condorelli G, et al. Apoptosis resistance in epithelial tumors is mediated by tumor-cell-derived interleukin-4. Cell death and differentiation. 2008; 15:762–772. [PubMed: 18202702]

Trauzold A, Siegmund D, Schniewind B, Sipos B, Egberts J, Zorenkov D, Emme D, Roder C, Kalthoff H, Wajant H. TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. Oncogene. 2006; 25:7434–7439. [PubMed: 16751802]

Varfolomeev E, Maecker H, Sharp D, Lawrence D, Renz M, Vucic D, Ashkenazi A. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. The Journal of biological chemistry. 2005; 280:40599–40608. [PubMed: 16227629]

Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nature medicine. 1999; 5:157–163.

Wu GS, Burns TF, Zhan Y, Alnemri ES, El-Deiry WS. Molecular cloning and functional analysis of the mouse homologue of the KILLER/DR5 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor. Cancer research. 1999; 59:2770–2775. [PubMed: 10383128]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Significance

Previous pre-clinical 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 non-apoptotic 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.

Europe PMC Funders Author Manuscripts

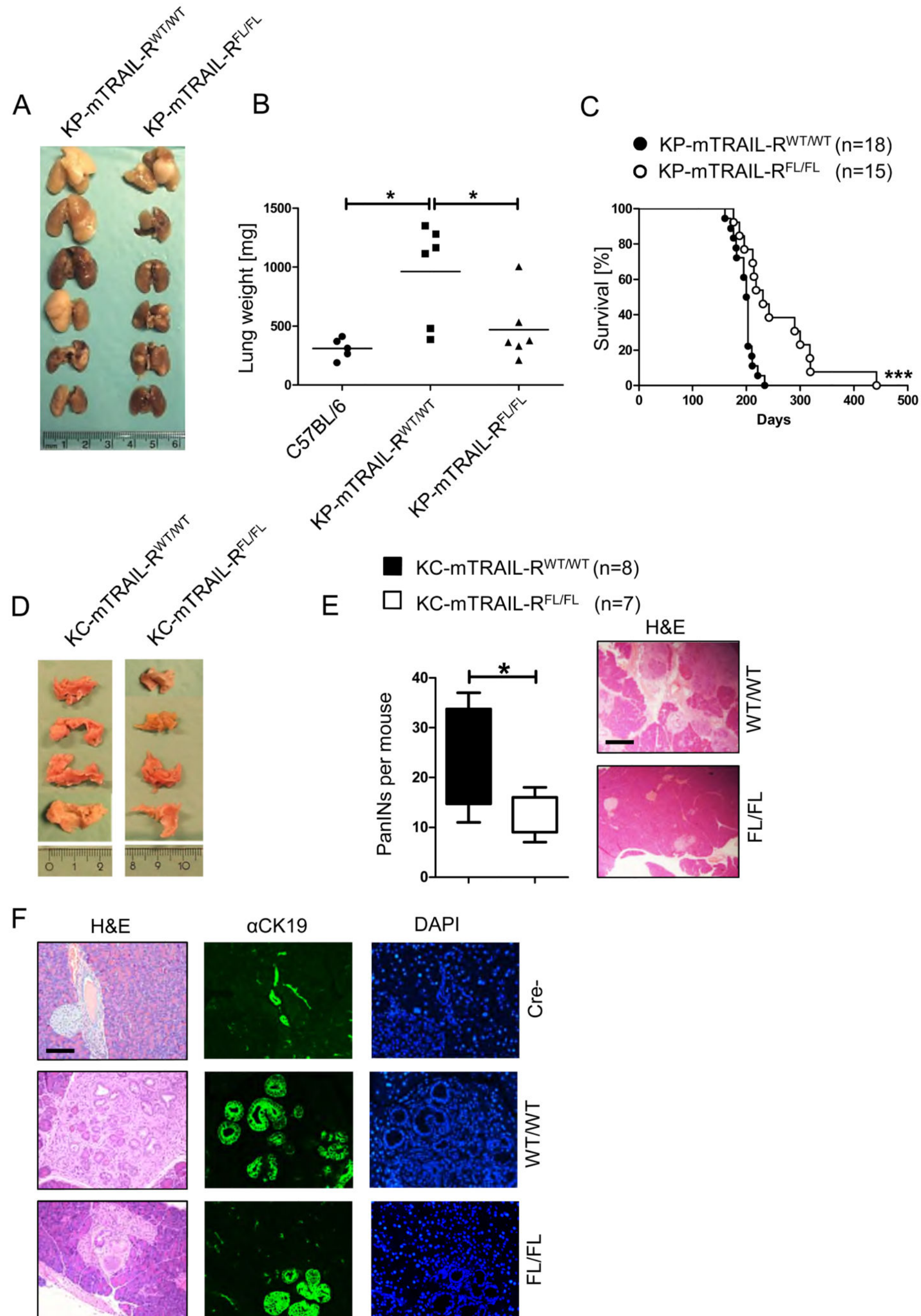

Figure 1. KRAS-driven lung and pancreatic cancers are promoted via endogenous mTRAIL-R

(A) Images of representative lungs of the indicated 6 months-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 months-old KC-mice.

Europe PMC Funders Author Manuscripts

(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, 100 μm.

(F) Representative H&E, αCK19 and DAPI stainings of pancreas of mice of indicated genotypes; scale bar, 25 μm.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

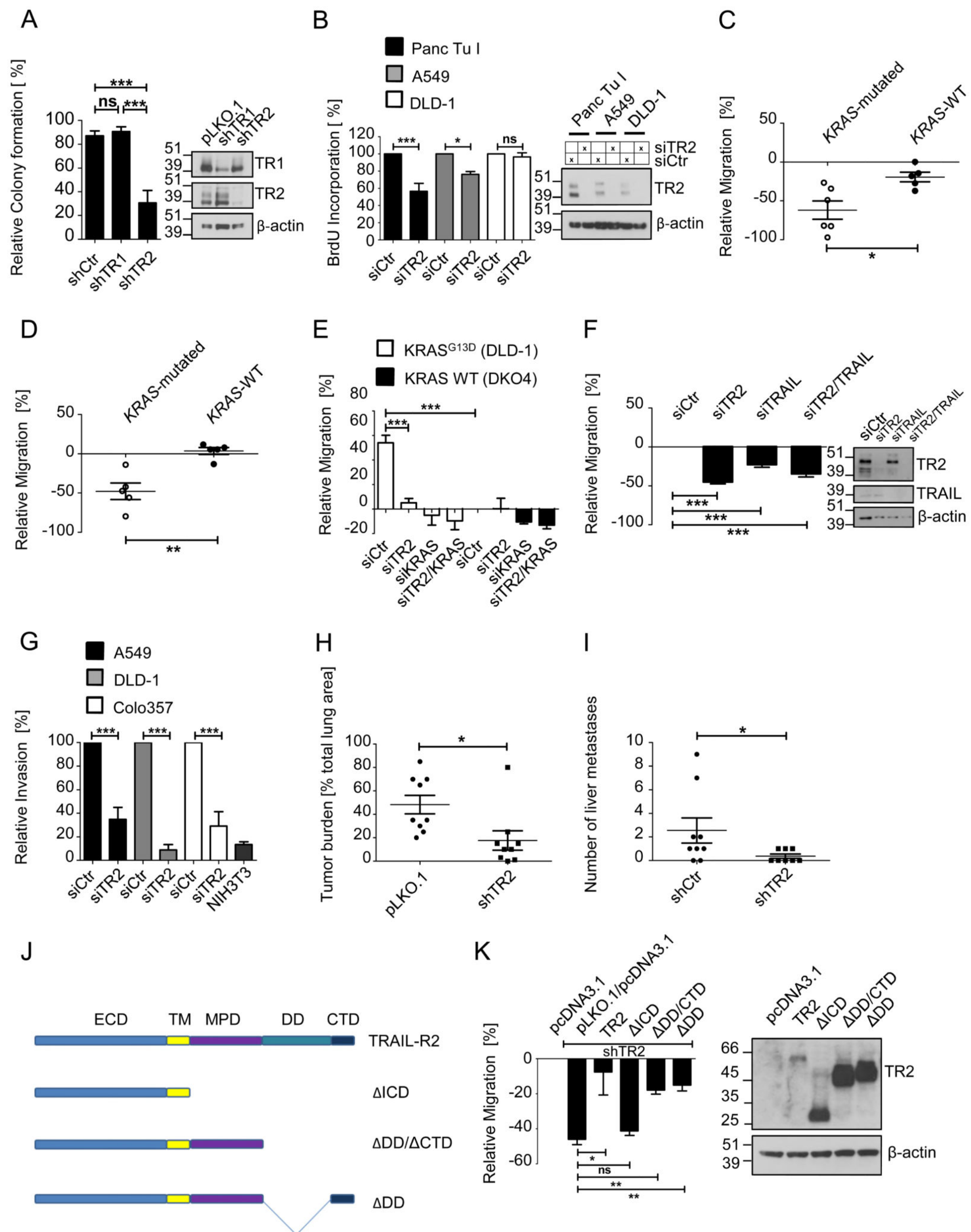

Figure 2. Human TRAIL-R2 drives proliferation, migration and invasion

(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 knockdown 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.
(H) 2 x 10^6 A549-luc pLKO.1 or shTRAIL-R2 were injected i.v. into SCID beige mice (n=9 mice/group). Tumor burden was quantified in paraffin sections of H&E-stained lungs by pathological inspection.
(I) 1 x 10^6 PancTu-I 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 +/- 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; DD, death domain; CTD, C-terminal domain. ns, not significant, * $p<0.05$; ** $p < 0.01$ and *** $p<0.001$ [ANOVA (A, B,E,F,G,K); Student’s t-test (C,D,H), Mann-Whitney test (I)]. See also Figure S1 and Table S1.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

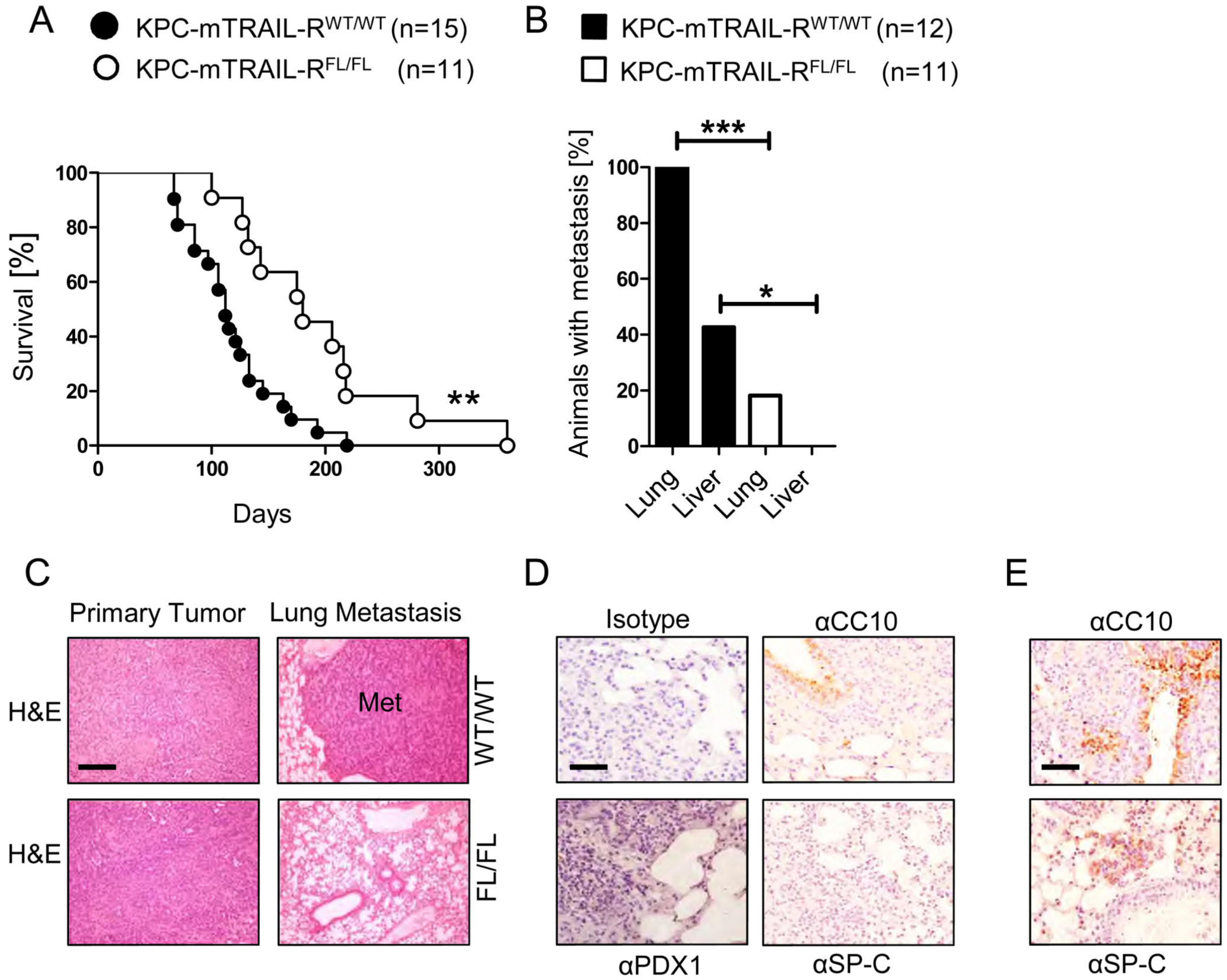

Figure 3. mTRAIL-R promotes KRAS-driven pancreatic cancer progression and metastasis

(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, 50 μm.

(D) Representative images of isotype, αPDX-1, αSp-C and αCC10 stainings of KPC lung metastases; scale bar, 25 μm.

(E) Representative images of αCC10 stainings of KP lung tumors; scale bar, 25 μm. See also Figure S2.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

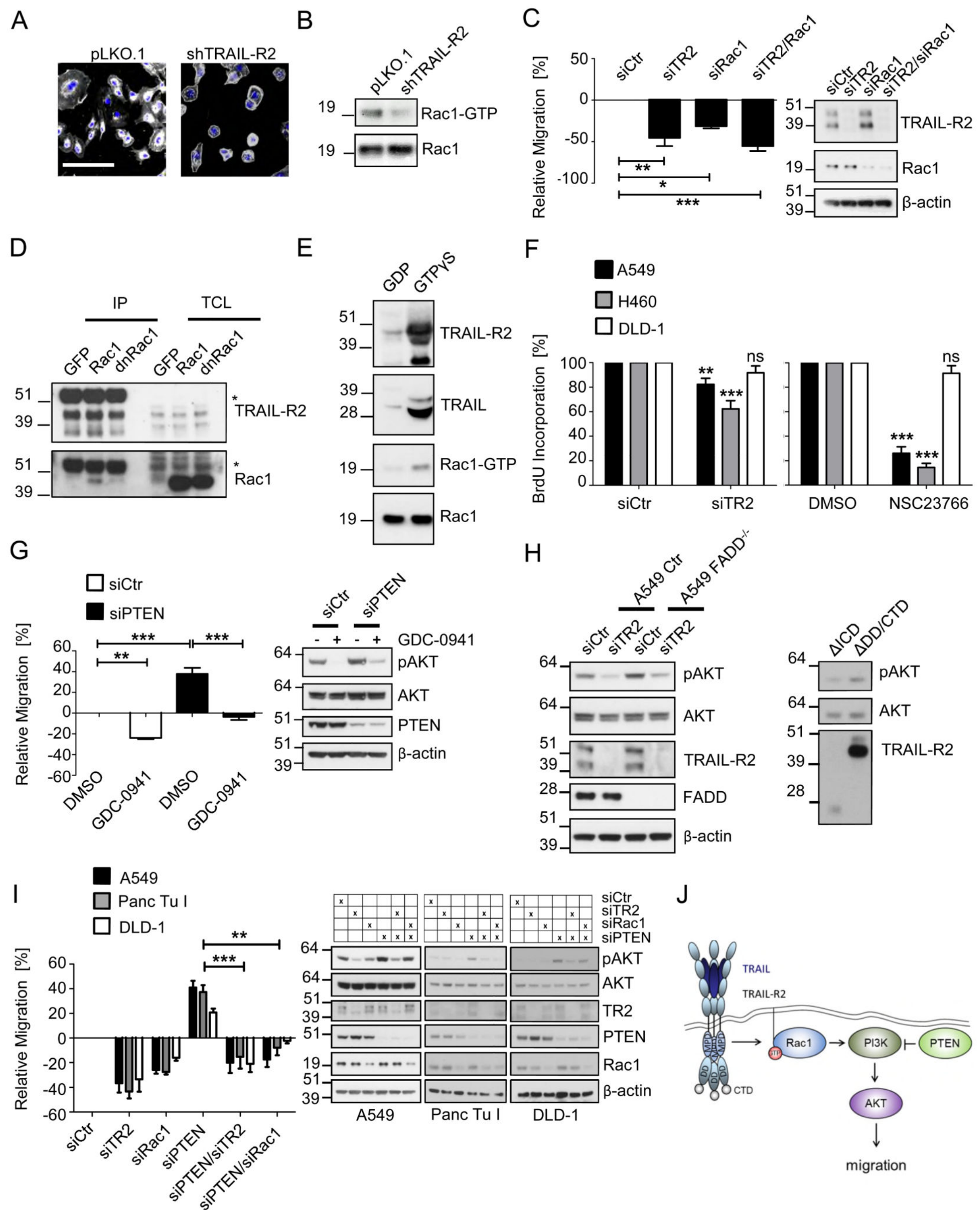

Figure 4. TRAIL-R2 promotes activation of a pro-migratory Rac1/PI3K signaling axis

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

(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 IP antibody heavy chain.
(E) Endogenous Rac1 in A549-luc cell lysates was loaded with GDP/GTPγS in vitro, and GTP-bound Rac1 was isolated using PAK1-RBD beads and analyzed by Western blot.
(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 ICD or DD/CTD. All cells were subjected to Western blotting.
(I) A549-luc, PancTu-I 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 +/- SEM of three independent experiments. ICD, intracellular domain; DD, death 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.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

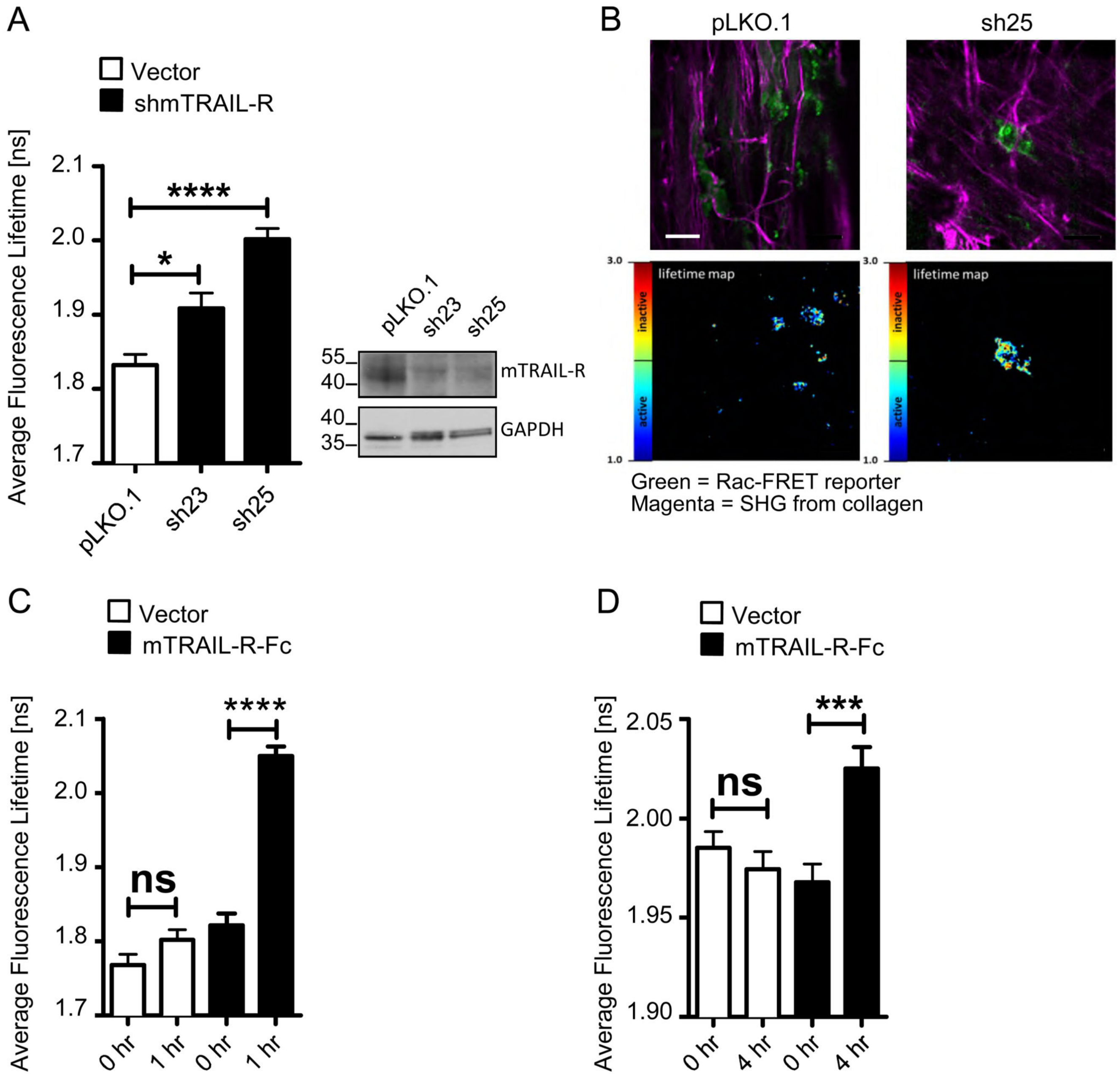

Figure 5. mTRAIL-R and mTRAIL promote Rac1 activity in KPC tumors in vivo

(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, 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.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

(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 +/- 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.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

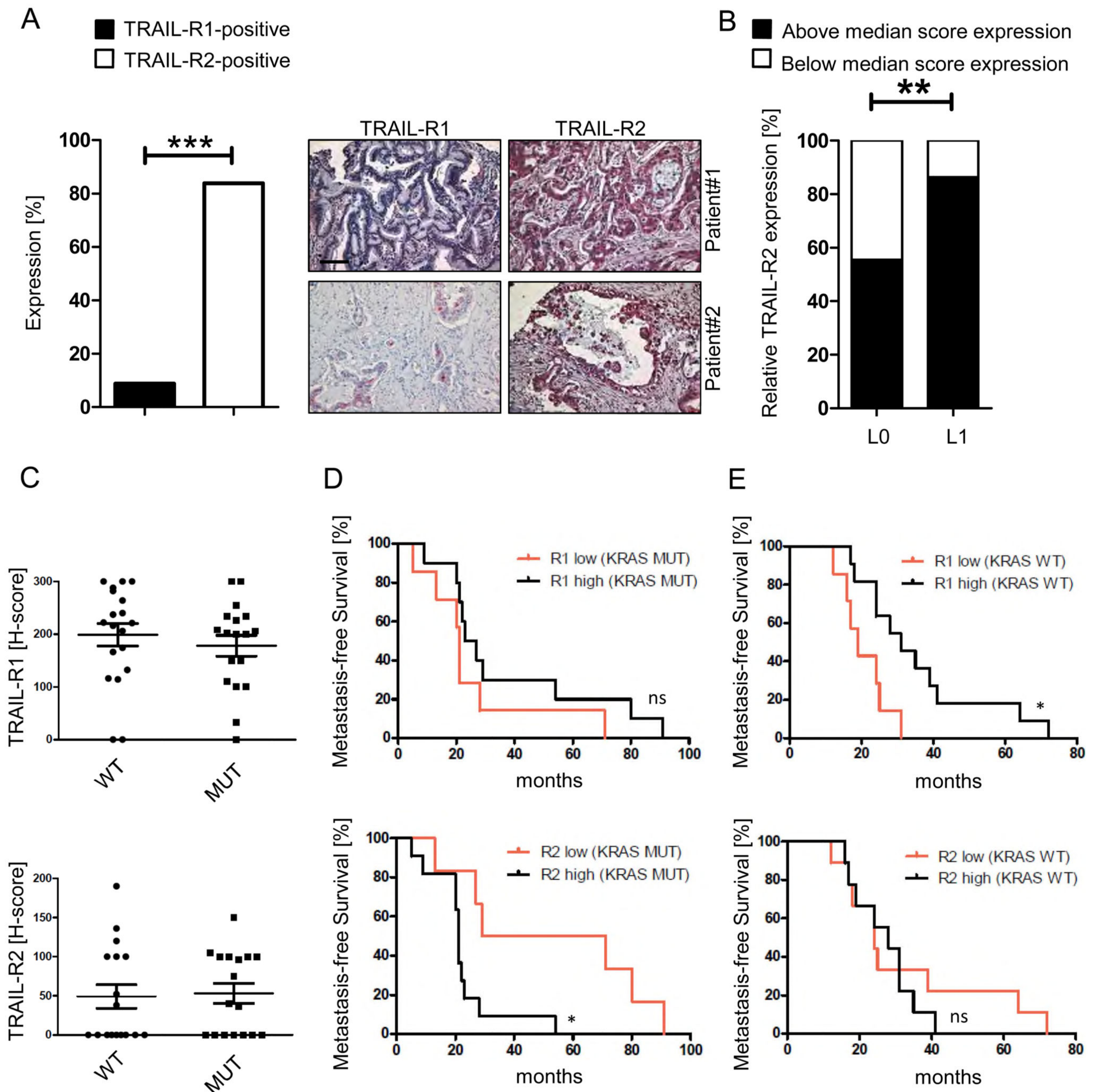

Figure 6. High TRAIL-R2 expression correlates with markers of malignancy in patients with *KRAS*-mutated cancers

(A) TRAIL-R1 and TRAIL-R2 expression in human PDAC samples of 95 patients was quantified. Percentage >20% of positive [intensity of a minimum of 1 on a scale of 1-3 (Ganten et al., 2009)] cancer cells within one patient sample was 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, 25 μm; Fisher's exact test *** p<0.0001.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

(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 5 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 +/- 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; * $p < 0.02$.