Published in final edited form as:

Sci Signal. ; 9(433): ra62. doi:10.1126/scisignal.aaf5106.

MET signaling in keratinocytes activates EGFR and initiates squamous carcinogenesis

Christophe Cattaïsson¹, Aleksandra M. Michalowski¹, Kelly Shibuya¹, Andrew Ryscavage¹, Mary Klosterman¹, Lisa Wright¹, Wendy Dubois¹, Fan Liu¹, Anne Zhuang¹, Kameron B. Rodrigues¹, Shelley Hoover¹, Jennifer Dwyer¹, Mark Simpson¹, Glenn Merlino¹, and Stuart H. Yuspa^{1,2}

¹Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD.

²To whom correspondence should be addressed at the Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, 37 Convent Drive, MSC-4255, Building 37, Room 4068, Bethesda, MD 20892-4255; Phone: 301-496-2162; Fax: 301-496-8709; yuspas@mail.nih.gov

Abstract

MET is commonly expressed in many human squamous skin cancers (SCC) while *Ras* mutations are infrequent. The number of carcinogen induced mouse skin squamous tumors increases by 5 fold in transgenic mice where cutaneous MET is activated by overexpressing HGF (MT-HGF mice). Without carcinogen, squamous tumors also erupt on MT-HGF mouse skin promoted by 12-O-tetradecanoyl-phorbol-13-acetate. Carcinogen initiated tumors have *Ras* mutations, but MET initiated tumors do not. *In vitro* MT-HGF and RAS transformed keratinocytes share identical phenotypic and biochemical features of initiation arising from autocrine activation of EGFR through elevated expression and release of EGFR ligands. EGFR inhibition ablates the initiated signature of MT-HGF keratinocytes *in vitro* and causes regression of MT-HGF induced tumors *in vivo*. EGFR activation by both MET and RAS requires the activity of ADAM17 mediated through both SRC and iRhom. Global gene expression profiles for MET and RAS transformed keratinocytes are highly concordant, and a core RAS/MET co-expression network is activated in pre-cancerous and cancerous human skin lesions. Tissue arrays reveal that many human skin SCC express abundant HGF transcripts and protein, and multiple human SCC cell lines are growth inhibited by blocking MET. Thus MET activation though EGFR parallels a RAS pathway to contribute to human and mouse cutaneous cancers.

One sentence summary:

Activated MET appropriates a pathway used by oncogenic RAS through ADAM17 and EGFR to initiate cutaneous carcinogenesis.

Author contributions: C.C., K.S., A.R., M.K., L.W., W.D., F.L., A.Z., K.B.R. performed experiments; A.M.M. performed computational work, S.H., J.D., M.S. and A.R. contributed IHC analysis, G.M. provided the MT-HGF mouse model, S.H.Y. designed experiments, analyzed data and wrote the paper.

Competing interests: The authors declare that they have no competing interests.

INTRODUCTION

Studies utilizing the multistage induction of squamous cancers on mouse skin have revealed much about the biology of tumor formation and first defined the operational and functional distinctions of initiation, promotion, premalignant progression and malignant conversion. Furthermore, the standard 7, 12 dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoyl-13-phorbol acetate (TPA) protocol confirmed the primacy of *Hras* mutations as an initiating event for squamous tumors *in vivo* and displayed the importance of regenerative hyperplasia and inflammation as the selective forces in tumor promotion leading to the emergence of HRAS initiated tumors. Through genetic modification of mice, the identification of potential initiating events for skin tumors has expanded to include *Kras* and *Nras* as well as known RAS targets in the EGFR pathway such as EGFR, ErbB2, or SOS and more downstream factors such as v-FOS, c-MYC, IGF1 and components of the NF- κ B pathway. In most of these cases a pro-inflammatory tumor promoter was also required for tumor formation (1). This requirement stimulated research to elucidate how promoter-induced inflammation provided the selection stimulus for tumor outgrowth leading to a broader understanding of the role of inflammatory cells, chemokines, and molecular pathways having both protective and negative consequences in carcinogenesis (2–4).

It is now recognized that initiating events themselves can have consequences on the inflammatory milieu, and this can be essential to their oncogenic potential. For example, transduction of keratinocytes with oncogenic *Hras* upregulates EGFR activity, leading to release of IL-1 α , activation of NF- κ B and elaboration of CXCR2 ligands that are essential components for HRAS mediated keratinocyte neoplastic transformation (5, 6). The magnitude of induction of these intermediate pathways is greatly enhanced by activation of PKC α , yet the overexpression and activation of PKC α in mouse skin is not sufficient to initiate tumors in the absence of *Ras* mutations (5, 7, 8). Nevertheless, transgenic mice that overexpress PKC α in the epidermis (K5-PKC α) are exquisitely sensitive to tumor promotion after DMBA initiation (5). This exquisite sensitivity to tumor promotion provides a model that could help identify initiating events of less potency than *Ras* mutations but of great relevance to human cancer. Signaling by hepatocyte growth factor (HGF) activating its receptor MET has been studied in multiple epithelial carcinomas (9, 10). Several studies suggest that MET is highly expressed in a subset of human skin cancers (11, 12) and a variety of other epithelial cancers (10, 13). The most frequent cause of MET's contribution in human cancers is protein overexpression resulting from transcriptional upregulation or gene amplification. Constitutively active *MET* mutations have been identified in hereditary papillary renal cell carcinoma patients (14, 15), but in general, activating MET mutations are infrequent in other human cancers (10). The downstream targets of activated MET include GAB1, PI3K, PLC, RAS, RAF, ERK, and MAPK leading to mitogenic, motogenic, and morphogenic responses in many cell types (9). Aside from intrinsic changes in MET, mutations in the regulatory region of the HGF gene leading to overexpression of the ligand and activation of MET contribute to both breast and bladder cancer (16, 17). HGF copy number amplification has been identified in head and neck squamous carcinoma (14), and HGF was described as a cancer driver gene across squamous cancers independent of tissue origin (18). HGF mutations have also been detected in about 20% of human cutaneous

squamous cell cancers (<http://www.cbioportal.org/>, (19, 20)), and elevated HGF is a primary response to ultraviolet light exposure in the skin (21, 22). In most normal tissues, HGF is produced predominantly by microenvironment stromal cells and has both paracrine and autocrine signaling (16, 23). Thus an experimental model that links HGF and MET to squamous cancer has relevance for human cancers. In transgenic mice overexpressing HGF driven by the MT (metallothionein 1) promoter (MT-HGF mice), melanocytes relocate to the epidermal-dermal junction, closely approximating the cellular distribution of human skin (24, 25). A single dose of UV irradiation or DMBA followed by TPA in MT-HGF neonates induces melanomas and squamous cell carcinomas through activation of MET (26, 27). In the current study we use the MT-HGF mouse in combination with the tumor promotion sensitive K5-PKC α mouse to explore the stage-specific contribution of increased MET activity to the development of squamous cell tumors in skin. In this setting we conclude that HGF activated MET is a fully functional tumor initiator co-opting many of the properties of oncogenic RAS and together HGF/MET comprise a relevant oncogenic pair for the propagation of human skin cancers.

RESULTS

Double transgenic (DT) MT-HGF/K5-PKC α mice develop significantly greater numbers of squamous tumors than wild-type (WT), K5-PKC α , and MT-HGF littermates

To generate the age-matched experimental and control genotypes to conduct this study, we crossed FVB/N K5-PKC α females with FVB/N MT-HGF transgenic males, to breed MT-HGF/K5-PKC α (DT) mice. In vitro fertilization of wild-type (WT) FVB/N females with sperm from DT males allowed for the generation of the four experimental genotypes of the same age. WT, K5-PKC α , MT-HGF, and DT littermates were subjected to a DMBA/TPA protocol initially reported to induce melanomas and squamous tumors in MT-HGF mice (27). In this protocol, 4 day old pups were initiated once with topical DMBA and starting at 6 weeks of age, when in telogen, promoted with TPA twice weekly for 5 weeks. Tumor growth was followed for up to 20 weeks (Fig. 1A). A maximum squamous tumor incidence of 40–60% was reached at 10 weeks in the DT and MT-HGF mice in contrast to 10% in K5-PKC α at that same time point (Fig. 1B) (DT vs K5-PKC α $p < 0.001$, MT-HGF vs K5-PKC α $p < 0.05$). By week 20, tumor bearing DT mice averaged 6 tumors per mouse, while K5-PKC α and MT-HGF mice averaged 1 tumor per mouse (Fig. 1C). Wild type mice did not develop tumors in this protocol as expected from the sub-optimal (5 weeks at low dose) TPA regimen (28). The malignant conversion rate (% squamous carcinoma among all squamous lesions) was significantly higher in MT-HGF (73%) versus DT mice (23%) (Fig. S1), suggesting that many of the excess tumors in the DT group were low risk (29). All tumors in the DT group and 70% of the K5-PKC α group contained the expected *Hras codon 61 A>T* transversion mutation characteristic of DMBA initiation/TPA promotion. In contrast, 6/18 tumors in the MT-HGF group had *Kras* mutations suggesting a distinct selection of initiated cells in the presence of excess HGF and absence of PKC α to facilitate promotion (Fig. S2A). WT, K5-PKC α , MT-HGF and DT littermates were also compared for squamous tumor formation in the absence of DMBA driven initiation by application of 1 μ g (1.6nmol) TPA twice weekly for up to 10 weeks starting at week 6 of age (Fig. 1D). At the termination of the experiment (week 16), 100% of DT mice had developed multiple

squamous papillomas (2.7 average number of tumor/mouse) while none of their WT, K5-PKC α , or MT-HGF littermates developed squamous lesions (Fig. 1, E and F). We further screened for *Kras*, *Hras* and *Nras* mutations at codons 12, 13 and 61 in the squamous lesions observed in DT animals without DMBA initiation and found none (Fig. S2B). This result suggests that activated MET is sufficient to drive skin carcinogenesis in a tumor promotion sensitive environment. To further confirm the initiated phenotype is conferred by activated MET on keratinocytes, we performed orthotopic grafting of MT-HGF or WT keratinocytes and primary dermal fibroblasts on syngenic MT-HGF or WT recipients without further treatment (6, 30). Four mice out of 5 developed squamous papillomas in the MT-HGF keratinocytes grafted to the MT-HGF recipients (Fig. S3, A and B) while none were observed with WT grafts on WT recipients. Mutations in *Ras* alleles were not detected in papillomas from orthografts (data not depicted). When WT keratinocytes were grafted to MT-HGF recipients, 40% of the mice formed tumors suggesting that paracrine HGF and a wound environment are sufficient stimulus to initiate and promote tumor formation (data not depicted). Collectively these results indicate that aberrant HGF signaling and MET activation can substitute for *Ras* mutation to initiate skin carcinogenesis.

HGF/MET does not sensitize mice to TPA responses.

The foregoing results suggest that MET activation is sufficient to initiate skin tumor formation but have not addressed a possible effect on tumor promotion. When TPA was applied to the skin of all 4 genotypes, only those genotypes expressing the K5-PKC α allele demonstrated enhanced promotion responses (hyperplasia, inflammation [MPO, COX-2, *Cxcl1*, *Areg*]) whereas MT-HGF and WT responded identically (Fig. S4). Thus, the TPA mediated promotion component responsible for the high tumor burden in DT mice is not enhanced by HGF activated MET signaling but is primarily a response to PKC α activation.

MT-HGF and DT primary keratinocytes exhibit a RAS-like phenotype *in vitro*

To examine cell autonomous effects of HGF/MET signaling in the skin tumor model, isolated keratinocytes from the various genotypes were studied *in vitro* and compared to the initiated phenotype produced by oncogenic RAS (RAS-keratinocytes) transduction *in vitro* (5, 6). WT RAS-keratinocytes exhibit refractile and elongated spindle-like morphology and grow to higher density at confluence than non-transduced keratinocytes (Fig. 2A). DT or MT-HGF but not K5-PKC α keratinocytes (not depicted) had similar morphology to RAS transduced cells. Indeed, treatment of MT-HGF keratinocytes with a MET inhibitor (PHA665752) reversed this phenotype *in vitro* (Fig. S5A). These observations suggest that MT-HGF and DT keratinocytes were cell-autonomously activated *in vitro* to resemble an initiated phenotype. MET is constitutively activated in cultured keratinocytes from MT-HGF or DT mice, and this activation is not further increased by oncogenic RAS transduction (Fig. 2B). As expected, RAS transduction activates MAPK signaling in all 4 genotypes, a change reproduced by constitutive MET signaling in MT-HGF and DT keratinocytes without RAS (Fig. 2B). Similar results are seen for activation of EGFR signaling, a main effector of RAS transformation in primary keratinocytes (6, 31) (Fig. 2B). While the MET inhibitor PHA665752 reduces the activation of EGFR in MT-HGF keratinocytes, the EGFR inhibitor AG1478 does not inhibit the activation of MET in DT keratinocytes indicating a unidirectional interaction of these two receptor kinases (Fig. S5, C and D). Induction of 4

EGFR ligands by oncogenic RAS is essential for the autocrine activation of EGFR in RAS-keratinocytes (31) (Fig. 2C). The expression of transcripts for those ligands is also elevated in MT-HGF and DT keratinocytes in the absence of RAS transduction (Fig. 2C) suggesting that autocrine MET signaling could mediate the activation of EGFR through the upregulation of its cognate ligands. The morphological and biochemical similarities between transduction by RAS and activation of MET in keratinocytes prompted us to examine the connection further. Both RAS transduction and MET activation in the absence of RAS transduction increase keratinocyte proliferation as measured by ^3H -thymidine incorporation (Fig. 3A, left panel). Furthermore, both RAS transduced MT-HGF and DT keratinocytes resist the growth inhibition produced by inducing keratinocyte differentiation with elevated calcium, a property of more advanced neoplastic keratinocytes (32) (Fig. 3A, right panel). Biochemically, resistance to differentiation induced growth inhibition is documented by the persistence of Cyclin D1 expression in differentiating MT-HGF keratinocytes with and without RAS transduction (Fig. 3B). Additional phenocopies of RAS and MET activity in keratinocytes are the suppression of suprabasal keratin production (K1 and K10) in response to elevated calcium (Fig. 3, C and D), *de novo* expression of keratin 8 (Fig. 3C) and upregulation of components of oncogenic RAS induced inflammation which are key parts of the gene signature (Fig. 3D) associated with initiation of keratinocyte neoplasia by oncogenic RAS (6). We determined the EGFR dependence of these signature changes of MET activation by treating EGFR null keratinocytes (33, 34) with HGF or by treating MT-HGF keratinocytes with small molecule inhibitors of EGFR (AG1478, PD168393) and monitoring signature responses (Fig. S6). In keratinocytes genetically depleted of EGFR the basal levels of transcripts for EGFR ligands, cytokines and keratins are essentially unchanged, but the induced signature alterations associated with HGF exposure are substantially reduced (Fig. S6A). An almost identical pattern in these signature markers is seen for *Hras* transduced EGFR null keratinocytes (Fig. S6B). Furthermore, blocking EGFR activity using the small molecule inhibitors (AG1478, PD168393) in MT-HGF keratinocytes reduces the elevated expression of *Cxcl1*, *Il1a* and restores the expression of K1 and K10 mRNA (Fig. S6C and Fig. S7), again reproducing studies on RAS-keratinocytes (5, 6, 31). No consistent association of these changes with MET directly activating an endogenous *Ras* allele could be discerned since knocking down each *Ras* allele with a specific siRNA in MT-HGF keratinocytes does not reverse the *Cxcl1*, *Krt1* and *Il1a* signature to control levels over several days of study (Fig. S8, A and B).

Activation of ADAM17 by HGF/MET and RAS is central to the initiated phenotype

The central role of EGFR activation in the MET signature indicated that the upregulation of EGFR ligands must also be associated with release from the cell surface to engage in receptor binding, a process catalyzed by the disintegrin and metalloprotease ADAM17 (35, 36). Consistent with this expectation, amphiregulin (AREG) is present in culture supernatants from MT-HGF keratinocytes at substantially higher levels than supernatants from the WT keratinocytes and this is reduced by blocking MET with PHA665752 (Fig. S5B). Treatment of MT-HGF keratinocytes with increasing concentrations of the ADAM17 inhibitor GM6001 also decreases free AREG in culture supernatant (Fig. 4A) and reduces EGFR activation (Fig. 4B). Targeting ADAM17 with several distinct siRNAs in MT-HGF keratinocytes also reduces the activation of EGFR (Fig. 4C). Furthermore, deleting

ADAM17 with adeno Cre in HGF treated ADAM17^{fl/fl} keratinocytes (37) reduces both the release of AREG in culture supernatants and EGFR activation (Fig. 4, D and E). Along with the other parallels among active MET and oncogenic RAS in keratinocytes, EGFR activation is also reduced in oncogenic RAS keratinocytes transduced with an siRNA targeting ADAM17 (Fig. 4F). Knockdown of ADAM17 by siRNA also effectively reversed the transcriptional signatures that characterize MT-HGF keratinocytes by 72 hours after transduction (Fig. 4G). Consistent with ADAM17 serving to release the EGFR ligands, neutralizing antibodies against TGF α and AREG in culture supernatants of MT-HGF keratinocytes also reduce EGFR activation (Fig. S9). Two predominant pathways controlling ADAM17 maturation and activation are SRC and iRhom 1 and 2 (38–41), and treating keratinocytes with HGF activates SRC (Fig. 5A). Using the release of AREG into culture supernatants of HGF treated keratinocytes as readout for ADAM17 activity, genetic (siRNA) inhibition of SRC reduces AREG release (Fig. 5B). HGF treatment of WT keratinocytes substantially elevates iRhom2 mRNA expression (Fig. 5C) and selective siRNA can reduce either iRhom1 or iRhom2 (Fig. S10). As seen for reduction of SRC, siRNA knockdown of iRhom1 and iRhom2 in WT keratinocytes reduces AREG release in response to HGF (Fig. 5D). The combination of siRNA to both SRC and iRhom essentially eliminates AREG release (Fig. 5D). Thus it appears that activation of MET sets in motion a SRC/iRhom mediated pathway to activate ADAM17, release EGFR ligands to stimulate EGFR activity and contribute to the initiated keratinocyte phenotype.

The cytokine signature of MT-HGF keratinocytes is IL-1 and NF- κ B dependent and active EGFR is required for tumor formation.

We have previously reported that the RAS-keratinocyte signature involves autocrine EGFR signaling and IL-1 α mediated activation of NF- κ B (6). Treatment of MT-HGF keratinocyte cultures with the IL-1 receptor antagonist (IL-1ra) or NF- κ B signaling blockade by transduction with I κ B α adenovirus reduces the expression of *Il1a* and *Cxcl1* (Fig. 6A). Blockade of IL-1 did not alter the expression of transgenic *Hgf* (data not depicted). Collectively, those results suggest that MET signaling in keratinocytes leads to the activation of an EGFR/IL-1/NF- κ B axis that contributes to the requirements for a cancer initiated phenotype previously shown for oncogenic RAS in keratinocytes (6). In order to test if EGFR activation is required for MET-driven skin carcinogenesis *in vivo*, we induced squamous papillomas by grafting MT-HGF keratinocytes to syngeneic hosts. Tumors from such grafts have elevated p-EGFR and activated p-ERK (Fig. 6B). Following tumor development we treated the mice daily with the oral competitive EGFR tyrosine kinase inhibitor gefitinib at a dose non-toxic to skin (100mg/kg) (42). Over 14 days of treatment, gefitinib nearly eliminated established squamous papillomas while tumors in the vehicle control continued to grow (Fig. 6, C and D). The regressing lesions display a reduced percentage of Ki67-positive basal keratinocytes and reduced microvessel density based on CD31⁺ cells compared to the vehicle group (Fig. S11).

MT-HGF and RAS transformed keratinocytes share strongly concordant global gene expression profiles

Since both MT-HGF and WT-RAS keratinocytes were able to initiate carcinogenesis that appeared to involve similar pathways, we considered that a more global view through a

microarray based gene expression analysis might reveal a common signature essential for tumor formation. Using ANOVA contrasts, we generated lists of differentially expressed genes in MT-HGF keratinocytes and keratinocytes transduced with RAS compared to normal controls all in 0.05 mM Ca^{++} proliferation medium (adjusted P-value < 0.05). Results presented in fig. 7A and B and table S1 show that both RAS and activated MET induced abundant and substantially overlapping transcriptional responses. A highly statistically significant overlap between the RAS and activated MET signatures (P-value<0.0001) comprises 6247 genes, where 93% of the mRNAs are concordantly up- or down-regulated relative to WT keratinocytes. Correlation analysis of fold changes in the shared signature (Fig. 7B) confirms that the gene regulation is highly concordant between the two (Pearson's correlation coefficient $r=0.88$, linear coefficient of determination $R^2=0.77$). For further in-depth analyses we selected from the concordant signature the top 372 genes with at least two fold changes in both experiments (287 up-regulated and 85 down-regulated mRNAs) whose estimated expression levels are shown in Fig. 7C. Notably, the majority of these genes (88%) are significantly affected by the EGFR inhibitor and negatively correlated ($r = -0.63$) with the changes in gene expression between MT-HGF and WT-Control (Fig. S7 and TableS1).

Gene set enrichment analysis (GSEA (43)) identified a total of 37 ontologies with 33 non-redundant gene functions significantly enriched in the 372-gene shared signature (see details in Table S2). Most significantly enriched terms in particular GO domains (Fig. 7D) were keratinization (NES = 2.1, Q-value < $2e-04$; biological process), serine-type endopeptidase activity (NES = 2.2, Q-value < $2e-04$; molecular function), and cornified envelope (NES = 2.0, Q-value = $4e-04$; cellular component), indicating up-regulation of these functions. Fig. 7E shows semantic similarity networks built from the enriched GO terms using REVIGO algorithm (44). The semantic similarities over the gene ontologies provide quantitative information on gene functional relationships, helping to gain a more holistic view on the processes affected. With this approach, we identify eight gene ontology networks with highly overlapping gene functions, which indicate that the top concordant RAS/MET signature captures the very essential genes related to keratinocyte biology; these include biological processes implicated in tissue development (epidermis and ectoderm development, epithelial differentiation), and extracellular matrix components. Peptidase/endopeptidase was revealed as a major category of concordant gene expression changes not previously studied in initiated keratinocytes. Included in this group are members of the TMPRSS11 family of membrane bound serine proteases not previously specifically linked to RAS or MET transformation. Several of these proteases have the capacity to release membrane bound growth factors to stimulate growth as well as migration of tumor cells. Lipid and fatty acid metabolism pathways emerged from this analysis as prominent associated biological and molecular functions consistent with recent associations of these pathways with EGFR activity (45). IPA's Upstream Regulator tool allows inferring the activity state of upstream factors responsible for the observed expression changes in a set of genes. Using Ingenuity Pathway Analysis (IPA) and its Ingenuity Pathway Knowledge Base (IPKB), we investigated the predicted upstream regulators of the MET/RAS signature. The IPA analysis (see Table S3) predicted inhibition (bias-corrected z-score < -1.96) of 17 and activation (bias-corrected z-score > $+1.96$) of 20 upstream regulators whose targets were

enriched in the 372 gene signature of RAS and activated MET (P-value 0.01). HRAS, KRAS and NUPR1 appear on this list of upstream regulators.

MET expression and MET signaling contribute to human cutaneous SCC

In a limited study of acute HGF treatment of normal human keratinocytes, both EGFR ligands and cytokine markers followed a similar pattern of change as seen in MT-HGF mouse keratinocytes (Fig. S12). To address the frequency of MET expression in human cutaneous SCC, we used human skin SCC tissue arrays (BIOmax) to probe for MET by immunohistochemical staining and histomorphometric analysis using the Aperio software. In the majority of the 76 cases examined, intense MET staining was readily detected (Fig. 8A) in many grade 1 lesions and intensity increased in grade 2 lesions when compared to grade 1 (Fig. 8B). Although the number of grade 3 lesions was too small to obtain statistical significance, the mean score of grade 2 and 3 lesions was the same. These data suggest that MET is commonly expressed in human cutaneous SCC development and could contribute to disease progression. We were unsuccessful in obtaining a signal for pMET in adjacent cutaneous SCC tissue arrays using multiple pMET antibodies and putative positive control samples. Instead we probed for expression of HGF transcripts by *in situ* hybridization (Fig. 8C). In this analysis 72% of 66 evaluable human cutaneous SCC tumor punches were positive for HGF expression with much of the signal detected in the tumor epithelium and individual stromal cells (Fig. 8C). Parallel immunohistochemical staining for HGF protein produced the same pattern. We also examined a series of 6 human SCC cell lines without *Ras* mutations to detect if MET was constitutively activated when probed for pMET by immunoblotting (Fig. 8D). While only SSC9 in this panel showed MET inhibitor responsive pMET activation, all lines responded biochemically to HGF indicating an intact MET responsive pathway. In fact, the growth of every line was reduced by a MET inhibitor in a dose dependent manner (Fig. 8E).

From the 372 most concordant genes in the gene set enrichment data we applied a systems approach, Denoising Algorithm based on Relevance network Topology (DART) (46), which allowed us to derive a cross-species, overall measure of the RAS/MET signature activity in an individual patient sample and compare the activity scores across the different tissues in the training and validation patient datasets. Fig. 9A presents the activity scores in patient-matched samples from the normal epidermis (NE), actinic keratosis (AK), and squamous cell carcinoma (SCC). In 10 out of 13 patients the activity score is higher in the AK sample than in the normal epidermis (NE) (P=0.003). In all but one patient the estimated level of the RAS/MET signature activity is higher in the SCC than in NE tissue (P=0.0005). Increased activity scores are also observed in the SCC compared to the AK samples (P=0.048). For further verification we computed the DART scores using the same network in two independent SCC test datasets (47, 48). As shown in Fig. 9B, higher activation of the RAS/MET signature in the AK and SCC samples than in the normal epidermis was again observed (P=0.001 and P=0.0001, respectively), although the difference between AK and SCC activity scores was not confirmed (P=0.68). Overall, the results support the conclusion that MET pathway upregulation occurs early in many squamous skin cancers and persists through the advanced stage.

DISCUSSION

Our work addresses the early changes required for the conversion of a normal keratinocyte into a premalignant tumor on the road to forming a cancer. The discoveries in the mid-1980's that an activated *Hras* allele was present in nearly all benign tumors induced on mouse skin by DMBA/TPA exposure together with experimental evidence that activated HRAS was sufficient to initiate normal keratinocytes to produce benign tumors were milestones in carcinogenesis research (49). In the interim decades numerous laboratories have filled in the intricate cascading signaling molecules downstream from oncogenic *Ras* alleles in multiple model systems (50). In keratinocytes, crucial biochemistry for HRAS initiation appears to involve the upregulation of ligands for and autocrine activation of EGFR. Since RAS is downstream from EGFR signaling, this requirement for EGFR activation may indicate that the signal strength of a single mutated *Ras* allele is not sufficient to drive early neoplasia. Following EGFR activation IL-1 α is released and activates IL-1R on keratinocytes creating a second autocrine loop leading to the activation of NF- κ B signaling that modifies expression of specific keratinocyte genes involved in tumor formation including increasing expression and release of CXC ligands such as CXCL1 (6). The consequences of cytokine release are both autocrine, stimulating tumor cell migration through activation of keratinocyte CXCR2, and paracrine, attracting immune cells into the tumor stroma (oncogene induced inflammation) (51). Inhibition of any one of these autocrine loops impairs tumor formation. We now show that activation of keratinocyte MET through elevated autocrine and/or paracrine HGF in the skin microenvironment uses these same pathways to be oncogenic for keratinocytes and is sufficient for tumor formation in the absence of RAS mutations but in the presence of a strong promoting stimulus such as wounding or enhanced cutaneous PKC α . The focal nature of tumor formation in the HGF/MET mice suggests that a subpopulation of keratinocytes in the skin epithelium is particularly sensitive to MET activation and the promoting stimulus. In normal mouse skin, HGF is expressed in the hair follicle dermal papilla and MET in the hair follicle including the hair follicle bulge where stem cells reside (52). In the MT-HGF mouse, the expression of HGF is more widespread. Therefore, it is reasonable to speculate that bulge stem cells give rise to MET induced squamous tumors but further studies are required to support this possibility. The combination of DMBA and MT-HGF favors the selection of *Kras* mutant tumors in addition to the expected *Hras* mutant tumors, potentially arising from the same cell compartment. The skin carcinogenesis literature is peppered with examples of KRAS mutant tumors emerging after carcinogen initiation when the promoting environment is modified from the standard TPA protocol (53–55). In our model it is notable that only mutant *Hras* tumors and no mutant *Kras* tumors were detected after DMBA in the DT group emphasizing the importance of context in the selection of incipient mutant tumor cells. Furthermore, many of the tumors in this group were at lower risk for malignant conversion (Fig. S1), while the increased frequency of KRAS mutations in the DMBA-MT-HGF group might have contributed to malignant conversion (54).

There is limited information regarding the precise contribution of the HGF/MET axis in normal skin homeostasis. Deletion of *Met* from the skin epithelium in mice does not produce a skin phenotype (52, 56) but does impair wound healing while HGF administration to

chronic wounds accelerates healing (56, 57). The MT-HGF mouse develops spontaneous internal tumors and cutaneous melanomas (25), but the epithelial skin compartment is not a particular target for spontaneous tumor development (58). Nevertheless, MET is highly expressed in a substantial fraction of human head and neck and cutaneous squamous cell cancers, and activating mutations of *MET*, although rare, may contribute to radioresistance in these tissues (11, 12, 59). An intact HGF/MET pathway was essential for skin tumor formation in a transgenic mouse model of skin targeted overexpression of matriptase, a serine protease that is capable of converting pro-HGF to the mature MET ligand (12). Furthermore, MET copy number increases are frequent in DMBA induced mouse skin papillomas and increase further during tumor progression to SCC (60). The sensitivity of human SCC cell lines to growth inhibition by a MET inhibitor, the frequent high MET and HGF expression detected in human skin SCC tissue arrays (Fig. 8), the higher intensity of MET expression in progressing human skin cancers (Fig. 8) and the increasing activity level of the RAS/MET activation signature derived from human skin pre-cancer and cancer data bases (Fig. 9) support a contribution of MET signaling to human skin cancer. Limited results suggest that both mouse and human keratinocytes respond similarly to HGF stimulation.

The mechanism through which cell autonomous or paracrine activation of MET in keratinocytes produced tumors became clearer when we determined that phenotypically and biochemically these keratinocytes reproduced the biology of RAS transformed keratinocytes. Like RAS, MET activates EGFR through enhancing expression of the EGFR cognate ligands and controlling their maturation through the membrane bound protease ADAM17, thus establishing the autocrine loops necessary for tumor formation. While previous studies have shown that EGFR can lead to MET activation (61–63), our data indicate that EGFR is an obligatory effector of MET driven skin carcinogenesis. Blocking EGFR reverses the MET biochemical signature and causes MET-driven tumors to regress. Pharmacological inhibition of EGFR causes major changes in the gene expression profile of MT-HGF keratinocytes (Fig. S7). This reliance on EGFR activity for tumor growth *in vivo* is also true for oncogenic RAS (31), further highlighting the commonalities among those two initiators of skin carcinogenesis. The cross-talk between MET and EGFR in our model is unidirectional since oncogenic RAS (and subsequent EGFR activation) does not cause MET activation (Fig. 2B) and treatment of DT keratinocytes with an EGFR inhibitor does not decrease phospho-MET levels (Fig. S5).

The activation of ADAM17 by oncogenic RAS has been recently reported in both pancreatic and colorectal cancers (64, 65), releasing EGFR ligands that are essential for tumor growth in both models. A previous report suggested HGF/MET induced ADAM17 activity in invading trophoblast cells (66), but this relationship in tumors has not been reported before as far as we can tell. We now show that ADAM17 is a central player in producing the EGFR activation critical for the oncogenic signature in both RAS and HGF/MET transformed keratinocytes, further linking these two tumor initiators. Our data have unexpectedly revealed that MET activation enhances SRC activity and iRhom expression, particularly iRhom2, leading to ADAM17 activation likely through translocation and maturation of ADAM17 proteolytic activity (40). Accumulating evidence suggests that iRhoms have important functions in cutaneous biology (67–69), but the connection to MET activation has not been made. When stabilized by mutation, the short lived iRhom2 protein enhances

cutaneous wound healing through activation of ADAM17 and release of EGFR ligands (69, 70). Of particular interest regarding cancer, stabilizing mutations in iRhom 2 cause tylosis esophageal cancer characterized by cutaneous dyskeratosis and inherited susceptibility to squamous esophageal cancer attributed to enhanced EGFR signaling through ADAM17 (71). This new link to MET suggests that exploring iRhom2 and ADAM17 in cancers associated with MET activation is worthy of further study.

Beyond the establishment of the autocrine loops emanating from EGFR activation, the pathways involved in both MET and RAS initiation of keratinocyte neoplasia converge on the expression of many genes and common pathways. The vast majority of the aberrantly expressed genes from keratinocytes initiated by activated RAS or MET overlap and is concordant. Selecting the 372 most modulated and concordant RAS and MET genes to produce a highly enriched tumor associated gene expression dataset coupled with gene ontology analyses derived from gene set enrichment data allowed us to identify biologically meaningful and coherent sets of gene functions involved. The concordant gene list (Table S1) confirmed the changes in cytokines, growth factors and differentiation markers that we have come to know as the signature of initiation. Not surprisingly the functional gene set analysis confirmed the importance of pathways associated with epidermal development and keratinocyte differentiation but revealed two unexpected highly relevant pathways. A strong functional association with endopeptidase/peptidase activity was revealed (Fig. 7E and Table S2). MMPs have been associated with transformation of keratinocytes and a number of other cancers, but the profiling revealed an association with a number of type II transmembrane serine proteases (TTSP). The functions of members of this family are not well known (72), but TMPRSS 13 is reported to activate pro-HGF to the mature ligand (73) and TMPRSS11E is reported to decrease in HNSCC (74). This is an area worthy of further study. Pathways controlling lipid biosynthesis, lipid transport and fatty acid synthesis were also unexpectedly revealed in the functional enrichment analysis. Recent studies indicate a strong association among EGFR signaling, lipid metabolism and cancer growth. Much of the data are derived from glioblastomas but our results suggest that the concept may be more widespread (45, 75, 76).

It is premature to speculate on what regulates the expression of downstream effectors in the RAS/MET profiles, but the IPA's Upstream Regulator tool identifies several transcriptional regulators previously associated with skin carcinogenesis or RAS transformation (Table S3). Among these, reduction in p53 regulated genes has been associated with skin tumor progression (77) and NUPR1 is required for RAS transformation of pancreatic cells (78). It is notable that HRAS, KRAS and RAF1 all appear on the upstream regulator algorithm as does TNF representing the key role of NF- κ B in the transformation process.

While our study focused on the role of HGF/MET in cutaneous cancer, MET signaling has been linked to a broader variety of human cancers, prompting the development of MET inhibitors as cancer therapeutics (10). Both preclinical and clinical experiences have revealed crosstalk of MET and EGFR in the therapeutic setting. In particular MET amplification is identified as a resistance mechanism for tumor cell lines and lung cancer patients treated with EGFR inhibitors (79). Conversely, EGFR inhibitors enhance the antitumor activity of MET inhibitors in cell lines, xenograft models and lung cancer patients

(80–82). Thus clinical trials of combined anti-MET and anti-EGFR treatment in advanced internal cancers show promise. Our data suggest that cutaneous cancers, perhaps in the setting of organ transplant patients where cutaneous SCC can be life threatening, should be considered as a model for combined MET/EGFR kinase inhibitor therapy where the dynamics of tumor response can be followed visually and sampled temporally.

MATERIALS AND METHODS

Mice and treatments.

Mouse studies were performed under a protocol approved by the National Cancer Institute (NCI) and NIH Animal Care and Use Committee. The construction and characterization of K5-PKC α and MT-HGF mice on an FVB/N background were previously described (58, 83). K5-PKC α and MT-HGF mice were crossed to produce F1 mice MT-HGF/K5-PKC α double transgenic (DT) mice. These DT mice were then crossed to wild-type FVB/N background mice to produce F2 mice of all four genotypes. At various time points post TPA application, skin biopsy samples were fixed in formalin (ED Biosciences) and embedded in paraffin for H&E analysis and immunohistochemistry. Epidermal hyperplasia was quantified by measuring the epidermal height at 5 randomly chosen sites per skin biopsy (x400 magnification; Nikon Eclipse E400). ADAM17^{fl/fl} (37) mice were purchased from Jackson laboratories.

Tumor induction experiments.

Initiation with DMBA (Sigma-Aldrich) was done by a single topical application of 20 μ g of DMBA in 0.1 mL acetone on the backs of 4-day-old mice. Twice a week TPA (LC Laboratories) treatments [1 μ g (1.6 nmoles) in 0.2mL of acetone] were started when mice reached 6 weeks of age and continued for 5 weeks. Squamous tumors induced by the initiation-promotion protocol were counted weekly until 20 weeks post DMBA treatment. For tumor studies where exogenous initiation was eliminated by excluding DMBA treatment, bi-weekly TPA treatment [1 μ g (1.6 nmoles) in 0.2mL of acetone] were started when mice reached 6 weeks of age and continued for 10 weeks. Portions of skin tumors were either frozen or formalin fixed at times and tumor type [squamous papilloma or squamous cell carcinoma (SCC)] was verified by visualizing H&E stained sections.

Syngeneic mouse grafting.

Confluent cultures of WT and MT-HGF primary keratinocytes were used for grafting as described previously (30). Six million keratinocytes (WT or MT-HGF) were respectively mixed with 6 million WT or MT-HGF mouse primary dermal fibroblasts (cultured for 1 week) and grafted onto syngeneic recipient back (WT or MT-HGF) on a prepared skin graft site located in the interscapular region. Three to four weeks post-grafting when tumors had developed on the MT-HGF grafts, daily gavage treatment with gefitinib (100mg/kg) or vehicle control (10% DMSO in water) was conducted for 2 weeks. This dosage and regimen was chosen based on efficacy and lack of side effect as reported previously (42). Tumor dimensions were measured weekly using calipers, and approximate tumor volumes were determined by multiplying tumor height $\times$ length $\times$ width. Tumors were collected in 10% formalin and processed for H&E staining (Histoserv Inc.), Ki67 and or anti-PECAM-1/

CD31 (Santa Cruz, sc-1506) immunostaining was performed by the Pathology/Histotechnology Laboratory (PHL, NCI-Frederick). The percentage of Ki67-positive cells was quantified by counting Ki67-labeled basal cells from at least three randomly chosen areas. The microvascular density was determined by counting the number of CD31 stained cells in at least five fields (x400 magnification, Nikon Eclipse E400).

Cell culture.

Primary mouse keratinocytes were isolated from newborn transgenic and WT littermate epidermis as described and cultured in MEM, 7% chelex treated fetal calf serum (Gemini Bio-Products) and 0.05mM calcium unless otherwise indicated (30). EGFR inhibitors AG1478, PD168393 (Calbiochem), gefitinib (LC Laboratories), MET inhibitor PHA 665752 (Tocris), MET inhibitor Capmatinib (Selleck Chem), ADAM17 inhibitor GM6001 (Calbiochem) were diluted in DMSO. Anti-TGF α antibody (Abcam), anti-AREG antibody (R&D systems) and IL-1 receptor antagonist (5 μ g/ml, IL-1ra or Anakinra, Division of Veterinary Resources, NIH) were diluted in culture medium and added to cell culture medium as indicated prior to cell harvesting. Non-silencing (control), Hras, Kras, Nras, Src, iRhom1, iRhom2 siRNAs (Qiagen), ADAM17 silencing siRNA (Qiagen and Thermo scientific) were transfected using RNAiMax (Life Technologies) at a final concentration of 20nM. At 24 h or 48h post-transfection with siRNA, cells were treated with HGF (Peprotech, 40ng/ml) or harvested for analysis. Primary mouse keratinocytes were isolated from newborn EGFR-deficient and wild-type mice and confluent cultures were treated for 3 hours with HGF. Human epidermal carcinoma cell line A431 (ATCC CRL-1555) was cultured in DMEM supplemented with 10% fetal bovine serum. SCC-4 (tongue, ATCC CRL-1624), SCC-9 (tongue, ATCC CRL-1629), SCC-15 (tongue, ATCC CRL-1623), and SCC-25 (tongue, ATCC CRL-1628) were cultured in DMEM:F12 (Lonza) supplemented with 0.5 mM sodium pyruvate, 400 ng/mL hydrocortisone and 10% fetal bovine serum. Cutaneous SCC-13 (84) was cultured in MEM (Lonza). Confluent cultures were treated with MET inhibitors, Capmatinib INCB28060 (Selleck) and PHA 665752 (Tocris) for 24 hours.

Retroviral and adenoviral constructs.

The v-*ras*^{Ha} replication defective ecotropic retrovirus was prepared using ψ 2 producer cells (85). Retrovirus titers were routinely 1×10^7 virus/ml. Cultured primary keratinocytes were infected with v-*ras*^{Ha} retrovirus (here referred to as RAS or oncogenic RAS) on day 3 at a MOI of 1 in medium containing 4 μ g/ml Polybrene (Sigma). The I κ B α super repressor (86) adenovirus was introduced into primary keratinocytes using an adenoviral construct driven by a cytomegalovirus (CMV) promoter and empty adenovirus was used as control (A-CMV). Keratinocytes were adenovirus infected for 30 minutes in serum-free medium with a multiplicity of infection of 10 viral particles/cell and 4 μ g/ml of Polybrene (Sigma) to enhance uptake. Serum containing medium was added to the cells for the next 48 hours after the infection. Primary keratinocytes from ADAM17^{fl/fl} (Jackson Laboratories) mice were cultured in 0.05 mM Ca⁺⁺ medium to confluence and transduced for 48h with Cre adenovirus (kind gift of Dr. Frank Gonzalez, NCI) and then challenged with HGF (Peprotech, 40 ng/ml).

Immunoblotting.

Cultured keratinocytes with or without v-ras^{Ha} transduction were lysed in MPER lysis buffer (Pierce) supplemented with 200 $\mu\text{mol/L}$ NaVO_3 , 10 mmol/L NaF , and Complete Mini tablets (Roche). Lysates prepared for ADAM17 analysis were supplemented with the metalloprotease inhibitor: 1,10-phenanthroline (Sigma) at 10 mM . Proteins were quantified by the Bradford method (Bio-Rad) and separated by 10.5–14 %, 4–20%, or 10% Tris-HCl gels (Bio-Rad). To prepare lysates for immunoblotting from skin biopsy samples, flash frozen skin biopsies in liquid nitrogen were processed using a ball mill pulverizer (Mikro-Dismembrator S, from Sartorius) for one minute at 2000 rpm. These samples were subsequently resuspended in RIPA buffer supplemented with a protease inhibitor cocktail (Halt, Thermo Scientific). For analysis of keratinocyte differentiation in vitro, cultures were washed once with PBS (Ca^{2+} and Mg^{2+} free), and total cell lysates were prepared in situ on the dish using 10 $\mu\text{l}/\text{cm}^2$ lysis buffer (5% SDS and 20% 5-mercaptoethanol in 0.25 M Tris, pH 6.8). Total EGFR (Santa Cruz Biotechnology), phospho-EGFR (Invitrogen), total MET and phospho MET (Tyr1234/5, Cell signaling) were detected after overnight incubation with 1:500 dilution of each antibody. Anti-TACE/ADAM17 (QED) and SRC (Cell Signaling) were incubated overnight at 1:1000 dilutions. COX-2 antibodies (Cayman Chemical) were incubated overnight at 1:750 dilutions. Anti-HSP90 antibodies (BD Transduction Laboratories) were incubated 2 hours at room temperature at 1:5000 dilutions. Anti-K8 (University of Iowa) was used at 1:100 and anti-K1 and anti-K10 (Covance) were used 1:10,000, both incubated overnight. Anti-ACTIN (Chemicon) was used 1:10,000 for one hour at room temperature. ECL SuperSignal (Pierce) system was used for detection. The intensities of immunoblots were quantified using ImageJ (NIH), and the relative expression of targeted proteins was normalized.

³H-Thymidine incorporation assay.

Keratinocytes and SCC cell lines were plated in 24 well plates and 3 days post plating ³H-thymidine (1 μCi per well) was added for 4hrs. Cultures were trypsinized and well content was transferred to glass fiber filters using a Brandel cell harvester and incorporated counts were read using a Wallac Trilux 1450 Microbeta scintillation counter (Perkin Elmer).

Isolation of Tumor DNA.

30 μm Sections were cut from formalin fixed, paraffin-embedded papillomas arising from DT mice treated with TPA only as described above. Genomic DNA was isolated using QIAamp DNA FFPE tissue kit (Qiagen) or DNAeasy Tissue kit (Qiagen) for tumors that were snap frozen (DMBA-TPA studies) in liquid nitrogen at collection time.

PCR Amplification and Sequencing of K-, H-, and N-Ras Genes.

Primers specific for mouse *Kras*, *Hras1*, and *Nras* were purchased from Invitrogen and are listed below. Nested primers were used for *Kras* and *Hras1* analysis. All PCR reactions used Taq master mix (Qiagen), and a PCR protocol previously described (54). After PCR amplification, product was purified using QIAquick PCR purification kit (Qiagen). Sequencing was performed using the ABI prism dye terminator kit, and was performed by NCI sequencing core.

Gene	Primer	Sequence
<i>Kras</i> (Codon 12+13)	External-Forward*	ACACACAAAGGTGAGTGTTAAA
	External-Reverse	GCAGCGTTACCTCTATCGTA
	Internal-Forward	TTATTGTAAGGCTGCTGAA
	Internal-Reverse	TCATACTCATCCACAAAGTG
<i>Kras</i> (Codon 61)	External-Forward	TTCTCAGGACTCCTACAGGA
	External-Reverse	ACCCACCTATAATGGTGAAT
	Internal-Forward	TACAGGAAACAAGTAGTAATTGATGGAGA
	Internal-Reverse	ATAATGGTGAATATCTTCAAATGATTTAGT
<i>Hras</i> (Codon 12+13)	External-Forward*	GGTGATCAACTGGGCCACTG
	External-Reverse	CCTCTGGCAGGTAGGCAGAG
	Internal-Forward	CTAAGTGTGCTTCTCATTGGCAGGT
	Internal-Reverse	CTCTATAGTGGGATCATACTCGTCC
<i>Hras</i> (Codon 61)	External-Forward	CCACTAAGCCGTGTGTTTTGCA
	External-Reverse	CTGTACTGATGGATGTCCTCGAAGGA
	Internal-Forward	GGACTCCTACCGGAAACAGG
	Internal-Reverse	GGTGTGTGTGATGGCAAATACA
<i>Nras</i> (Codon 12+13)	Forward	GACTGAGTACAACTGGTGG
	Reverse	GGGCCTCACCTCTATGGTG
<i>Nras</i> (Codon 61)	Forward	GGTGAGACCTGCCTGCTGGA
	Reverse	ATACACAGAGGAACCCCTTCG

Primers marked with * were designed for this experiment, all non-marked primers were previously reported (54).

Reverse transcription-PCR analysis.

RNA was isolated from cultured cells and post-homogenized tissue biopsies with Trizol using manufacturer's protocol (Invitrogen). cDNA synthesis and real-time PCR analysis were conducted as previously described (87). Predesigned Quantitect primers (Qiagen) were used for all genes except for *Gapdh*, where the following primers were designed: forward 5'-CATGGCCTTCCGTGTTTCCTA-3' and reverse 5'-GCGGCACGTCAGATCCA-3'.

MPO assay.

Skin samples were homogenized in potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB), sonicated, and freeze thawed 3 times, after which sonication was repeated. The suspension was centrifuged at 40,000 *g* for 15 minutes, and 10 μ l of supernatant was added to 290 μ l of potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml *o*-dianisidine dihydrochloride (Sigma-Aldrich) and 0.0005% hydrogen peroxide. Changes in OD were monitored at 460 nm at 25°C, over a 4-minute period.

IHC analysis.

Human skin cancer tissue arrays were obtained from US Biomax (SK802a) and were used for MET expression and localization studies. Immunohistochemical staining used the MET antibody (Cell Signaling D1C2; 1:100 dilution) and procedures described by the array manufacturers. Target retrieval (DAKO) was performed in a microwave for 10 min. Secondary antibody (anti-rabbit 1:300; Vector) and Avidin/Biotinylated conjugation was performed with ABC kit (Vector) according to procedures described by the manufacturer. Slides were scanned and analyzed using a ScanScope XT scanner and ImageScope viewing software version 11.0.2.725 (Aperio Technologies Inc., Vista, CA).

HGF in situ hybridization.

This procedure was performed by a commercial vendor (Phylogeny, Inc.) according to the published method (88). FFPE TMAs from US Biomax were deparaffinized for 5 minutes in xylene, immersed in 100% ethanol for 5 minutes then air-dried. Treatment was with Bond Epitope Retrieval Solution 2 from Leica (AR9640) for 30 minutes. LNA probes HGF-1-1, HGF-1-2 and Scramble-miR, were prepared according to the manufacturer's recommended conditions (Exiqon) and each was labeled at the 5' end with digoxigenin. Probes were diluted to 25 fmol per μ l in Exiqon hybridization buffer (208022). Probe solutions were placed on tissue sections, covered with polypropylene coverslips and heated to 60°C for 5 minutes, followed by hybridization at 37°C overnight. Sections were washed in intermediate stringency solution (0.2X SSC with 2% bovine serum albumin) at 55°C for 10 minutes. Sections were treated with anti-digoxigenin-alkaline phosphatase conjugate (1:150 dilutions in pH 7 Tris buffer; Roche) at 37°C for 30 minutes. Development was carried out with NBT/BCIP from ThermoFisher (34042). Development was closely monitored and stopped when the control sections appeared light blue. Development time with the chromogen was between 15–30 minutes. Sections were counterstained with nuclear fast red for 3 to 5 minutes, rinsed and mounted with coverslips. Slides were scanned using Aperio ScanScope XT System and analyzed using ImageScope (version 12.1.0.5029, Leica). To distinguish signal from counter-stain, a color deconvolution algorithm (Aperio) was used to separate chromagen generated signal from counter stain and pseudo-colored using Adobe Photoshop.

ELISA.

Quantikine ELISA kits for CXCL1 and Amphiregulin were used according to the manufacturer's protocol (R&D Systems).

Microarray.

Total RNA was extracted from keratinocyte cultures with TRIzol (Invitrogen) using manufacture's protocol. Three independent biological replicates were evaluated. Gene expression profiling was done using Affymetrix Mouse Gene 1.0 ST array platform. RNA quality-testing, microarray hybridization and processing were done by the Laboratory of Molecular Technology in Frederick, MD. Raw gene expression data (.CEL files) were processed with the Robust Multichip Average algorithm (RMA) and quantile normalization (89) -implemented in the BRB-ArrayTools version 4.3.2 (software developed by Dr. Richard Simon and BRB-ArrayTools Development Team; <http://linus.nci.nih.gov/BRB->

[ArrayTools.html](#)). Gene annotation and mapping of the mouse-human orthologs were imported from mAdb (<https://madb.nci.nih.gov/>). For genes with multiple probesets, average expression was calculated from the two most correlated probesets if the Pearson correlation reached at least 0.8, otherwise the probeset with the highest median was selected. A total of 17,114 mouse-human orthologues were input into the statistical analysis. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession GSE58671.

Genomic analyses.

Analysis of variance.—One-way RVM-ANOVA and contrast comparisons (90) were used to identify differential expression between WT and MT-HGF-, MT-HGF-AG1478-, and RAS-keratinocytes. False discovery rate (91) less than 1% was chosen as the significance cutoff. In addition, genes showing at least 2-fold change and the same direction of change in both RAS-keratinocytes and MT-HGF keratinocytes were selected to the model RAS/MET initiation signature. The calculations were performed in R programming environment (*R version 3.0.2*) and R/Bioconductor *qvalue 1.36.0* library (92).

Gene ontology (GO) analyses.—Gene Set Enrichment Analysis (GSEA) was conducted using GO gene sets collections with the broadest terms eliminated (GO_FAT) from DAVID database (). In GSEA, genes were ranked by the average ANOVA t-statistic and gene sets were considered enriched at 5% false discovery rate. Furthermore, subsets of enriched gene sets were selected if the core genes (GSEA 'leading edge') included at least five genes from the top RAS/MET signature and were summarized with semantically non-redundant terms using REVIGO algorithm (44). REVIGO interaction maps were visualized using R/CRAN *igraph 0.7.0* library (93).

Ingenuity Pathways Analysis (IPA).—Genes from the RAS/MET signature were also used as input to the Upstream Regulator tool in the Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). The enrichment p-value (Fisher's exact test) less than 0.01 and bias-corrected z-score (predicted activation state) exceeding ± 1.96 were used to select candidate upstream regulators.

Denoising Algorithm based on Relevance network Topology (DART).—An unsupervised strategy of Jiao et al. (46) for inferring molecular activation of a model perturbation signature was used to evaluate expression of the model RAS/MET signature in squamous cell carcinoma patients. Microarray datasets available in GEO were utilized in the analysis, namely patient data from Hameetman et al. (94), Mitsui et al. (47), and Nindl et al. (48). Gene expression in each dataset was standardized by means of z-score (the mean of zero and unit standard deviation). Predicted activity levels of RAS/MET signature were compared between normal epidermis, AK and SCC samples using the non-parametric Wilcoxon signed rank test (paired data) or Wilcoxon rank sum test (unpaired data). The computations were performed using R/Bioconductor *DART 1.8.0* version (46).

Statistics.

Unless otherwise specified, biochemical data were analyzed by prism software and significance values assigned through Student's t-test or One-Way ANOVA with Tukey post-test. $P < 0.05$ was considered to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

The authors thank Susanna Walters for care of the mouse colonies, Donna Butcher and Miriam Anver from the Pathology/Histotechnology Laboratory (SAIC-Frederick) for excellent technical assistance and Chuck Ostermeier from the Cryopreservation & Assisted Reproduction Laboratory (SAIC-Frederick).

Funding: This work was funded by the intramural program of the Center for Cancer Research of the National Cancer Institute.

References and Notes

1. Rundhaug JE, and Fischer SM. Molecular mechanisms of mouse skin tumor promotion. *Cancers (Basel)*. 2010;2(2):436–82. [PubMed: 21297902]
2. Coussens LM, and Werb Z. Inflammation and cancer. *Nature*. 2002;420(6917):860–7. [PubMed: 12490959]
3. Mueller MM. Inflammation in epithelial skin tumours: old stories and new ideas. *EurJCancer*. 2006;42(6):735–44.
4. Johansson M, Denardo DG, and Coussens LM. Polarized immune responses differentially regulate cancer development. *Immunol Rev*. 2008;222(145–540).
5. Cataisson C, Ohman R, Patel G, Pearson A, Tsien M, Jay S, Wright L, Hennings H, and Yuspa SH. Inducible cutaneous inflammation reveals a protumorigenic role for keratinocyte CXCR2 in skin carcinogenesis. *Cancer Res*. 2009;69(1):319–28. [PubMed: 19118017]
6. Cataisson C, Salcedo R, Hakim S, Moffitt BA, Wright L, Yi M, Stephens R, Dai RM, Lyakh L, Schenten D, et al. IL-1R-MyD88 signaling in keratinocyte transformation and carcinogenesis. *J Exp Med*. 2012;209(9):1689–702. [PubMed: 22908325]
7. Wang HQ, Kim MP, Tiano HF, Langenbach R, and Smart RC. Protein kinase C- α coordinately regulates cytosolic phospholipase A(2) activity and the expression of cyclooxygenase-2 through different mechanisms in mouse keratinocytes. *MolPharmacol*. 2001;59(4):860–6.
8. Jansen AP, Dreckschmidt NE, Verwiebe EG, Wheeler DL, Oberley TD, and Verma AK. Relation of the induction of epidermal ornithine decarboxylase and hyperplasia to the different skin tumor-promotion susceptibilities of protein kinase C α , δ and ϵ transgenic mice. *IntJCancer*. 2001;93(5):635–43.
9. Knudsen BS, and Vande Woude G. Showering c-MET-dependent cancers with drugs. *Curr Opin Genet Dev*. 2008;18(1):87–96. [PubMed: 18406132]
10. Graveel CR, Tolbert D, and Vande Woude GF. MET: a critical player in tumorigenesis and therapeutic target. *Cold Spring Harbor perspectives in biology*. 2013;5(7).
11. Lee YJ, Kim DH, Lee SH, Kim DW, Nam HS, and Cho MK. Expression of the c-Met Proteins in Malignant Skin Cancers. *Annals of dermatology*. 2011;23(1):33–8. [PubMed: 21738360]
12. Szabo R, Rasmussen AL, Moyer AB, Kosa P, Schafer JM, Molinolo AA, Gutkind JS, and Bugge TH. c-Met-induced epithelial carcinogenesis is initiated by the serine protease matriptase. *Oncogene*. 2011;30(17):2003–16. [PubMed: 21217780]
13. Danilkovitch-Miagkova A, and Zbar B. Dysregulation of Met receptor tyrosine kinase activity in invasive tumors. *J Clin Invest*. 2002;109(7):863–7. [PubMed: 11927612]

14. Martin CL, Reshmi SC, Ried T, Gottberg W, Wilson JW, Reddy JK, Khanna P, Johnson JT, Myers EN, and Gollin SM. Chromosomal imbalances in oral squamous cell carcinoma: examination of 31 cell lines and review of the literature. *Oral Oncol.* 2008;44(4):369–82. [PubMed: 17681875]
15. Schmidt L, Junker K, Nakaigawa N, Kinjerski T, Weirich G, Miller M, Lubensky I, Neumann HP, Brauch H, Decker J, et al. Novel mutations of the MET proto-oncogene in papillary renal carcinomas. *Oncogene.* 1999;18(14):2343–50. [PubMed: 10327054]
16. Ma J, DeFrances MC, Zou C, Johnson C, Ferrell R, and Zarnegar R. Somatic mutation and functional polymorphism of a novel regulatory element in the HGF gene promoter causes its aberrant expression in human breast cancer. *J Clin Invest.* 2009;119(3):478–91. [PubMed: 19188684]
17. Chiba S, Tsuchiya N, Horikawa Y, Narita S, Inoue T, Akihama S, Saito M, Numakura K, Tsuruta H, Huang M, et al. Functional mononucleotide repeat polymorphism in the promoter region of HGF is associated with risk and malignant aggressiveness of bladder cancer. *International journal of oncology.* 2014;44(3):678–84. [PubMed: 24366484]
18. Hoadley KA, Yau C, Wolf DM, Cherniack AD, Tamborero D, Ng S, Leiserson MD, Niu B, McLellan MD, Uzunangelov V, et al. Multiplatform analysis of 12 cancer types reveals molecular classification within and across tissues of origin. *Cell.* 2014;158(4):929–44. [PubMed: 25109877]
19. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Science signaling.* 2013;6(269):p11.
20. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer discovery.* 2012;2(5):401–4. [PubMed: 22588877]
21. Mildner M, Mlitz V, Gruber F, Wojta J, and Tschachler E. Hepatocyte growth factor establishes autocrine and paracrine feedback loops for the protection of skin cells after UV irradiation. *J Invest Dermatol.* 2007;127(11):2637–44. [PubMed: 17597814]
22. Choi W, Miyamura Y, Wolber R, Smuda C, Reinhold W, Liu H, Kolbe L, and Hearing VJ. Regulation of human skin pigmentation in situ by repetitive UV exposure: molecular characterization of responses to UVA and/or UVB. *J Invest Dermatol.* 2010;130(6):1685–96. [PubMed: 20147966]
23. Knowles LM, Stabile LP, Egloff AM, Rothstein ME, Thomas SM, Gubish CT, Lerner EC, Seethala RR, Suzuki S, Quesnelle KM, et al. HGF and c-Met participate in paracrine tumorigenic pathways in head and neck squamous cell cancer. *Clin Cancer Res.* 2009;15(11):3740–50. [PubMed: 19470725]
24. Takayama H, La Rochelle WJ, Anver M, Bockman DE, and Merlino G. Scatter factor/hepatocyte growth factor as a regulator of skeletal muscle and neural crest development. *Proceedings of the National Academy of Sciences of the USA.* 1996;93(12):5866–71. [PubMed: 8650184]
25. Takayama H, Larochelle WJ, Sharp R, Otsuka T, Kriebel P, Anver M, Aaronson SA, and Merlino G. Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. *Proceedings of the National Academy of Sciences of the USA.* 1997;94(2):701–6. [PubMed: 9012848]
26. Noonan FP, Recio JA, Takayama H, Duray P, Anver MR, Rush WL, De Fabo EC, and Merlino G. Neonatal sunburn and melanoma in mice. *Nature.* 2001;413(6853):271–2. [PubMed: 11565020]
27. Tormo D, Ferrer A, Gaffal E, Wenzel J, Basner-Tschakarjan E, Steitz J, Heukamp LC, Gutgemann I, Buettner R, Malumbres M, et al. Rapid growth of invasive metastatic melanoma in carcinogen-treated hepatocyte growth factor/scatter factor-transgenic mice carrying an oncogenic CDK4 mutation. *American Journal of Pathology.* 2006;169(2):665–72. [PubMed: 16877364]
28. Abel EL, Angel JM, Kiguchi K, and DiGiovanni J. Multi-stage chemical carcinogenesis in mouse skin: fundamentals and applications. *NatProtoc.* 2009;4(9):1350–62.
29. Glick A, Ryscavage A, Perez-Lorenzo R, Hennings H, Yuspa S, and Darwiche N. The high-risk benign tumor: evidence from the two-stage skin cancer model and relevance for human cancer. *Mol Carcinog.* 2007;46(8):605–10. [PubMed: 17538943]

30. Lichti U, Anders J, and Yuspa SH. Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for in vitro analysis and for grafting to immunodeficient mice. *NatProtoc.* 2008;3(5):799–810.
31. Dlugosz AA, Hansen L, Cheng C, Alexander N, Denning MF, Threadgill DW, Magnuson T, Coffey RJ, Jr., and Yuspa SH. Targeted disruption of the epidermal growth factor receptor impairs growth of squamous papillomas expressing the v-ras Ha oncogene but does not block in vitro keratinocyte responses to oncogenic ras *Cancer Res.* 1997;57(3180–8).
32. Morgan D, Welty D, Glick A, Greenhalgh D, Hennings H, and Yuspa SH. Development of an in vitro model to study carcinogen-induced neoplastic progression of initiated mouse epidermal cells. *Cancer Res.* 1992;52(3145–56).
33. Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, LaMantia C, Mourton T, Herrup K, Harris RC, et al. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science.* 1995;269(230–4).
34. Mascia F, Lam G, Keith C, Garber C, Steinberg SM, Kohn E, and Yuspa SH. Genetic ablation of epidermal EGFR reveals the dynamic origin of adverse effects of anti-EGFR therapy. *Science translational medicine.* 2013;5(199):199ra10.
35. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, et al. A metalloproteinase disintegrin that releases tumour-necrosis factor- α from cells. *Nature.* 1997;385(6618):729–33. [PubMed: 9034190]
36. Peschon JJ, Slack JL, Reddy P, Stocking KL, Sunnarborg SW, Lee DC, Russell WE, Castner BJ, Johnson RS, Fitzner JN, et al. An essential role for ectodomain shedding in mammalian development. *Science.* 1998;282(5392):1281–4. [PubMed: 9812885]
37. Horiuchi K, Kimura T, Miyamoto T, Takaishi H, Okada Y, Toyama Y, and Blobel CP. Cutting edge: TNF- α -converting enzyme (TACE/ADAM17) inactivation in mouse myeloid cells prevents lethality from endotoxin shock. *J Immunol.* 2007;179(5):2686–9. [PubMed: 17709479]
38. Maretzky T, Zhou W, Huang XY, and Blobel CP. A transforming Src mutant increases the bioavailability of EGFR ligands via stimulation of the cell-surface metalloproteinase ADAM17. *Oncogene.* 2011;30(5):611–8. [PubMed: 20871631]
39. Adrain C, Zettl M, Christova Y, Taylor N, and Freeman M. Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. *Science.* 2012;335(6065):225–8. [PubMed: 22246777]
40. Li X, Maretzky T, Weskamp G, Monette S, Qing X, Issuree PD, Crawford HC, McIlwain DR, Mak TW, Salmon JE, et al. iRhoms 1 and 2 are essential upstream regulators of ADAM17-dependent EGFR signaling. *Proc Natl Acad Sci U S A.* 2015;112(19):6080–5. [PubMed: 25918388]
41. Maney SK, McIlwain DR, Polz R, Pandya AA, Sundaram B, Wolff D, Ohishi K, Maretzky T, Brooke MA, Evers A, et al. Deletions in the cytoplasmic domain of iRhom1 and iRhom2 promote shedding of the TNF receptor by the protease ADAM17. *Science signaling.* 2015;8(401):ra109. [PubMed: 26535007]
42. Yan Y, Lu Y, Wang M, Vikis H, Yao R, Wang Y, Lubet RA, and You M. Effect of an epidermal growth factor receptor inhibitor in mouse models of lung cancer. *Mol Cancer Res.* 2006;4(12): 971–81. [PubMed: 17189387]
43. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A.* 2005;102(43):15545–50. [PubMed: 16199517]
44. Supek F, Bosnjak M, Skunca N, and Smuc T. REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS One.* 2011;6(7):e21800. [PubMed: 21789182]
45. Guo D, Bell EH, Mischel P, and Chakravarti A. Targeting SREBP-1-driven lipid metabolism to treat cancer. *Current pharmaceutical design.* 2014;20(15):2619–26. [PubMed: 23859617]
46. Jiao Y, Lawler K, Patel GS, Purushotham A, Jones AF, Grigoriadis A, Tutt A, Ng T, and Teschendorff AE. DART: Denoising Algorithm based on Relevance network Topology improves molecular pathway activity inference. *BMC Bioinformatics.* 2011;12(403).

47. Mitsui H, Suarez-Farinas M, Gulati N, Shah KR, Cannizzaro MV, Coats I, Felsen D, Krueger JG, and Carucci JA. Gene Expression Profiling of the Leading Edge of Cutaneous Squamous Cell Carcinoma: IL-24-Driven MMP-7. *J Invest Dermatol.* 2014;134(5):1418–27. [PubMed: 24270662]
48. Nindl I, Dang C, Forschner T, Kuban RJ, Meyer T, Sterry W, and Stockfleth E. Identification of differentially expressed genes in cutaneous squamous cell carcinoma by microarray expression profiling. *Molecular cancer.* 2006;5(30).
49. Balmain A, and Yuspa SH. Milestones in skin carcinogenesis: the biology of multistage carcinogenesis. *J Invest Dermatol.* 2014;134(e1):E2–7. [PubMed: 25302469]
50. Pylayeva-Gupta Y, Grabocka E, and Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer.* 2011;11(11):761–74. [PubMed: 21993244]
51. Salcedo R, Cataisson C, Hasan U, Yuspa SH, and Trinchieri G. MyD88 and its divergent toll in carcinogenesis. *Trends in immunology.* 2013;34(8):379–89. [PubMed: 23660392]
52. Lindner G, Menrad A, Gherardi E, Merlino G, Welker P, Handjiski B, Roloff B, and Paus R. Involvement of hepatocyte growth factor/scatter factor and met receptor signaling in hair follicle morphogenesis and cycling. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology.* 2000;14(2):319–32. [PubMed: 10657988]
53. Megosh L, Halpern M, Farkash E, and O'Brien TG. Analysis of ras gene mutational spectra in epidermal papillomas from K6/ODC transgenic mice. *Mol Carcinog.* 1998;22(3):145–9. [PubMed: 9688139]
54. Rehman I, Lowry DT, Adams C, Abdel-Fattah R, Holly A, Yuspa SH, and Hennings H. Frequent codon 12 Ki-ras mutations in mouse skin tumors initiated by N-methyl-N'-nitro-N-nitrosoguanidine and promoted by mezerein. *Molecular Carcinogenesis.* 2000;27(4):298–307. [PubMed: 10747294]
55. List K, Szabo R, Molinolo A, Sriuranpong V, Redeye V, Murdock T, Burke B, Nielsen BS, Gutkind JS, and Bugge TH. Deregulated matriptase causes ras-independent multistage carcinogenesis and promotes ras-mediated malignant transformation. *Genes Dev.* 2005;19(16):1934–50. [PubMed: 16103220]
56. Chmielowiec J, Borowiak M, Morkel M, Stradal T, Munz B, Werner S, Wehland J, Birchmeier C, and Birchmeier W. c-Met is essential for wound healing in the skin. *J Cell Biol.* 2007;177(1):151–62. [PubMed: 17403932]
57. Bevan D, Gherardi E, Fan TP, Edwards D, and Warn R. Diverse and potent activities of HGF/SF in skin wound repair. *The Journal of pathology.* 2004;203(3):831–8. [PubMed: 15221943]
58. Noonan FP, Otsuka T, Bang S, Anver MR, and Merlino G. Accelerated ultraviolet radiation-induced carcinogenesis in hepatocyte growth factor/scatter factor transgenic mice. *Cancer Res.* 2000;60(14):3738–43. [PubMed: 10919643]
59. Aebersold DM, Landt O, Berthou S, Gruber G, Beer KT, Greiner RH, and Zimmer Y. Prevalence and clinical impact of Met Y1253D-activating point mutation in radiotherapy-treated squamous cell cancer of the oropharynx. *Oncogene.* 2003;22(52):8519–23. [PubMed: 14627992]
60. McCreery MQ, Halliwill KD, Chin D, Delrosario R, Hirst G, Vuong P, Jen KY, Hewinson J, Adams DJ, and Balmain A. Evolution of metastasis revealed by mutational landscapes of chemically induced skin cancers. *Nat Med.* 2015;21(12):1514–20. [PubMed: 26523969]
61. Jo M, Stolz DB, Esplen JE, Dorko K, Michalopoulos GK, and Strom SC. Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. *J Biol Chem.* 2000;275(12):8806–11. [PubMed: 10722725]
62. Bergstrom JD, Westermarck B, and Heldin NE. Epidermal growth factor receptor signaling activates met in human anaplastic thyroid carcinoma cells. *Experimental cell research.* 2000;259(1):293–9. [PubMed: 10942601]
63. Fischer OM, Giordano S, Comoglio PM, and Ullrich A. Reactive oxygen species mediate Met receptor transactivation by G protein-coupled receptors and the epidermal growth factor receptor in human carcinoma cells. *J Biol Chem.* 2004;279(28):28970–8. [PubMed: 15123705]
64. Ardito CM, Gruner BM, Takeuchi KK, Lubeseder-Martellato C, Teichmann N, Mazur PK, Delgiorno KE, Carpenter ES, Halbrook CJ, Hall JC, et al. EGF receptor is required for KRAS-induced pancreatic tumorigenesis. *Cancer Cell.* 2012;22(3):304–17. [PubMed: 22975374]

65. Van Schaeuybroeck S, Kyula JN, Fenton A, Fenning CS, Sasazuki T, Shirasawa S, Longley DB, and Johnston PG. Oncogenic Kras promotes chemotherapy-induced growth factor shedding via ADAM17. *Cancer Res.* 2011;71(3):1071–80. [PubMed: 21148749]
66. Yang Y, Wang Y, Zeng X, Ma XJ, Zhao Y, Qiao J, Cao B, Li YX, Ji L, and Wang YL. Self-control of HGF regulation on human trophoblast cell invasion via enhancing c-Met receptor shedding by ADAM10 and ADAM17. *The Journal of clinical endocrinology and metabolism.* 2012;97(8):E1390–401. [PubMed: 22689693]
67. Etheridge SL, Brooke MA, Kelsell DP, and Blaydon DC. Rhomboid proteins: a role in keratinocyte proliferation and cancer. *Cell Tissue Res.* 2013;351(2):301–7. [PubMed: 23263464]
68. Leilei Y, Bing L, Yang L, Shaoxia W, Yuan X, Dongping W, Huahu Y, Shichen S, Guangzhou Z, Ruiyun P, et al. iRhom2 mutation leads to aberrant hair follicle differentiation in mice. *PLoS One.* 2014;9(12):e115114. [PubMed: 25546423]
69. Brooke MA, Etheridge SL, Kaplan N, Simpson C, O'Toole EA, Ishida-Yamamoto A, Marches O, Getsios S, and Kelsell DP. iRHOM2-dependent regulation of ADAM17 in cutaneous disease and epidermal barrier function. *Hum Mol Genet.* 2014;23(15):4064–76. [PubMed: 24643277]
70. Hosur V, Johnson KR, Burzenski LM, Stearns TM, Maser RS, and Shultz LD. Rhbdf2 mutations increase its protein stability and drive EGFR hyperactivation through enhanced secretion of amphiregulin. *Proc Natl Acad Sci U S A.* 2014;111(21):E2200–9. [PubMed: 24825892]
71. Blaydon DC, Etheridge SL, Risk JM, Hennies HC, Gay LJ, Carroll R, Plagnol V, McDonald FE, Stevens HP, Spurr NK, et al. RHBDF2 mutations are associated with tylosis, a familial esophageal cancer syndrome. *Am J Hum Genet.* 2012;90(2):340–6. [PubMed: 22265016]
72. Antalis TM, Bugge TH, and Wu Q. Membrane-anchored serine proteases in health and disease. *Progress in molecular biology and translational science.* 2011;99(1–50).
73. Hashimoto T, Kato M, Shimomura T, and Kitamura N. TMPRSS13, a type II transmembrane serine protease, is inhibited by hepatocyte growth factor activator inhibitor type 1 and activates pro-hepatocyte growth factor. *The FEBS journal.* 2010;277(23):4888–900. [PubMed: 20977675]
74. Lang JC, and Schuller DE. Differential expression of a novel serine protease homologue in squamous cell carcinoma of the head and neck. *Br J Cancer.* 2001;84(2):237–43. [PubMed: 11161383]
75. Guo D, Prins RM, Dang J, Kuga D, Iwanami A, Soto H, Lin KY, Huang TT, Akhavan D, Hock MB, et al. EGFR signaling through an Akt-SREBP-1-dependent, rapamycin-resistant pathway sensitizes glioblastomas to antilipogenic therapy. *Science signaling.* 2009;2(101):ra82. [PubMed: 20009104]
76. Cheng C, Ru P, Geng F, Liu J, Yoo JY, Wu X, Cheng X, Euthine V, Hu P, Guo JY, et al. Glucose-Mediated N-glycosylation of SCAP Is Essential for SREBP-1 Activation and Tumor Growth. *Cancer Cell.* 2015;28(5):569–81. [PubMed: 26555173]
77. Kemp CJ, Donehower LA, Bradley A, and Balmain A. Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors. *Cell.* 1993;74(813–22).
78. Hamidi T, Algul H, Cano CE, Sandi MJ, Molejon MI, Riemann M, Calvo EL, Lomberg G, Dagorn JC, Weih F, et al. Nuclear protein 1 promotes pancreatic cancer development and protects cells from stress by inhibiting apoptosis. *J Clin Invest.* 2012;122(6):2092–103. [PubMed: 22565310]
79. Niederst MJ, and Engelman JA. Bypass mechanisms of resistance to receptor tyrosine kinase inhibition in lung cancer. *Science signaling.* 2013;6(294):re6. [PubMed: 24065147]
80. Zhang YW, Staal B, Essenburg C, Lewis S, Kaufman D, and Vande Woude GF. Strengthening context-dependent anticancer effects on non-small cell lung carcinoma by inhibition of both MET and EGFR. *Mol Cancer Ther.* 2013;12(8):1429–41. [PubMed: 23720767]
81. Zhang YW, Staal B, Essenburg C, Su Y, Kang L, West R, Kaufman D, Dekoning T, Eagleson B, Buchanan SG, et al. MET kinase inhibitor SGX523 synergizes with epidermal growth factor receptor inhibitor erlotinib in a hepatocyte growth factor-dependent fashion to suppress carcinoma growth. *Cancer Res.* 2010;70(17):6880–90. [PubMed: 20643778]
82. Spigel DR, Ervin TJ, Ramlau RA, Daniel DB, Goldschmidt JH, Jr., Blumenschein GR, Jr., Krzakowski MJ, Robinet G, Godbert B, Barlesi F, et al. Randomized phase II trial of Onartuzumab

- in combination with erlotinib in patients with advanced non-small-cell lung cancer. *J Clin Oncol*. 2013;31(32):4105–14. [PubMed: 24101053]
83. Cataisson C, Joseloff E, Murillas R, Wang A, Atwell C, Torgerson S, Gerdes M, Subleski J, Gao JL, Murphy PM, et al. Activation of cutaneous protein kinase C alpha induces keratinocyte apoptosis and intraepidermal inflammation by independent signaling pathways. *J Immunol*. 2003;171(5):2703–13. [PubMed: 12928424]
 84. Rheinwald JG, and Beckett MA. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. *Cancer Res*. 1981;41(1657–63).
 85. Roop DR, Lowy DR, Tambourin PE, Strickland J, Harper JR, Balaschak M, Spangler EF, and Yuspa SH. An activated Harvey ras oncogene produces benign tumours on mouse epidermal tissue. *Nature*. 1986;323(822–4).
 86. Guttridge DC, Albanese C, Reuther JY, Pestell RG, and Baldwin AS, Jr. NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. *MolCell Biol*. 1999;19(8):5785–99.
 87. Cataisson C, Pearson AJ, Tsien MZ, Mascia F, Gao JL, Pastore S, and Yuspa SH. CXCR2 ligands and G-CSF mediate PKCalpha-induced intraepidermal inflammation. *J Clin Invest*. 2006;116(10):2757–66.
 88. Nuovo GJ. In situ detection of precursor and mature microRNAs in paraffin embedded, formalin fixed tissues and cell preparations. *Methods*. 2008;44(1):39–46. [PubMed: 18158131]
 89. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, and Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003;4(2):249–64. [PubMed: 12925520]
 90. Wright GW, and Simon RM. A random variance model for detection of differential gene expression in small microarray experiments. *Bioinformatics*. 2003;19(18):2448–55. [PubMed: 14668230]
 91. Storey JD, and Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A*. 2003;100(16):9440–5. [PubMed: 12883005]
 92. Dabney A, and Storey JD. Bioconductor R package; 2003.
 93. Csardi G, and Nepusz T. igraph version 0.7.0: The igraph software package for complex network research. *Interjournal Complex Systems*. 2006.
 94. Hameetman L, Commandeur S, Bavinck JN, Wisgerhof HC, de Gruijl FR, Willemze R, Mullenders L, Tensen CP, and Vrieling H. Molecular profiling of cutaneous squamous cell carcinomas and actinic keratoses from organ transplant recipients. *BMC cancer*. 2013;13(58).

Fig.1: Responsiveness of WT, K5-PKCα, MT-HGF and DT mice to chemically-induced skin carcinogenesis.
(A) Breeding scheme and DMBA-TPA timeline; mice were initiated by topical application with 20μg DMBA/0.1mL acetone at day 4 after birth and promoted by treating with 1μg (1.6nmol) TPA/0.2mL acetone twice a week from week 6 to 11. Tumors were counted every week. (B) Panel represents the percentage of mice with tumors. (C) Panel represents the mean number of skin tumors per tumor-bearing animal (mean ± SEM). WT (n=7), K5-PKCα (n=18), MT-HGF (n=14) and DT (n=9). Comparisons of DT and MT-HGF (*, p<0.05) and DT and K5-PKCα (*, p<0.05) were analyzed by Mann-Whitney U test. (D) TPA timeline in the absence of initiation, mice were promoted with 1μg (1.6nmol) TPA/0.2mL acetone twice a week from weeks 6 to 16. (E) Tumor incidence at week 16. (F) Photograph of representative DT mice at week 16.

Fig. 2: MT-HGF and DT keratinocytes exhibit activated MET and EGFR signaling.

Primary keratinocytes from wild-type (WT), K5-PKCα, MT-HGF and DT newborns were cultured in 0.05 mM Ca⁺⁺ medium and transduced with v-ras^{Ha} for 3 days. (A) HGF and DT keratinocytes display a v-ras^{Ha}-keratinocyte like elongated morphology in the absence of RAS transduction. (B) total cell extract from primary keratinocytes untreated or transduced for 3 days with v-ras^{Ha} were analyzed by immunoblotting for phospho-MET, total MET, phospho-ERK and total ERK or phospho-EGFR, total EGFR and HSP90. Values below the phospho-EGFR blot represent the densitometric analysis of the phospho-EGFR/total EGFR ratio after normalization with HSP90 expression for input relative to non-RAS WT control. (C) real-time PCR analysis of amphiregulin (*Areg*), betacellulin (*Btc*), heparin-binding EGF-like growth factor (*Hbegf*), and transforming growth factor α (*Tgfa*) mRNA expression in control and v-ras^{Ha}-keratinocytes 3 days after transduction. Data shown are representative of

three independent experiments, and bars represent the mean $\pm$ SEM of three replicates. ** P < 0.01 vs. WT control. *** P < 0.001 vs. WT control.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3: MT-HGF and DT keratinocytes exhibit a RAS-like phenotype.

(A) Tritiated thymidine incorporation for 24 hours was measured in control and RAS-transduced WT, K5-PKCα, MT-HGF and DT keratinocyte cultures grown under proliferative (0.05 mM Ca^{2+} , left panel) or differentiating conditions (0.12 mM Ca^{2+} , right panel). Right panel = percent inhibition of thymidine incorporation compared to respective culture maintained under proliferative conditions. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. WT control. # $P < 0.001$ vs. WT RAS. (B) and (C) Total SDS cell extracts from control and RAS-transduced keratinocytes were immunoblotted with specific antibodies for the expression of Cyclin D1 and ACTIN (panel B) or (panel C) early markers of differentiation (K1 and K10) and simple epithelia marker (K8). SDS lysates were analyzed from 3-d post RAS transduction cultures that were switched to 0.12 mM Ca^{2+} media for an extra 24 h. (D) real-time PCR analysis of CXCL1 (*Cxcl1*), CXCL2 (*Cxcl2*), K1 (*Krt1*), K10 (*Krt10*), TNFα (*Tnf*), MMP9 (*Mmp9*), GM-CSF (*Csf2*), SLPI (*Slpi*) and IL-1α (*Il1a*) mRNA expression in

control and RAS-keratinocytes 3 days after transduction. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. WT control. Bars represent the mean $\pm$ SEM of three replicates.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4: MET transactivates EGFR through ADAM17 mediated release of EGFR ligands. Keratinocytes from MT-HGF mice were treated for 24h at confluence with (panel A and B) ADAM17 inhibitor (GM6001) or (panel C and G) siRNA targeting ADAM17 (Qiagen (5 and 6) or Dharmacon (Dhar.)). (A) Amphiregulin (AREG) concentrations in culture supernatant. Bars are mean $\pm$ SEM of triplicate determinations. *** P < 0.001 vs. MT-HGF DMSO (0 uM). (B) and (C) Immunoblotting of cell extracts for phospho-EGFR, total EGFR, HSP 90 and ACTIN showing densitometric values for phospho-EGFR/total EGFR ratio normalized by HSP90 or ACTIN. Control is set to 1. (D) and (E) Keratinocytes from ADAM17^{fl/fl} mice were cultured to confluence and transduced for 48h with control (Cont.) or Cre adenovirus and challenged with HGF for 6h (panel D) or 10 min. (panel E), AREG in culture supernatants and ADAM17 expression (insert) are shown in panel D. *** P < 0.001 vs control treated with HGF. (F) WT or RAS-keratinocytes were transduced with ADAM17-targeting siRNA for 48h and lysates were immunoblotted for phospho-EGFR, ADAM17, HSP 90. (G) Expression of signature transcripts (PCR) after ADAM17 siRNA transduction. ** P < 0.01, **** P < 0.0001 vs. MT-HGF control. Bars represent the mean $\pm$ SEM of triplicate determinations.

Fig.5: MET activates SRC and iRhom to mediate the release of AREG.

(A) Total cell extract from primary keratinocytes treated for the indicated periods of time with HGF were analyzed by immunoblotting for pSRC, total SRC and HSP90. (B) Primary keratinocytes were cultured in 0.05 mM Ca^{++} medium to confluence and treated for 6h with HGF in the presence of siRNAs targeting Src (#1 to 4). AREG concentrations in culture supernatant and SRC/HSP90 expression (insert) are shown. * $P < 0.05$, *** $P < 0.001$ vs. Cont. HGF. (C) and (D), Primary keratinocytes were cultured in 0.05 mM Ca^{++} medium to confluence and treated for 6h with HGF in the presence of siRNAs targeting iRhom 1, iRhom2, Src and combinations thereof. Cells were harvested and both iRhom1 (*Rhbdf1*) and iRhom2 mRNAs (*Rhbdf2*) were quantified by real-time PCR (C) while AREG concentrations in culture supernatant was determined by ELISA (D). *** $P < 0.001$, **** $P < 0.0001$ vs control. Bars represent the mean $\pm$ SEM of quadruplicate determinations.

Fig. 6: RAS-like phenotype and tumor development by HGF overexpressing keratinocytes is mediated by EGFR.

(A) Real-time PCR analysis of IL-1 α (*Il1a*) and CXCL1 (*Cxcl1*) mRNA expression in WT and MT-HGF keratinocytes 3 days after adenoviral transduction with the NF- κ B dominant-negative (IkBsr) adenovirus or treatment with the IL-1R antagonist (IL-1ra). # $P < 0.001$ vs. WT PBS. *** $P < 0.001$ vs. MT-HGF PBS. & $P < 0.001$ vs. MT-HGF Null Ad. (B) MT-HGF tumors from orthotopic grafts on MT-HGF host and MT-HGF normal skin were analyzed by immunoblotting for phospho-EGFR, phospho-ERK, total EGFR and HSP90. (C) and D, Six million MT-HGF keratinocytes were mixed respectively with 6 million MT-HGF primary dermal fibroblasts prior to grafting in the interscapular region of syngeneic hosts. Once squamous papillomas were clearly established, mice were treated daily by oral gavage with vehicle control or gefitinib at 100mg/kg for two weeks. (C) Waterfall plot of tumor response to gefitinib, data are expressed as % change of tumor volume after 2 weeks of treatment. (D) Representative photographs of orthotopic squamous papillomas at the start of the treatment (day 0) and termination (day 14).

Fig. 7: Identification of the model RAS/MET signature.

(A) Venn diagram depicting the number of differentially expressed genes in the MT-HGF and WT-RAS experiments (false discovery rate < 1%). (B) Scatter plot of the genes from the MT-HGF and WT-RAS overlapping signature (6247 genes); linear regression between the two experiments is shown by the solid black line ($R^2=0.77$); 93% out of 6247 genes in the overlap are concordantly up- or down-regulated between the two experiments. (C) Heatmap visualization of estimated expression levels in 372 genes selected for the model RAS/MET signature (concordant, at least 2-fold change in both MT-HGF and WT-RAS); genes (columns) and samples (rows) are ordered by hierarchical clustering using Euclidean distance and complete linkage. (D) GSEA-enrichment plots are shown for top enriched gene sets in the model RAS/MET signature. The green line is the running enrichment score calculated along the ranked gene list represented by the red-blue horizontal bar (average t-statistic from MT-HGF and WT-RAS comparisons ranked from the highest positive to the

highest negative value); the vertical black bars in the plot indicate the position of the genes from the respective GO terms, which are mostly situated within up-regulated genes. (E) GSEA-enrichment map for non-redundant and overlapping GO gene sets generated with REVIGO algorithm ($\text{SimRel} < 0.9$); Highly similar GO terms (3% of the strongest pairwise semantic similarities) are linked to each other with edges weighted by the semantic similarity; GO nodes are color-coded by the significance of enrichment (GSEA false discovery rate $< 5\%$) and sized proportionally to the percentage of genes annotated to the term; in addition, unweighted edges connect genes from the model RAS/MET signature, which are also among the GSEA core genes ('leading edge'), to the GO terms they are annotated to. The gene nodes are color-coded by the average difference in expression of MT-HGF and WT-RAS versus WT-Control. See Supplementary Table 2 for detailed statistical results of GSEA analysis.

Fig. 8: MET is highly expressed in human cutaneous SCC.

(A) Human skin squamous cell cancer tissue array was immunostained with anti-MET antibody and visualized by bright-field microscopy. (B) Quantification of the epithelial compartment staining intensity using the Aperio software ImageScope according to tumor grade. Grade 1 (n=49), grade 2 (n=20) grade 3 (n=5). * $P < 0.05$ vs. grade 1. (C) RNA *in situ* and protein IHC in human squamous cell carcinomas. RNA *in situ* signal (red) deconvoluted with Aperio ScanScope algorithm and pseudo-colored with Adobe imaging software. Bar = 200 μ m. Both positive and negative tumors are shown for ISH and IHC. (D) Human SCC cell lines were grown to confluence and treated for 24h with DMSO, Capmatinib (Cap.), PHA665752 (PHA) or recombinant HGF. Total cell extracts were analyzed by immunoblotting for phospho-MET, total MET and HSP 90 (D) and in separate vessels tritiated-thymidine incorporation was measured (E). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

*** $P < 0.001$, **** $P < 0.0001$ vs control (0). Bars represent the mean $\pm$ SEM value of quadruplicates.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 9: Evaluation of activity of the model RAS/MET signature in cancer patients.

(A) Predicted DART scores of the signature activity level in patient-matched samples (GSE32979, N=13) from normal epidermis (NE), actinic keratosis (AK) and squamous-cell carcinoma (SCC). (B) Predicted DART scores of the signature levels in patients and healthy controls (combined data from GSE2505 and GSE42677; normal epidermis (NE), 16 samples; actinic keratosis (AK), 9 samples; squamous-cell carcinoma (SCC), 15 samples).