The Ectodysplasin receptor EDAR acts as a tumor suppressor in melanoma by conditionally inducing cell death

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Abstract

Ectodysplasin receptor EDAR is seen as a typical Tumor Necrosis Factor receptor (TNFR) family member known to interact with its ligand Eda-A1, and signaling mainly through the nuclear factor-kappaB (NF-κB) and c-jun N-terminal kinases pathways. Mutations in genes that encode proteins involved in EDAR transduction cascade cause anhidrotic ectodermal dysplasia. Here, we report an unexpected pro-apoptotic activity of EDAR when unbound to its ligand Eda-A1, which is independent of NF-κB pathway. Contrarily to other death receptors, EDAR does recruit caspase-8 to trigger apoptosis but solely upon ligand withdrawal, thereby behaving as the so-called dependence receptors. We propose that pro-apoptotic activity of unbound EDAR confers it a tumor suppressive activity. Along this line, we identified loss-of-pro-apoptotic function mutations in EDAR gene in human melanoma. Moreover, we show that the invalidation of EDAR in mice promotes melanoma progression in a B-Raf mutant background. Together, these data support the view that EDAR constrains melanoma progression by acting as a dependence receptor.

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Introduction

The Ectodysplasin A receptor (EDAR) is a typical TNF receptor (TNFR) family member with a signal peptide, three cysteine-rich (CRDs), a transmembrane and an intracellular domains, the latest comprising a so-called “death domain” (DD) [1, 2]. The TNFRs family members that display such a DD have been termed death receptors. However, these receptors can be separated into two main categories, depending on the primary signal that they activate upon ligand binding: the prototypical death receptors such as

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CD95 (Fas/APO-1), TRAIL-R1, or TRAIL-R2 primarily triggering cell death upon ligand binding via recruitment of the DD-containing adapter FADD and subsequent activation of caspases, and the TNFR1 transducing a signaling

cascade leading to genes activation through NF- κ B [3–5]. Similarly to TNFR1, binding of the EDAR ligand Eda-A1 was shown to induce NF- κ B activation, via successive recruitment of the DD-containing protein EDAR-associated

◀ **Fig. 1** EDAR induces apoptosis via an EDARADD and caspase-8 dependent signaling pathway. **a** EDAR induces caspase-3 activation in HEK293T cells. HEK293T cells were transiently transfected with EDAR-encoding vector (EDAR) or adequate empty one (Ctl). Caspase-3 activity measurements are presented as means $\pm$ std; $n = 3$; $*p \leq 0.05$; two-sided Mann–Whitney *U*-test. **b** EDAR induces DNA fragmentation in HEK293T cells. DNA fragmentation is detected by TUNEL staining in HEK293T cells transiently transfected with EDAR-encoding vector (EDAR) or adequate empty one (Ctl). Left panel: representative images are shown. TUNEL positive cells are labeled in red. Nuclei are counterstained in blue by Hoechst staining. Right panel: quantification of one representative experiment out of three performed is exposed. Results are presented as percentage of TUNEL-positive cells per field; $n = 5$; $*p \leq 0.05$; two-sided Mann–Whitney *U*-test. **c** EDAR Death-Domain (DD) is required for apoptosis induction. HEK293T cells were transiently transfected with a vector encoding full-length EDAR (EDAR), EDAR deleted from its DD (EDAR- Δ DD) or adequate empty one (Ctl). Caspase-3 activity measurements are presented as means $\pm$ std; $n = 5$; $*p \leq 0.05$; two-sided Mann–Whitney *U*-test. **d** FADD is not necessary for EDAR-mediated apoptosis. HEK293T cells were transiently transfected with a vector encoding full-length EDAR, with or without a dominant-negative form of FADD (FADD-DN). Caspase-3 activity measurements are presented as means $\pm$ std; $n = 3$; $*p \leq 0.05$; two-sided Mann–Whitney *U*-test. *NS* non-significant. **e** Transduction of EDAR apoptotic signaling requires its adaptator EDARADD. HEK293T cells were transiently transfected with a vector encoding full-length EDAR, with or without a siRNA targeting its adaptator EDARADD (siEDARADD). Caspase-3 activity measurements are presented as means $\pm$ std; $n = 3$; $*p \leq 0.05$; two-sided Mann–Whitney *U*-test. **f** Caspase-8 mediates EDAR apoptotic cascade in type I cells. HEK293T cells were transiently transfected with a vector encoding full-length EDAR, with or without a dominant-negative form of caspase-8 (caspase-8-DN) or 9 (caspase-9-DN). Caspase-3 activity measurements are presented as means $\pm$ std; $n = 5$; $*p \leq 0.05$; two-sided Mann–Whitney *U*-test. **g** EDAR apoptotic cascade goes through mitochondrial pathway in type II cells. A549 cells were transiently transfected with a vector encoding full-length EDAR, with or without a siRNA targeting either Bid (siBid), Bax (siBax) or caspase-9 (siCaspase-9). Caspase-3 activity measurements are presented as means $\pm$ std; $n = 3$; $*p \leq 0.05$; two-sided Mann–Whitney *U*-test

death domain (EDARADD), and of a complex composed of TRAF6 (TNF-receptor associated 6), TAB2 (TAK1/MAP3K7 binding protein 2), and TAK1 (TGF β -activated protein kinase 1) [6, 7].

EDAR is expressed by epithelial cells of ectodermic derivatives [1, 2, 8, 9]. Disruption of Eda-A1/EDAR signaling is the underlying cause of a genetic condition called anhidrotic ectodermal dysplasia, characterized, among other defects, by the abnormal development of teeth, hair, and sweat glands [7].

While overexpressing EDAR in cells, we observed that this receptor is intriguingly able to induce cell death, but solely in absence of its ligand Eda-A1, thereby functioning as a dependence receptor. This functional family gathers very distinct transmembrane receptors, sharing the unique common property to trigger apoptosis when disengaged from their ligand [10, 11]. Prototypical members of the dependence receptor family include the netrin-1 receptor

DCC and the SHH receptor Patched, which have been shown to recruit and activate the apical caspase-9 in absence of their ligand [12–15]. We demonstrate here that EDAR-mediated cell death requires the DD-containing adapter EDARADD and goes through the recruitment of caspase-8. Dependence receptors such as DCC have been described to function as tumor suppressor, by conditioning tumor cell survival to ligand availability [11, 16]. Along this line, we report hereby that EDAR prevents melanoma progression, using both xenograft and EDAR conditional knockout (KO) murine models. Moreover, EDAR expression is altered in human melanoma tumors, quantitatively and qualitatively, with subsequent impairment of its pro-apoptotic function. Thus, EDAR appears to be an atypical TNFR, inducing cell death like dependence receptors, and acting consequently as a conditional tumor suppressor.

Results

EDAR induces apoptosis via an EDARADD and caspase-8 dependent signaling pathway

While EDAR is usually seen as a NF- κ B signaling receptor, the presence of the DD and the mention of cell death observed upon EDAR expression in Kumar et al., led us to investigate the ability of EDAR to trigger cell death [17]. We first transiently transfected EDAR in HEK293T cells. Expression of EDAR is sufficient to significantly increase caspase-3 activity and DNA fragmentation (Fig. 1a, b and Supplementary Figure 1a–b), which are two hallmarks of apoptosis. Consistently with the implication of EDAR DD, its deletion prevents cell death induction (Fig. 1c). As the DD-containing adapter FADD was shown to be necessary for death-transduction signal induced by most death receptors, being either recruited immediately at the membrane or in a secondary cytoplasmic complex [3, 4], we used both siRNA and dominant-negative strategies to evaluate its involvement in EDAR apoptosis. As shown in Figure 1d and Supplementary Figures 1c–e, we failed to observe any impact of FADD-activity blockade on EDAR ability to induce death. Conversely, silencing of EDAR specific adapter EDARADD significantly alters EDAR-apoptotic cascade (Fig. 1e and Supplementary Figure 1f). Death receptors induce apoptosis mainly via caspase-8 and activation of the so-called extrinsic cascade. In HEK293T that are type I cells, the extrinsic signal is sufficient by itself to trigger cell death, without requirement of signal amplification via the intrinsic death pathway [18, 19]. Consistently, in those cells, EDAR-induced cell death is impaired when blocking caspase 8, either via use of a dominant-negative form of this enzyme or by siRNA silencing of its expression (Fig. 1f and Supplementary

Figure 1g), whereas blockade of caspase-9 does not significantly impair EDAR-induced cell death: this indicates that direct activation of caspase-3 by caspase-8 is sufficient in such type I cells (Fig. 1f and Supplementary Figure 1g). On the contrary, in type II A-549 cells, Bax, Bak, Bid, and caspase-9 are all necessary for transduction of the EDAR apoptotic signal until caspase-3 activation (Fig. 1g and Supplementary Figure 1h). Together, these results support the view that when overexpressed, EDAR transduces a cell death pathway sharing similarities with death receptors, with the particularity that it relies on the recruitment of its own adapter EDARADD, but not FADD.

EDAR functions as a dependence receptor rather than as a death receptor

Death receptors are known to activate apoptosis upon ligand binding. For example, TRAIL-R2 but also for TNFR-1, are able to engage cell death independently of their NF- κ B inducer function. Therefore, we analyzed whether in the above settings, addition of EDAR ligand, Eda-A1, is associated with a marked increase in cell death. EDAR was thus transiently expressed in HEK293T cells, in presence of increasing doses of Eda-A1. Contrary to what is observed for classical death receptors, EDAR-induced cell death is inhibited by Eda-A1, in a dose-dependent manner (Fig. 2a, b and Supplementary Figures 2a-b). Consistent with the above, the interaction between EDAR and caspase-8 is reversed rather than induced by Eda-A1 (Fig. 2c, d). Contrary to classical death receptors but consistently to dependence receptors, the engagement of the EDAR pro-apoptotic program is then directly and oppositely related to the amount of Eda-A1 in the extracellular medium.

Transduction of signals mediated by the TNF-like receptors relies on their trimerization via the pre-ligand binding assembly domain (PLAD) contained in their extracellular domain. EDAR does display such a PLAD domain. However, deletion of this domain has no impact on EDAR ability to activate caspase-3 (Fig. 2e and Supplementary Figure 2c). This suggests that EDAR may induce apoptosis as a monomer, as shown previously for other dependence receptors, such as UNC5B or DCC [20].

Upon ligand binding, trimeric TNFR can transduce two signals. First, TNFR1 recruits at the plasma membrane the complex I, containing proteins such as TRADD, TRAF2 or 5, and cIAP1 or 2, which mediates NF- κ B activation and then survival; but, RIP1 can also dissociate from this complex to form the complex II with FADD and caspase-8 notably, which on the contrary engages the apoptotic program [5, 21]. The shift from the complex I to the pro-apoptotic complex II was shown to be notably tuned by NF- κ B pathway itself via activation of the caspase-8 inhibitor c-FLIP: if NF- κ B pathway is activated above a minimum

threshold, then complex II formation is turned off through blockade of this cystein-protease [5, 22, 23]. As a consequence, inhibition of NF- κ B tends to promote complex II formation and cell death upon TNFR1 activation. However, we observed that the inhibition of NF- κ B pathway, either by expression of a dominant-negative form of I κ B α (I κ B α - Δ N) or by treatment with its chemical inhibitor Bay 11-7082, does not potentiate EDAR-induced cell death (Fig. 2f and Supplementary Figures 2d-e). Reciprocally, its activation by expression of an active mutant of IKK (CA-IKK), which degrades I κ B α , is not sufficient to block apoptosis induced by EDAR (Fig. 2g and Supplementary Figures 2f). These results suggest that there is no interdependency between these signaling cascades, or that in other words, induction of apoptosis and NF- κ B activation are not mutually exclusive in settings where EDAR is expressed in absence of Eda-A1.

From these data, it seems possible to conclude that EDAR functions as a dependence receptor, whose pro-apoptotic activity is regulated by the presence of its Eda-A1 ligand, rather than by mechanisms classically observed for TNFRs.

EDAR behaves as a conditional tumor suppressor in melanoma

Dependence receptors have been shown to function as conditional tumor suppressors, thanks to their ability to trigger cell death in absence of their ligand. As a result, these receptors could restrict cell proliferation and migration to the availability of ligand in the environment [10, 11]. Reciprocally, loss of receptor expression or of its pro-apoptotic function is hypothesized to act as a selective mechanism for tumor growth and progression [16, 24]. Melanoma is a malignant form of skin cancer, which is readily curable by surgical excision when confined to the epidermis, but often becomes resistant to treatment when tumors progress and invade surrounding tissues [25]. Consequently, elucidating mechanisms underlying this neoplasia is a key issue. Melanoma tumors derive from melanocytes [25]. More precisely, they could result from the transformation of melanocytes stem cells found in hair follicles in a specific niche called bulge, ensuring renewal and maintenance of melanin-producing cells contingent. Bulge is localized in the outer root sheath, a region in which EDAR and Eda-A1 were shown to be highly expressed, notably during hair follicle cycle and involution [26]. Taking into account these data and a case report suggesting an increase propensity of patients suffering from anhidrotic ectodysplasia to develop this kind of malignancies [27], we decided to investigate the putative tumor suppressor role of EDAR in melanomagenesis.

We first quantified expression of EDAR in a panel of human melanoma and benign naevi lesions. Interestingly,

we observed a significant loss of EDAR expression in malignant melanoma tumors, as compared to benign naevi lesions (Fig. 3a and Supplementary Figure 3a). This result was further confirmed by utilizing the data from

whole-genome expression profiling of benign and atypical naevi on one side, and early and advanced-stage melanoma on the other side (Supplementary Figure 3b, GSE4587, [28]). We then analyzed the expression of EDAR in

◀ **Fig. 2** EDAR functions as a dependence receptor, since its pro-apoptotic activity is blocked by its ligand Eda-A1. **a** Caspase-3 activation induced by EDAR is blocked in a dose-dependent manner by Eda-A1 in HEK293T cells. HEK293T cells were transiently transfected with EDAR-encoding vector (EDAR) or adequate empty one (Ctl), in presence or not of soluble Eda-A1 (+: 1 ng/mL; ++: 10 ng/mL), or with TNFR1-encoding vector, with or without soluble TNF α (+: 1 ng/mL), or with TRAIL-R2-encoding vector with or without soluble TRAIL (+: 1 ng/mL). Caspase-3 activity measurements are presented as means $\pm$ std; $n = 3$; $*p \leq 0.05$; two-sided Mann–Whitney *U*-test. **b** Eda-A1 prevents EDAR-induced DNA fragmentation in HEK293T cells. DNA fragmentation is detected by TUNEL staining in HEK293T cells transiently transfected with EDAR-encoding vector (EDAR) or adequate empty one (Ctl), in combination Eda-A1. Results are presented as percentage of TUNEL-positive cells per field; $n = 5$; $***p = 0.008$, $**p = 0.012$; two-sided Mann–Whitney *U*-test. **c, d** Caspase-8 is recruited by EDAR in a pro-apoptotic complex, solely in absence of Eda-A1. **c** Immunoprecipitation of endogenous caspase-8 was carried out on HEK293T cells lysates, transiently transfected with EDAR-encoding vector (EDAR) or adequate empty one (Ctl), in presence or not of soluble Eda-A1 (+: 1 ng/mL; ++: 10 ng/mL). Representative blots of one experiment out of three are shown. **d** Interaction between transfected EDAR and endogenous caspase-8 evaluated by PLA-assay (Duolink), in presence or not of soluble Eda-A1. Green dots indicate interaction between EDAR and caspase-8. Nuclei are counterstained in blue by Hoechst staining. **e** Trimerization of EDAR via its extracellular domain (EC) is not required for apoptosis induction. HEK293T cells were transiently transfected with a vector encoding full-length EDAR (EDAR), EDAR deleted from its EC (EDAR- Δ EC) or adequate empty one (Ctl). Caspase-3 activity measurements are presented as means $\pm$ std; $n = 5$; $***p = 0.005$; two-sided Mann–Whitney *U*-test. **f** Blockade of NF- κ B pathway does not potentiate EDAR-induced caspase-3 activation. HEK293T cells were transiently transfected with a vector encoding full-length EDAR (EDAR), with or without a dominant-negative form of IKB α (IKB α -DN). Caspase-3 activity measurements are presented as means $\pm$ std; $n = 3$; *NS* non-significant; two-sided Mann–Whitney *U*-test. **g** Activation of NF- κ B pathway does not potentiate EDAR-induced caspase-3 activation. HEK293T cells were transiently transfected with a vector encoding full-length EDAR (EDAR), with or without a constitutively active form of IKK (CA-IKK). Caspase-3 activity measurements are presented as means $\pm$ std; $n = 3$; *NS* non-significant; two-sided Mann–Whitney *U*-test

cutaneous melanoma samples from the TCGA dataset [29]. Although there were large variations in expression of EDAR, its expression is totally lost in a significant number of melanoma samples (expression = 0 in 159/472 samples, 34%, range 1–1001) strengthening the observation that EDAR loss is a frequent event in melanoma. Loss of EDAR did not, however, correlate with tumor size (T stage), lymph node invasion (N stage), or survival in subsequent analyses (data not shown). Data regarding DNA alterations in the MAPK pathway, which are common in cutaneous melanoma, were available for 363 samples. In this subgroup of patients, we did not identify any correlation between the expression of EDAR and the presence of alterations in BRAF or NRAS mutations.

As COSMIC and cBioPortal databases report the existence of EDAR somatic mutations in 3.55% of skin tumors, we introduced the first 4 point-mutations described in these

tumors in EDAR sequence and studied their impact on EDAR ability to induce cell death. Interestingly, each of these mutations (T167I, E254K, P409L, and V416M) was sufficient to significantly impair EDAR pro-apoptotic activity, as shown by caspase-3 measurement (Fig. 3b and Supplementary Figure 3c–d). Altogether, these results support the tumor suppressor function of EDAR during melanomagenesis.

As a first approach to assay this in an animal system, we used a murine xenograft model. 1205-Lu melanoma cells were grafted in immunocompromised mice and we performed intra-tumoral transfection of an EDAR expressing construct. As shown in Fig. 3c, intra-tumoral transfection of EDAR is sufficient to prevent tumor growth in vivo (Fig. 3c and Supplementary Figure 3e). To investigate this point in a more pathologically relevant loss-of-function model, we generated a conditional KO murine model of EDAR, in which the fourth exon of the gene was flanked by two Lox excision sites (Fig. 3d and Supplementary Figure 3f). To study the impact of EDAR loss in melanomagenesis, we backcrossed these mutant mice with Tyr::Cre-ER ones, allowing expression of the recombinase Cre specifically in melanocytes after 4HT treatment, in a B-Raf^{V600E} mutant context (Fig. 3e). Indeed, nearly 60% of human melanoma display somatic mutations that constitutionally activate the serine/threonine-protein kinase B-Raf. B-Raf^{CA/+} murine model, conditionally expressing the dominant oncogenic form B-Raf^{V600E} of B-Raf protein, was shown to be prone to develop melanoma neoplasia upon ageing [30]. Accordingly, when treated with 4HT, 20% of the Tyr::CreER; B-Raf^{VE/+} developed cutaneous lesions that were categorized as melanoma by pathologists (Fig. 3f, g). Remarkably we observed that, upon 4HT treatment, loss of EDAR expression in the B-Raf^{V600E} background is associated with an increased propensity to develop melanoma (Fig. 3f, g and Supplementary Figure 3g): 63.6% of Tyr::CreER; B-Raf^{VE/+}; EDAR^{-/-} mice developed melanoma lesions over the first 400 days after 4HT application, compared to 20% of their control littermates Tyr::CreER; B-Raf^{VE/+}; EDAR^{+/+}. Moreover, this phenotype is associated with a decreased animal survival (Fig. 3h). Together, these results suggest that EDAR likely constrains melanoma development. Loss of its expression or of its pro-apoptotic activity via mutations, observed in the human pathology, appears as selective advantages for melanoma progression.

Discussion

The data presented here support the idea that EDAR could be envisaged as a player at the crossroad between TNFR family members and dependence receptors. Indeed, we provide evidence that, besides its well-characterized ability

to trigger NF- κ B activation upon ligand binding, EDAR induces cell death, via a caspase-dependent mechanism. Similarly to death receptors, EDAR-dependent cell death is mediated by activation of caspase-8, which is sufficient by

itself to trigger apoptosis in type I cells and requires subsequent activation of mitochondrial pathway in type II ones. However, and unlike what has been reported so far for TNFR, fine-tuning of the complex I to complex II transition

◀ **Fig. 3** EDAR behaves as a conditional tumor suppressor in melanoma. **a** Analysis of EDAR expression in a cohort of benign naevi ($n = 29$) and malignant melanoma ($n = 30$). Quantification of EDAR expression was performed by Q-RT-PCR, relatively to *HPRT* housekeeping gene. $*p = 0.02$; two-sided Mann–Whitney *U*-test. **b** Impact of point-mutation introduction on EDAR-ability to induce apoptosis in A375 melanoma cells. Caspase-3 activity measurements are presented as means $\pm$ std; $n = 3$; $*p \leq 0.05$; one-sided Mann–Whitney *U*-test. **c** Re-expression of EDAR by in vivo transfection using JetPEI prevents tumor growth in a murine xenograft model of melanoma cells. Nude mice were engrafted with 1205-Lu melanoma cells by subcutaneous injection. When tumor size reached around 100 mm^3 , EDAR-encoding plasmid was complexed with in vivo JetPEI and injected every 2 days into tumors. Results are presented as mean tumor volumes $\pm$ std; $*p < 0.05$, $**p \leq 0.01$; two-sided Mann–Whitney *U*-test. **d** Scheme of the EDAR conditional knock-out allele construct. FLP1 (FRT) and Cre recombinases recognition sites are indicated. **e–h** Incidence and characterization of melanoma in EDAR knock-out mice. **e** Breeding strategy used to generate Tyr:CreERT2; BRAF^{V600E}; EDAR^{+/+}, and Tyr:CreERT2; BRAF^{V600E}; EDAR^{-/-} mice. **f** Histological analysis of melanoma in Tyr:CreERT2; BRAF^{V600E}; EDAR^{-/-} mutant mice. Dark granulations could be seen in some tumor cells cytoplasm (arrow). **g, h** Incidence of melanoma (**g**) and survival (**h**) in Tyr:CreERT2; BRAF^{V600E}; EDAR^{+/+} ($n = 10$) and EDAR^{-/-} ($n = 11$) mice. Tumors classification was performed blinded to genotype according to anatomicopathologists consensus. $*p = 0.05$; two-sided Fisher's exact test

seems here regulated by ligand availability. Indeed, EDAR-induced apoptosis is blocked in a dose-dependent manner by Eda-A1. Contrary to other death receptors, trimerization of EDAR via PLAD does not seem to be required for cell death induction, suggesting that switch of its oligomerization status could be also associated with transduction of downstream cascade of events, as previously reported for other dependence receptors such as UNC5B [20]. FADD is necessary for apoptotic signaling induced by most death receptors, whereas TRADD is required for the gene activation signaling [5]. However, the recruitment here of the death-domain adapter EDARADD seems necessary for both signals induced by EDAR, indicating that shift from one complex to the other must rely on downstream recruitment of different partners. Further characterization of the nature of the apoptotic platform associated to EDAR will be required to improve understanding of EDAR-induced cascades. In other words, even if it is tempting to speculate that switch from a tetramerized status in presence of Eda-A1 to a monomeric one in its absence could lead to recruitment of different intracellular partners, mechanisms associated with the switch from complex leading to NF- κ B activation in presence of Eda-A1 on one side and cell death in its absence on the other side remain to be fully elucidated.

EDAR is mainly known for its role during development, especially in controlling the number, size, and morphogenesis of placodes giving rise to ectodermal organs [6, 7]. EDAR expression was long correlated with placodal cells fate, and it was shown more recently that it does so by promoting the centripetal migration of cells forming the

placode [31, 32]. Although most of EDAR-mediated effects have been shown to result from NF- κ B activation in response to Eda-A1 binding, its dependence receptor function could also serve during placode formation and expansion. For example, this dependence receptor function could act at the placode boundary, eliminating cells with a placodal fate (e.g., expressing EDAR) but located in an incorrect position regarding the source of ligand. In line with a receptor-dependent function in vivo, apoptosis is elevated in the tabby mutant mice, in which the Eda ligand is absent [33]. Together, these observations envision of a potential fine-tuning role of the dependence receptor function of EDAR during the development of ectodermal organs.

Besides its physiological roles, we also provide evidence that EDAR may be considered as a new tumor suppressor. Its expression appears altered qualitatively and/or quantitatively in human biopsies, and these alterations likely result in apoptosis silencing. Death receptors have been generally considered as guardians against tumorigenesis [34]. Impaired expression or function of death receptors was shown to confer an oncogenic advantage to tumor cells [35–37]. Loss of TRAIL-R in mice is notably associated with an increase propensity to tumor initiation/progression in some tissues [38, 39]. However, our data support the fact that EDAR may act as tumor suppressor in a rather distinct manner as tumor cell death induction is induced by ligand withdrawal rather than ligand engagement. Accordingly, whereas loss of TRAIL favors tumorigenesis in mice [40], it is expected that the loss of Eda-A1 should have the opposite effect. Similarly to other dependence receptors ligands [41–44], upregulation of Eda-A1 could be envisaged as a selective advantage for tumor growth. Its oncogenic activity could result both from inhibition of cell death induced by unbound EDAR and from genes activation via classical signaling: it is worth mentioning, for example, that the chemokines CXCL10 and CXCL11, which are activated by the pair EDAR/Eda-A1 via NF- κ B, have been shown to play an oncogenic role in melanoma [45–48]. Future works will have to investigate whether Eda-A1 is indeed gained in some cancers and whether it may be worth considering therapeutic approaches aiming at inhibiting its interaction with EDAR.

Material and methods

Patients and RNA samples

Naevi ($n = 30$) and primary melanoma ($n = 26$) biopsies were collected by the Dermatology Department of the Hospices Civils de Lyon (France). Tissues banking and researches conducted were performed according to national

ethical guidelines. Anatomopathologic characterization of samples was made according to standard international recommendations by Dr S. Dalle. Four sections of 10 µm in thickness were taken from each paraffin-embedded block, deparaffinized, and each sample was retrieved in a 2 mL collection tube. Tissues were then lysed in 200 µL of ATL buffer (Qiagen) with 2 µL Proteinase K (Roche). 1 mL of Trizol (Life Technologies) was then added to isolate total RNA, before classical purification using 200 µL chloroform and 500 µL isopropanol with 1 µL of Glycoblue (Thermo-Fisher). After washing in ethanol 75%, digestion of contaminant genomic DNA was performed 1 h at 37°C by resuspending the nucleic acid pellets in 15 µL of 1× buffer containing 0.25 µL of 100 mM DTT, 2 µL of 1 M DNase and 1 µL of RNAsine. A new step of precipitation by isopropanol/ethanol was then performed again to eliminate DNase and RNA samples were frozen (-80°C) before use.

Plasmid constructs and siRNA

Plasmids encoding human Eda-A1 and EDAR in pcDNA3 are a kind gift from Dr V. Laudet. Each of the four point-mutations was introduced in EDAR using Quick-change strategy (Stratagene®), according to manufacturer's instructions. Dominant-negative forms of caspase-8, caspase-9, FADD, IKBα-ΔN, and CA-IKK, and TRAIL-R2 and TNFR1 encoding plasmids have already been described [12, 49, 50].

siRNAs targeting either human EDAR, EDARADD, caspase-9, BID, and Bax were designed by Dharmacon as a pool of 4 target-specific 20–25nt siRNAs.

Cell culture

Human embryonic kidney HEK293T and melanoma cell lines A375 and 1205-Lu were cultured in DMEM medium (Gibco®, Invitrogen) supplemented with 10% fetal bovine serum. 1 or 5 µM Bay 11-7082 diluted in DMSO (Sigma-Aldrich), 1 ng/mL of TNF-α (Roche) and 1 ng/mL of recombinant his-tagged human TRAIL produced as described previously [50] were respectively added to cell culture medium for inhibition of NF-κB, activation of TNFR1 or TRAIL-R2. Recombinant Eda-A1 (3944-ED-010, Roche) was used at concentrations indicated in Results section.

Cell Death Assays

For each cell line, 1×10⁵ of HEK293T or A375 cells were transfected with 22 pmol of siRNA and/or 1 µg of plasmid DNA in 4 µL JetPrime transfectant diluted in 200 µL buffer, according to manufacturer's instructions (Polyplus

Transfection). 48 h later, cells were collected and lysed in 60 µL of adequate buffer, using the procedure described in caspase-3/CPP32 Colorimetric Assay kit (Biovision). Lysate of 50 µL was added to an equal volume of reaction buffer, according to manufacturer's guidelines, and caspase-3 activity was monitored by measuring fluorescence emitted by DEVD cleavage. Alternatively, active caspase-3 positive cells were quantified after immunofluorescence, using an antibody targeting the cleaved form of this protease (5A1E, Cell Signaling), using the procedure described below. Terminal deoxynucleotidyl transferase mediated dUTP-biotin Nick End Labeling (TUNEL) immunostaining was performed 72 h after transfection on cytopun cells as described previously [51].

Quantitative RT-PCR

Total RNA from murine xenografted tumors and cell lines was extracted using the Nucleospin RNAII kit (Macherey-Nagel) and 1 µg was reverse-transcribed using the iScript cDNA Synthesis kit (BioRad). Expression of EDAR, FADD, EDARADD, Bid, Bax, caspase-9, and Cox-2 was assessed by real-time quantitative RT-PCR with specific primers available upon request, on a LightCycler 480 apparatus (Roche) using the LightCycler® TaqMan® Master kit (Roche). Reaction conditions for optimal amplification of each gene, as well as primers selection were determined as already described [52]. The ubiquitously expressed HPRT was used as internal calibrator.

Immunoprecipitation, western-blot, and immunofluorescence

In total, 1×10⁶ HEK-293-T cells were seeded in 10 cm large dishes and transfected with 4 µg of plasmids combination diluted in JetPrime, according to standard protocol (Polyplus Transfection). 24 h later, cells were lysed in 500 µL of a pH7.6 solution containing 50 mM Hepes-Sodium, 150 mM NaCl, 5 mM EDTA, 0.1% NP40 in the presence of protease inhibitors (Roche). Each lysate of 50 µL was withdrawn as total extract. Remaining lysates were further incubated with anti-caspase-8 (sc-6136, Santa Cruz) and protein G-Sepharose (GE Healthcare) to pull down caspase-8 complexes. Immunoprecipitated protein extracts were then analysed by immunoblot. Briefly, proteins were loaded onto 10% SDS-polyacrylamide gels and blotted onto PVDF sheets (Millipore Corporation) using TurboBlot technology (BioRad). Filters were blocked with 10% non-fat dried milk and 5% BSA in PBS/0.1% Tween 20 (PBS-T) for one hour and then incubated overnight with anti-EDAR (sc-271627, Santa Cruz), anti-TNFR1 (ab19139, Abcam), or anti-caspase-8 (sc-6136, Santa Cruz). After three washes with PBS-T, filters were incubated with the appropriate

HRP-conjugated secondary antibody (1:5000, Jackson ImmunoResearch) for 1 h. Detection was performed using West Dura Chemiluminescence System (Pierce).

Same immunoblot procedure was followed to control the expression of proteins after transfection using anti-EDAR (sc-271627, Santa Cruz), anti- β -actin (mAB1501R, Sigma), anti-HA for FADD, caspase-8, caspase-9 or Eda-A1 (H6908, Sigma), or anti-FLAG for TRAIL-R2 (F3165, Sigma).

Expression of EDAR and its mutants was assessed by immunofluorescence on cells fixed 20 min in 4% paraformaldehyde and permeabilized in PBS1x/Triton0.2%. According to mutations, EDAR expression was analyzed using two specific antibodies directed against different epitopes (R&D, AF157, 1:500; Santa Cruz, sc-271627; 1:500). Fluorescence labelings were obtained using corresponding secondary antibodies coupled to alexa-Cy3 at a dilution of 1:500 (Jackson ImmunoResearch).

Duolink assay

The anti-EDAR and the anti-caspase-8 antibodies were respectively conjugated with Duolink PLA probe PLUS and MINUS separately using Duolink In Situ Probemarker kit following the instructions of the manufacturer. In total, 1×10^4 cells HEK-293-T were transfected with 0.4 μ g of relevant plasmids using JetPrime Lab-Tek chambers. 48 h after transfection, cells were fixed 30 min in 4% PFA. PLUS-probe-coupled antibody of 5 μ g mL⁻¹ and 5 μ g mL⁻¹ MINUS-probe-coupled antibody were then added on fixed cells. Ligation, amplification, and detection with Duolink In Situ Detection Reagents Orange were done according to the manufacturer's instructions. In this procedure, bright fluorescent dots are observed when two probes are in close proximity. Nuclei were labeled with Hoechst. Images were captured using a Zeiss Axiovert 200 M inverted microscope equipped with Zen image capture system.

Tumor engrafted nude mice

Seven-weeks-old (20–22 g body weight) female athymic nu/nu mice were obtained from Charles River animal facility. The mice were housed in sterilized filter-topped cages and maintained in a pathogen-free animal facility. 1205-Lu cells were implanted by s.c. injection of 1×10^6 cells in 200 μ L of PBS into the right flank of the mice. Once tumors were established ($V \approx 100$ mm³), mice were treated by intra-tumoral injection of 5 μ g of EDAR ($n = 6$) or empty pcDNA3 plasmid ($n = 6$) diluted in JetPEI (Polyplus transfection) in vivo transfectant. Tumor sizes were measured with a caliper. The tumor volume was calculated with the formula $v = 0.5(\text{length} \times \text{width}^2)$.

Generation and analysis of EDAR conditional knock-out mutant mice

ES cells containing an EDAR transgene consisting in a reporter-tagged insertion of lacZ and neo cassettes after the third exon and two Lox sites flanking the fourth exon of the gene were obtained from KOMP Repository Resources Bases. Cells were then injected in mice blastocytes by the Mice Experimental Platform AniRA-PBES (Lyon, France). Chimaeric mice were then selected for germline transmission by successive mating in C57BL/6 background. Removal of FRT sites surrounding lacZ and neo cassettes of EDAR conditional knock-out mice (EDAR^{lox/+}) was achieved using mice expressing the FLP1 recombinase under the control of actin promoter. EDAR^{lox/lox} and BRaf^{CA/+} mice (kindly provided by Dr L Larue, Institut Curie, Paris) were then each separately mated with Tyr::CreER ones, all in C57BL/6 background. Double heterozygous Tyr::CreER^{+/-};BRaf^{CA/+} and Tyr::CreER^{+/-};EDAR^{lox/lox} were then interbred to generate Tyr::CreER^{+/-};BRaf^{CA/+};EDAR^{lox/lox} in the offspring. Triple heterozygous mice were finally mated to generate mice with the genotypes of interest, i.e., Tyr::CreER^{+/-};BRaf^{CA/+};EDAR^{lox/lox} and Tyr::CreER^{+/-};BRaf^{CA/+};EDAR^{+/+}. Routine genotyping of mice was performed by PCR assay on DNA purified from tails biopsies (Viagen, Biotech; Supplementary Figure 3c). Cre expression and local melanomagenesis induction were activated by applying 200 μ L of 2 mg/mL solution of 4-HT (70% Z-isomer, Sigma) diluted in 100% ethanol on the back skin of 6 weeks-old mice for 4 consecutive days. All experiments were performed in accordance with relevant guidelines and regulations of animal ethics committee (Authorization n°CLB-2014-010; accreditation of laboratory animal care by CECCAPP, ENS Lyon-PBES).

Statistical methods

Statistical significance of differences between groups was evaluated by Mann–Whitney test or Fisher's exact test. *P* values <0.05 were considered to be statistically significant.

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Authors contribution: JV and AR have performed experimental design and work, with the help of PC, SD, DM, AS, and LGH. AT has realized anatomopathological analyses of murine samples. AF, SD, and LD have provided human biopsies and performed their anatomopathological characterization, with the technical support of SL and GT. OM on one side, and SP and VL on the other side, have provided some scientific insights and technical expertise respectively for death receptors or EDAR signaling dissection. PM and MC proposed the project, did experimental design and wrote the manuscript.

Conflict of interest PM declares to have conflict of interest as shareholder of Netris Pharma. The remaining authors declare that they have no conflict of interest.

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