Targeted expression of RALT in mouse skin inhibits epidermal growth factor receptor signalling and generates a Waved-like phenotype

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Although it has been clearly established that negative feedback loops have a fundamental role in the regulation of epidermal growth factor receptor (EGFR) signalling in flies, their role in the regulation of mammalian EGFR has been inferred only recently from *in vitro* studies. Here, we report on the forced expression of RALT/MIG-6, a negative feedback regulator of ErbB receptors, in mouse skin. A RALT transgene driven by the K14 promoter generated a dose-dependent phenotype resembling that caused by hypomorphic and antimorphic *Egfr* alleles—that is, wavy coat, curly whiskers and open eyes at birth. *Ex vivo* keratinocytes from K14-RALT mice showed reduced biochemical and biological responses when stimulated by ErbB ligands. Conversely, knock-down of RALT by RNA interference enhanced ErbB mitogenic signalling. Thus, RALT behaves as a suppressor of EGFR signalling in mouse skin.

Keywords: ErbB; RALT; keratinocyte; feedback inhibition; RNA interference

EMBO reports (2005) 6, 755–761. doi:10.1038/sj.embor.7400458

INTRODUCTION

Receptor tyrosine kinases (RTKs) regulate a range of cellular functions by delivering signals of appropriate robustness within defined spatial and temporal boundaries (Freeman, 2000). This sharp configuration of RTK output is achieved by means of dynamically balanced generation (positive signalling) and

extinction (negative signalling) of signals (Fiorini *et al*, 2001). Epidermal growth factor receptor (EGFR), the founder member of the ErbB family of RTKs, has served for a long time as a model to study negative signalling to RTKs. On activation, the EGFR undergoes rapid endocytosis, a process that is involved in spatial regulation of EGFR signalling, but ultimately down-regulates EGFR activity (Di Fiore & De Camilli, 2001).

Whereas endocytosis serves as a housekeeping-type mechanism of negative signalling to activated EGFR, feedback inhibition (FI) provides a further, transcriptionally induced layer of regulation of EGFR activity (Fiorini *et al*, 2001). In finalistic terms, FI allows cells to sense the accumulation of EGFR signals over time and generate a commensurate level of negative signalling to EGFR. By integrating these processes, FI is thought to provide EGFR signalling with an essential element of stability (Freeman, 2000). In mammalian cells, ErbB-specific FI is carried out by LRIG1, which is structurally related to *Drosophila* Kek1 (Gur *et al*, 2004), and RALT (receptor associated late transducer)/MIG-6 (mitogen-inducible gene; Fiorentino *et al*, 2000; Hackel *et al*, 2001), which can be traced down to lower vertebrates (O.S., unpublished data). Transcription of the *RALT* gene is driven by the RAS-ERK (extracellular-signal-regulated kinase) pathway in a quasilinear fashion (Fiorini *et al*, 2002). The RALT protein in turn binds to activated ErbB RTKs, including the EGFR, by means of its EBR (ErbB-binding region) and restricts ErbB signals through mechanisms that are still poorly defined.

Herein, we exploited the model of mouse skin morphogenesis to test whether RALT acts as a suppressor of EGFR function in a developmental context. Although *Egfr*^{-/+} mice show no obvious phenotype (Sibilia & Wagner, 1995), further reductions of EGFR function, as determined by expression of skin-targeted DN (dominant-negative)-EGFR (Murillas *et al*, 1995), homozygous inheritance of hypomorphic *Egfr* alleles (Luetteke *et al*, 1994; Sibilia *et al*, 2003) or heterozygous inheritance of the antimorphic *wa5/velvet* *Egfr* allele (Lee *et al*, 2004) cause the Waved phenotype, characterized by wavy coat, curly whiskers and open eyelids at birth (OEB). Thus, skin morphogenesis in mice becomes

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abnormal under conditions in which <50% EGFR activity is still tolerated in most EGFR target tissues, in fact providing a sensitive readout of reduced EGFR activity (Lee *et al*, 2004). We report here that overexpression of RALT in the mouse skin suppresses EGFR signalling and causes a Waved-like phenotype.

RESULTS AND DISCUSSION

K14-RALT phenocopies EGFR deficiency

In the mouse, skin EGFR expression is confined to follicular and interfollicular keratinocytes (Hansen *et al*, 1997). Administration of pharmacological doses of epidermal growth factor (EGF) to mice promotes tyrosine phosphorylation of EGFR in the skin (Luetteke *et al*, 1994; Lee *et al*, 2004; supplementary Fig S1 online). We used this approach to test whether EGFR triggering

was followed by induction of RALT in the skin of 3- and 14-day-old pups. As shown in supplementary Fig S1 online, the induction of *Ralt* messenger RNA peaked at 90 min after administration of EGF, whereas RALT protein accumulation peaked between 3 and 5 h afterwards, consistent with data obtained in various cultured cell types (Fiorentino *et al*, 2000).

We directed RALT expression in basal and outer root sheath skin keratinocytes by placing the *Ralt* open reading frame under the control of a K14 promoter cassette (Vassar & Fuchs, 1991) and obtained K14-RALT transgenic mice in the BDF1 strain. By PCR (not shown) and Southern and western blot analysis (Fig 1A,B), we identified six founder mice, which reached adulthood and gave rise to offspring. K14-RALT mice segregated into two classes (Fig 1C–F). One class showed a severe phenotype that resulted in

Fig 1 | Generation of K14-RALT mice. (A) Southern blot analysis of genomic DNA isolated from founder mice. The sizes of the hybridized fragments from the endogenous *RALT* gene (9 kb) and transgenic K14-*RALT* (4.6 kb) are indicated. (B) Expression of RALT protein in the dorsal skin from wild-type (wt) mice and the heterozygous progeny of selected founders. Expression levels are compared with those in control and RALT-overexpressing NIH 3T3 cells. Immunoblotting was performed on total protein extracts using a polyclonal antiserum that recognizes rat and mouse RALT. (C) Photograph of 4-week-old A9 and littermate control mice showing that A9 mice have a typical Waved phenotype. (D) Newborn pups of the A9 line show defective closure of eyelids. (E) Adult mice from the A9 line show fuzzy and wavy hair with areas of alopecia and inflammation of the skin. (F) A 3-week-old mouse of the A24 line showing a wavy first hair coat. (G) Segments of hairs from wt (upper) and heterozygous (lower) adults of the A9 line. Note septulation and irregular pattern of pigmentation in the medulla.

OEB (e.g. K14-RALT A9; Fig 1D), curly whiskers and an altered initial coat characterized by wavy and fuzzy hairs (Fig 1C; data not shown). In adulthood, skin abnormalities progressed to extensive skin inflammation and alopecia (Fig 1E). Moreover, by 2 weeks of age, the offspring of mice belonging to this phenotypic class were smaller than littermates and only rarely did they reach adulthood. The second class of transgenic mice had a milder phenotype, characterized solely by a wavy hair coat (exemplified by strain A24; Fig 1F). Altered hair morphogenesis in K14-RALT mice resulted in the disruption of the ordered arrangement of pigmented medullary cells in hairs (Fig 1G).

The skin was isolated from A24 and A9 K14-RALT mice at different ages. The skin from 3-day-old K14-RALT mice had a normal histological appearance; that is, its thickness, and number and diameter of hair follicles were comparable with those of controls (Fig 2A). Likewise, the intense cell proliferation typical of anagen hair follicles was not perturbed in K14-RALT mice (Fig 2B). The interfollicular epidermis of transgenic mice also appeared normal (Fig 2A), as shown by the percentage of Ki67 staining (29.5 ± 6.8 in wild type (wt) versus 25.2 ± 4.2 in A9 mice; Fig 2B) and by the normal distribution of markers identifying proliferating basal (p63; Fig 2B) and differentiating suprabasal keratinocytes (keratin 1 (K1), Fig 2B; filaggrin, data not shown).

By 3 weeks of age, hair follicles enter telogen, a quiescent phase that follows the rapid vectorial apoptosis and ensuing follicle shrinkage of catagen. In skin samples obtained at day 18, telogen hair follicles of control mice were typically confined to the upper layer of the dermis (Fig 2A). By contrast, although with variable severity (compare A24 with A9), hair follicles in K14-RALT mice appeared locked in anagen (Fig 2A). This caused a marked increase in skin thickness, due to the persistence of a large number of follicles in the subdermal adipose layer. Moreover, K14-RALT follicles appeared larger than their normal counterparts and hyperplastic. Intriguingly, in A9 mice, the percentage of Ki67-positive cells was increased in the interfollicular epithelium (34.8 ± 5.4 versus 18.2 ± 2.4 , $P < 0.001$), which, however, did not show altered K14 and K1 expression (Fig 2B). Only a few ($< 1\%$) TdT-mediated dUTP nick end-labelling (TUNEL)-positive keratinocytes were observed in the interfollicular epidermis of both A9 and wt mice at 18 days (Fig 2C). Consistent with other studies, these TUNEL-positive cells were often present in the outermost layer of the epidermis. Occasionally, TUNEL-positive keratinocytes were detected in the outer root sheath of deep follicles in A9 mice (not shown). This negligible level of TUNEL staining indicates that apoptosis has no significant role in determining the K14-RALT phenotype and supports the conclusion that follicles in K14-RALT mice are blocked in anagen. Finally, no appreciable differences in the expression level of ErbB receptors and cognate ligands (Fig 2D; supplementary Fig S2 online) were detected in the skin of K14-RALT mice at 3 and 14 days of age.

At 50 days of age, hair follicles of control mice were again in telogen (supplementary Fig S3A online), having completed their second cycle. In contrast, the skin of A9 mice showed a high density of hair follicles in an aberrant anagen phase. These follicles contained proliferating cells in the hair bulb matrix and outer root sheath, as shown by Ki67 labelling (supplementary Fig S3B online). This histology was frequently associated with hair follicle degeneration and massive infiltration of inflammatory cells into the dermis and subdermal fat tissue. Additionally, the skin of

A9 mice showed a prominent hyperplasia in interfollicular regions (supplementary Fig S3A online), with keratinocytes arranged in several layers expressing basal cell markers, namely K14 (supplementary Fig S3B online) and p63 (not shown). Ki67-positive cells were found at high frequency in the basal layer, whereas suprabasal cells coexpressed the differentiation markers K1 and filaggrin (supplementary Fig S3B online). Basal keratinocytes also stained for keratin 6 (supplementary Fig S3B online), which is induced in the interfollicular epidermis only after skin injury/inflammation. Epidermal hyperplasia in an inflammatory skin background was also reported in mice expressing a K5-DN-EGFR allele (Murillas *et al*, 1995), and in *Egfr*^{-/-} skin grafts (Hansen *et al*, 1997). In all of the above genetic contexts, epidermal hyperplasia is apparently at odds with the increased rate of cell proliferation observed in the mouse epidermis on overexpression of EGFR ligands (Vassar & Fuchs, 1991) and with the role of RALT as a suppressor of EGFR signalling (Anastasi *et al*, 2003). We posit that the hyperplasia of interfollicular epidermis in adult K14-RALT A9 mice is secondary to the dermal inflammation and the ensuing synthesis of cytokines able to trigger keratinocyte proliferation independently of ErbB function. This is supported by the finding that *IL-1* (interleukin-1) and *IL-6* mRNAs were aberrantly expressed in the skin of K14-RALT mice (supplementary Fig S3C online). It is noteworthy that IL-6 is a known mitogen for keratinocytes (Sato *et al*, 1999) and IL-1 can induce fibroblasts to produce keratinocyte growth factor (KGF), a potent keratinocyte mitogen (Maas-Szabowski *et al*, 1999). *KGF* mRNA was indeed induced in the skin of A9 mice (supplementary Fig S3C online).

In summary, the K14-RALT phenotype closely resembles that of mice with a defective transforming growth factor- α /EGFR signalling axis (Luetteke *et al*, 1994; Sibilio *et al*, 2003; Lee *et al*, 2004). We also note that skin defects in K14-RALT mice somehow recapitulate the spectrum of skin abnormalities present in mutant mice with graded deficit of EGFR function. Thus, low doses of the K14-RALT transgene (as in A24 mice; Fig 2D) generate a phenotype similar to that determined by the mild *wa2* allele (Luetteke *et al*, 1994), whereas higher doses of K14-RALT (as in A9 animals; Fig 2D) seem to phenocopy the dominant alleles *wa5/velvet* (Lee *et al*, 2004) and K5-DN-EGFR (Murillas *et al*, 1995). Interestingly, it has been proposed (Lee *et al*, 2004) that the severity of the *wa5*^{-/+} phenotype may be due to the ability of *wa5* receptors to trans-dominantly inhibit all ErbB dimers in the skin (Stoll *et al*, 2001). RALT is a pan ErbB inhibitor (Anastasi *et al*, 2003), and as such it is expected to dampen signalling by all ErbB dimers in K14-RALT keratinocytes (Fig 3C).

RALT levels influence the phenotype of keratinocytes

The genetic evidence discussed above is compatible with RALT acting as a suppressor of EGFR signalling in keratinocytes. This was validated further in short-term cultures of basal keratinocytes. Although barely detectable in mitogen-deprived keratinocytes, RALT immunoreactivity was evident in lysates of quiescent cells exposed to mitogens, including EGF, neuregulin 1 (NRG1), basic fibroblast growth factor (bFGF) and serum (Fig 3A). Similar observations were made in HaCaT human keratinocytes (supplementary Fig S4A online). In both mouse and human keratinocytes, RALT was also induced by the differentiating agent 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Fig 3A; supplementary

Fig 2 | Hair-follicle cycle and morphogenesis are severely impaired in K14-RALT mice. (A) Histological sections through the skin of control and A24 and A9 lines at different ages. The skin of newborn K14-RALT mice and that of age-matched controls appear similar. In 18-day-old K14-RALT mice, hair follicles fail to progress to catagen and accumulate in the subdermal tissue, whereas follicles from control mice are in telogen. As a result, overall skin thickness is markedly different in the two groups. Scale bar, 100 μ m. (B) Sections of the dorsal skin from newborn and 18-day-old mice (control and A9 littermates) were immunostained with the indicated antibodies. In some cases, H33258 was used to stain nuclei. Note normal proliferation and differentiation patterns in the newborn skin; however, Ki67 staining is increased in the basal layer of epidermis in 18-day-old A9 mice. The dotted line is superimposed on the basement membrane. Scale bars, 100 μ m. (C) Representative micrographs of TdT-mediated dUTP nick end-labelling (TUNEL)-positive cells in cryosections from the skin of 18-day-old A9 mice. The blue H33258 image is merged with the green FITC image (TUNEL). Scale bar, 50 μ m. (D) Expression levels of RALT and ErbB family members detected by immunoblotting in skin extracts from 3-day-old and 14-day-old pups of the indicated genotypes.

Fig S4A online) but not by high calcium (supplementary Fig S4B online). As RALT expression is under the control of the ERK pathway (Fiorini *et al*, 2002), this observation is probably explained by the finding that calcium, unlike EGF and TPA,

elicits a short-lived activation of ERK (supplementary Fig S4 online). As expected, keratinocytes derived from A24 (Fig 3B) and A9 mice (not shown) expressed RALT constitutively. When compared with explants from normal littermates, keratinocytes

Fig 3 | RALT regulates ErbB-driven keratinocyte proliferation. (A) Western blot analysis of RALT expression in normal keratinocytes and NIH 3T3 cells after treatment with the indicated agents. (B) Expression levels of RALT protein in keratinocytes explanted from individual littermates (b–i) from crossing of heterozygous A24 mice; the two rows of numbers below each lane refer to the degree of inhibition of basal and EGF-induced (0.5 ng/ml) S-phase entry measured by bromodeoxyuridine (BrdU) incorporation in sister cultures. (C) Effect of ectopic RALT expression on BrdU incorporation of primary keratinocytes (wild type (wt), A9 and adenovirus-infected) and adenovirus-infected HaCaT cells stimulated with the indicated agents (see Methods). Note that the degree of inhibition is comparable with that shown by 0.2 μ M PD168393. The data are mean $\pm$ s.d. from three independent experiments. (D) Stable ablation of endogenous RALT in HaCaT cells by RNA interference (short hairpin RALT, shRALT; left panel) leads to threefold enhancement in S-phase entry after treatment with EGF (0.3 ng/ml; centre panel). In the same cells, EGF-induced (5 ng/ml) tyrosine phosphorylation of epidermal growth factor receptor (EGFR), evaluated by immunoblotting with anti-PY1068, is enhanced (right panel). The data are representative of three independent experiments. bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; FBS, fetal bovine serum; NRG1, neuregulin 1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

derived from newborn A9 mice showed a marked reduction in proliferation. This was observed both under basal conditions (i.e. mitogen-free medium) and after either EGF or NRG1 stimulation (Fig 3C). Because in keratinocytes NRG1 is expected to activate ErbB-2.ErbB-3 dimers (Stoll *et al*, 2001), these data confirm the ability of RALT to act as a pan-ErbB suppressor. Importantly, basal proliferation was still largely dependent on EGFR signalling (presumably because of autocrine receptor stimulation), as it could be inhibited by 70%–80% on addition of the EGFR kinase inhibitor PD168393 (Fig 3C). Conversely, neither ectopic RALT nor PD168393 inhibited bFGF-dependent proliferation of control and A9 keratinocytes (Fig 3C). The suppressive function of RALT on EGFR-dependent proliferation of A9 keratinocytes was not due to secondary adaptation of cells to ectopic RALT. This was proved by short-term experiments in which Ad-RALT infection specifically inhibited basal and EGF-driven proliferation of primary mouse and HaCaT keratinocytes (Fig 3C). Deletion of its EGFR-binding region (EBR; Anastasi *et al*, 2003) caused RALT to lose

80% of its activity (i.e. RALT and RALT Δ EBR suppressed EGF-induced proliferation by 75% and 14%, respectively).

The phenotype of K14-RALT mice varied depending on the expression of the RALT transgene, which is compatible with a dose-dependent suppressive activity of RALT. This was addressed more directly by measuring EGF-driven proliferation in keratinocyte cultures derived from individual F₂ siblings of the A24 line (Fig 3B). In these cultures, the level of ectopic RALT was three- to tenfold higher than that of the endogenous RALT protein, a range compatible with heterozygous versus homozygous inheritance of the K14-RALT allele in the F₂ offspring. Significantly, the entity of suppression of EGF mitogenic signalling in these cultures correlated well with the level of expression of ectopic RALT both in mitogen-free and in EGF-stimulated cultures (Fig 3B).

We surmised that a reduced rate of autonomous EGFR-dependent proliferation could accelerate the commitment of K14-RALT keratinocytes to differentiation. Compared with keratinocytes explanted from control littermates, cultured A9 keratinocytes

expressed much higher levels of K1, keratin 10 and profilaggrin, at the mRNA (supplementary Fig S5A online) and protein (supplementary Fig S5B online) level. Acute suppression of EGFR signalling in normal keratinocytes, as obtained by either infection with Ad-RALT or PD168393 treatment, yielded comparable results, both qualitatively and quantitatively (supplementary Fig S5A,B online). Compared with wt keratinocytes, Ad-RALT keratinocytes showed a minor increase of TUNEL positivity in mitogen-free conditions (from $7.7 \pm 2.1\%$ to $10.1 \pm 2.2\%$), which was poorly rescued by EGF ($6.4 \pm 1.8\%$ for wt and $8.3 \pm 2.3\%$ for A9 cells).

We also addressed the impact of altered RALT signalling on EGF-driven keratinocyte migration. The velocity of directional migration in wound re-epithelialization *in vitro* assays was markedly reduced in A9 keratinocyte cultures (wt: $42.3 \pm 6.5 \mu\text{m/h}$; A9: $9.2 \pm 1.8 \mu\text{m/h}$; supplementary Fig S6 online). As OEB is caused by defective keratinocyte migration at the protruding ridges of the eyelids (Zenz *et al*, 2003), our data provide a rationale for the OEB phenotype of K14-RALT mice.

Our attempts to knock down RALT expression by RNA interference in mouse cells were not met with success; however, we managed to target *RALT* efficiently in human keratinocytes after retroviral expression of a *RALT*-specific short hairpin RNA (shRALT; Fig 3D). When compared with controls, shRALT-HaCaT cells showed a marked reduction of basal and mitogen-induced RALT expression. Remarkably, shRALT cells showed an increased rate of S-phase entry at suboptimal EGF concentrations (Fig 3D). Thus, knock down of RALT expression in keratinocytes relieves an element of negative signalling to EGFR.

Phosphorylation of EGFR Y1068 was attenuated in EGF-treated A9 keratinocytes (Fig 4A) and in skin extracts from EGF-injected A9 mice (supplementary Fig S1C online). Comparable results were obtained in Ad-RALT keratinocytes (Fig 4C). Conversely, no significant inhibition of the kinetics of EGFR phosphorylation was observed in A24 keratinocytes (Fig 4B) and in the skin from A24 mice (not shown). Thus, RALT is able to attenuate EGFR tyrosine phosphorylation, although this mechanism may not fully account for its ability to suppress the EGFR signal. ERK activation in the epidermis is essentially confined to the basal layer, is inhibited by agents that block EGFR activity (Albanell *et al*, 2001) and, if sustained, may antagonize keratinocyte differentiation (Evans *et al*, 2003). Significantly, the kinetics of EGF-stimulated ERK activation in K14-RALT keratinocytes was much shorter than that detected in control cultures (Fig 4A) and paralleled the extent of EGFR phosphorylation in the various cell types (Fig 4A–C). This suggests that reduced signalling through the EGFR–ERK pathway

could have a role in curbing proliferation and enhancing differentiation of cultured K14-RALT keratinocytes.

CONCLUSIONS

EGFR activity is required during the morphogenesis of murine hair follicles to direct their polarized orientation along the anterior–posterior body axis and to direct anagen-to-catagen transition during their growth and differentiation cycle. Excess RALT activity in the skin of K14-RALT mice causes a disordered spatial

Fig 4 | Expression levels of transgenic K14-RALT correlate with autophosphorylation of EGFR and downstream pathway activation. Primary keratinocytes explanted from A9 (A) and A24 (B) mice were mitogen-starved for 1 day and exposed to epidermal growth factor (EGF) for various lengths of time. Lysates were immunoblotted with anti-PY1068 for detection of phosphorylated EGFR and with the other antibodies indicated. Levels of activation of extracellular-signal-regulated kinase (ERK) were determined by immunoblotting with anti-P-ERK. Note that concentrations of EGF were 10 ng/ml (A) and 1.0 ng/ml (B). wt, wild type. (C) Primary keratinocytes from wt littermates were infected with Ad-RALT, mitogen-starved and subjected to the same treatment and analysis as in (A,B). GFP, green fluorescent protein.

arrangement of hair follicles and halts their anagen-to-catagen transition. Hence, this phenotype mimics the developmental defects caused by reduced EGFR function in the mouse skin. Cultured keratinocytes from K14-RALT mice show reduced EGFR autophosphorylation, impaired ERK activation and a proliferation deficit restricted to ErbB-driven mitogenic programmes. Our data provide the first evidence that a mammalian feedback inhibitor of ErbB RTKs is able to suppress EGFR activity in a developmental context.

METHODS

Generation of transgenic mice. The rat *Ralt* open reading frame (NCBI NM 001014071) was PCR cloned into the K14 cassette (Vassar & Fuchs, 1991). Purified DNA fragments were injected into the pronucleus of BDF1 mice. Transgenic mice were identified by PCR analysis (see supplementary information online for primer sequence) and Southern blotting of tail genomic DNAs.

Keratinocyte culture and proliferation assay. Primary keratinocytes were isolated from newborn mice and cultured in low calcium (0.05 mM CaCl_2) medium and EGF (10 ng/ml; Calautti *et al*, 1998). HaCaT cells were propagated in high-calcium DMEM plus 10% FCS. For induction of RALT expression, inducers were EGF and bFGF (10 ng/ml), NRG1 (30 ng/ml), 10 nM TPA or 10% FCS. For proliferation assays (Fig 3), primary keratinocytes and HaCaT cells were allowed to grow to 50% confluence and were rendered quiescent by cultivation in 0.5% FCS for 24 h. Cells were then stimulated with mitogens (EGF (2 ng/ml), bFGF (2 ng/ml), NRG1 (2 ng/ml) or 5% FBS) for 18 h, allowing for bromodeoxyuridine (20 μM) incorporation during the last 6 h. Cells were processed using a cell proliferation assay kit (Amersham Biosciences, UK).

RNA interference. Stable HaCaT derivatives were obtained by infection with either pSuper Retro or pSuper Retro shRALT. The pSuper-shRALT vector targets nucleotides 1323–1341 of human RALT mRNA (NCBI accession NM_018948).

In vivo phosphorylation assay. Control and K14-RALT A9 pups (3–4 days old) were injected subcutaneously with 10 $\mu\text{l/g}$ body weight of PBS or 1.0 $\mu\text{g/g}$ body weight of EGF in PBS. After 10 min, skin was collected and frozen in liquid nitrogen. Frozen tissue was extracted in five volumes of homogenization buffer and extracts were immunoblotted with anti-pTyr 1068 EGFR.

In vivo induction of RALT was assayed after subcutaneous injection of control and K14-RALT A9 mice (3 and 14 days old) with PBS or 5.0 $\mu\text{g/g}$ body weight of EGF in PBS. Skin samples were collected at various times after injection and lysates were subjected to analysis of RALT protein by immunoblotting and *Ralt* mRNA by reverse transcription–PCR.

Immunoblotting. Skin samples were extracted in 20 mM HEPES, pH 7.4, 150 mM NaCl, 2% glycerol, 2 mM EGTA, 1% Triton X-100 and a cocktail of protease and phosphatase inhibitors. Cultured cells were lysed in radio-immunoprecipitation assay buffer. Immunoblot studies were carried out as described previously (Fiorini *et al*, 2002). **Supplementary information** is available at *EMBO reports* online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We thank C. Talora, A. Bartolazzi and A. Usiello for advice. We are indebted to G. Sala, C. Talora, E. Fuchs, M. Crescenzi and C. Marchese for providing reagents. This work was supported by MIUR-FIRB (Ministero Istruzione Università—Ricerca/Fondo Investimenti Ricerca Base), Telethon (B55 to C.T.) and AIRC (Associazione Italiana per la Ricerca sul Cancro).

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