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ORIGINAL PAPER

Tiam1-deficiency impairs mammary tumor formation in MMTV-c-*neu* but not in MMTV-c-*myc* mice

K. Strumane · T. Rygiel · M. van der Valk · J. G. Collard

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Abstract
Background Rho-like small GTPases, including RhoA, Rac1 and Cdc42, are crucial for the regulation of a large variety of biological processes such as the cytoskeletal organization and gene transcription. The activities of Rho GTPases are predominantly controlled by guanine nucleotide exchange factors (GEFs), which activate GTPases by catalyzing the exchange of bound GDP for GTP. Earlier, we have identified the *Tiam1* gene as an invasion-inducing gene that encodes a specific activator (GEF) of the Rac GTPase. We found that Tiam1-mediated Rac signaling functions in various aspects of tumorigenicity including the formation and progression of Ras-induced skin tumors and Wnt-induced intestinal tumors. Here, we further distinguish the oncogenic pathways that depend on Tiam1 signaling in the mammary gland.
Material and methods We crossed Tiam1 knockout mice with MMTV-c-*myc* and MMTV-c-*neu* transgenic mice, in which the expression of both oncogenes is targeted to the mammary gland leading to mammary tumorigenesis.
Results We found Tiam1 important for Neu-induced tumor formation and progression but not for Myc-induced tumors. Tiam1-deficiency delayed Neu-induced tumor initiation and reduced metastasis but had no effect on the growth of the MMTV-c-*neu* tumors.
Conclusion Our data indicate that the Rac activator Tiam1 contributes to tumorigenicity induced by specific oncogenic signaling pathways only.

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Introduction

The activity of Rho-like GTPases in response to receptor stimulation is strictly controlled to stimulate, locally and temporally, specific downstream signaling pathways in cells. The regulators of the activity of Rho GTPases consist of three classes of proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). To date, over 70 Rho GEFs have been identified (Schmidt and Hall 2002). In earlier studies, we have identified the *Tiam1* gene (T-cell invasion and metastasis gene 1), which encodes a specific GEF and thus activator of the Rho-like GTPase Rac (Habets et al. 1994; Michiels et al. 1995). Rho GTPases are best characterized for their regulation of actin cytoskeleton dynamics, but they also control various other processes including apoptosis, cell proliferation and gene transcription (Bishop and Hall 2000). It is therefore not surprising that Rho GTPases and their regulators may contribute to various aspects of tumorigenicity (Malliri et al. 2002a; Sahai and Marshall 2002).

The activators of Rho GTPases not only catalyze GDP/GTP exchange but also contribute to RhoGTPase downstream signaling by connecting active GTPases to specific scaffold and effector proteins (Mertens et al. 2003; Rossman et al. 2005). Scaffold proteins that complex Tiam1 with components of specific Rac effector pathways include IB2 and spinophilin, which direct Tiam1-mediated Rac activation towards the p38 MAPK and p70S6 K cascades implicated in transcription and translation, respectively (Buchsbaum et al. 2002, 2003). Tiam1 also binds to different

components of the Par polarity complex and thereby regulates polarity processes in various cell types. Tiam1 associates with Par3 and PKCξ and connects Tiam1-mediated Rac signaling to the establishment of apical–basal cell polarity in contacting epithelial cells as well as front–rear polarity in freely migrating epithelial cells (Chen and Macara 2005; Mertens et al. 2005; Pegtel et al. 2007). Tiam1 also associates with activated Rap1 and in conjunction with the Par polarity complex controls chemokine-induced T-cell polarization (Gerard et al. 2007). The association of Tiam1 with IRSp53 and p21Arc provides a direct link between Tiam1-mediated Rac activation and Arp2/3 complex-controlled actin polarization, which is required for cytoskeletal dynamics during cell migration and cell polarization (Connolly et al. 2005; ten Klooster et al. 2006). In receptor signaling, activated Ras may activate Rac by direct binding to Tiam1 (Lambert et al. 2002) or indirectly by activation of phosphoinositide 3-kinase (PI3-kinase) that may recruit and activate Tiam1 and thereby Rac downstream of Ras (Fleming et al. 2000; Zondag et al. 2000).

Tiam1 is expressed in human and rodent tumor cells of different tissue origin and has been shown to affect various aspects of tumorigenicity (Habets et al. 1995; Minard et al. 2004). The influence of Tiam1 on different stages of tumor development is illustrated in previous studies using mouse tumor model systems and Tiam1 knockout (*Tiam1*$^{-/-}$) mice. Tiam1-deficiency inhibits Ras-induced mouse skin tumor initiation and growth in a two-stage DMBA/TPA carcinogenesis model (Malliri et al. 2002b). These skin tumors arise by DMBA-induced Ras mutations in keratinocytes of the epidermis. *Tiam1*$^{-/-}$ mice are more resistant to the Ras-induced skin tumor development because epidermal keratinocytes are more susceptible for Ras-induced apoptosis during tumor initiation. Although the few Ras-induced skin tumors that do occur in *Tiam1*$^{-/-}$ mice grow much slower than wild type tumors, they convert more frequently to a malignant phenotype, presumably as a result of the function of Tiam1 in maintenance of E-cadherin-based cell–cell adhesions (Malliri et al. 2002b, 2004). Such a bifunctional effect of Tiam1-deficiency on tumor formation and progression was also found for intestinal tumors in APC Min (multiple intestinal neoplasia) mutant mice (Malliri et al. 2006). Tiam1 is a Wnt-responsive gene in colon cells and its deficiency reduces the formation and growth of polyps in APC Min mutant mice but promotes invasion of progressed malignant intestinal tumors (Malliri et al. 2006). These in vivo data indicate that Tiam1 functions downstream of at least two independent oncogenic signaling pathways, i.e., the Ras and the Wnt pathway.

Tiam1 is thus a potential therapeutic target, and chemical inhibitors have been developed to inhibit the function of Tiam1 and Rac in tumors in vivo (Gao et al. 2004; Shutes et al. 2007). In this context, it is important to decipher the specificity of Tiam1 as a modifier of tumor development and progression in the context of different oncogenic signaling pathways and of tumor cell types. Therefore, we investigated the function of Tiam1 in mammary tumorigenesis induced by two alternative oncogenic signaling pathways. We crossed *Tiam1*$^{-/-}$ mice with two strains of breast cancer prone transgenic mice that express oncogenic Myc or Neu under the control of the mouse mammary tumor virus (MMTV) promoter. Interestingly, we found that Tiam1-deficiency did not influence Myc-induced tumorigenesis but specifically impaired c-*neu* induced mammary tumor formation in mice, illustrating that Tiam1-mediated Rac signaling is required for only specific oncogenic signaling pathways that lead to tumorigenesis.

Materials and methods

Mice

A congenic line of FVB/*Tiam1*$^{-/-}$ mice was used, which was generated as described earlier (Malliri et al. 2002b). Transgenic MMTV-c-*neu*, line TG.NK (Muller et al. 1988) and MMTV-c-*myc* (Stewart et al. 1984) mice were purchased from Charles River Laboratories. All transgenic mice were on FVB background. Transgenic male mice were crossed with *Tiam1*$^{-/-}$ females. The resulting *Tiam1*$^{\pm}$/MMTV-oncogene males were backcrossed with female *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ mice. From their offspring, *Tiam1*$^{+/+}$/MMTV-oncogene and *Tiam1*$^{\pm}$/MMTV-oncogene males were backcrossed to *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ females, respectively, to yield the experimental groups: *Tiam1*$^{+/+}$/MMTV-oncogene, *Tiam1*$^{\pm}$/MMTV-oncogene, *Tiam1*$^{-/-}$/MMTV-oncogene. Only females were used for subsequent analyses. MMTV-c-*neu* females were kept as virgins throughout the entire observation period. MMTV-c-*myc* females underwent forced breeding for two pregnancies to promote tumorigenesis. Mice were monitored by palpation for tumors and killed when they harbored a mammary tumor that reached a size of 10 mm. Local ethics committee for animal experiments approved the mouse experiments according to the Dutch law that implements the European guideline 86/609/EEG.

Histology and immunohistochemistry

H&E stainings and immunohistochemistry were performed on 4 μm paraffin-embedded tissue sections as described (Strumane et al. 2005). Antibodies used are as follows: an anti-DH Tiam1-specific rabbit polyclonal antibody (Habets et al. 1994), polyclonal anti-Keratin 1 (Covance/Babco, 1:250), anti-Keratin 8 (troma-1; University of Iowa, Department of Biological Sciences, Iowa City, Iowa USA; 1:400) and anti-Keratin 14 (Covance/Babco; 1:10,000).

Whole mounts of mammary glands

Inguinal mammary fat pads were excised from euthanized mice and stretched on a glass slide for fixation in methanol:chloroform:acidic acid (6:3:1) for at least 24 h. After washing in 70% ethanol for 1 h, the slides were rinsed in water and stained in carmine for 24 h. All incubations were performed at room temperature (RT). The fat pads were dehydrated in a graded series of alcohols and kept in methyl salicylate to make photographic images.

Isolation mammary epithelial cells

The left and right mammary fat pads 2 and 3 were excised from euthanized mice at 16 weeks of age. The isolated tissues were washed three times in 70% ethanol and transferred to L15 medium (Gibco BRL) with 10% fetal calf serum (FCS). The fat pads were chopped using a scalpel and digested in a 0.3% collagenase–0.15% trypsin mix in serum-free L15 medium at 37°C for 1 h with periodic shaking. Cells were pelleted and washed four times with L15 medium with serum and subsequently incubated in DMEM containing 10% FCS, 2 mM L-glutamine (L-Gln) and 100 U/ml penicillin/100 µg/ml streptomycin (P/S) at 37°C, 5% CO_2, 5% O_2 to remove contaminating fibroblasts. After 1 h, the epithelial cells were still floating in the medium and could be easily separated from the fibroblasts that had attached to the bottom of the culture flasks. The pelleted epithelial cells were resuspended and cultured for maximal 3 days in DMEM:F12 (1:1; Gibco-BRL) medium containing 10% FCS and P/S and supplemented with 5 µg/ml insulin, 5 ng/ml choleratoxin and 5 ng/ml EGF.

Western blotting

Lysates were prepared using standard SDS or RIPA lysis buffers as indicated. Cultured cells were washed with PBS and scraped in lysis buffer. Snap frozen tumor material was first grinded in a mortar and than lysed. Proteins were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). After blocking with 5% skimmed milk, the blots are probed using the indicated antibodies. Primary antibodies used were anti-DH [(Habets et al. 1994);1/500] and C-16 (sc-872, Santa Cruz, Venendaal, The Netherlands; 1/1.000) against Tiam1, anti-α-tubulin (Clone B-5-1-2, Sigma, 1/5.000), anti-c-erbB2/HER-2/neu (Ab-17, Neomarkers, 1/2.000) and anti-Rac1 (clone 23A8, Upstate Biotechnology, Venendaal, The Netherlands; 1/1.000). As secondary antibodies, peroxidase-conjugated IgGs were used followed by enhanced chemiluminiscence (ECL) detection (Amersham).

Apoptosis quantification

MDA-MB-361 cells were cultured in DMEM:F12 (1:1; Gibco-BRL) medium supplemented with P/S, 10% FCS, 10 ng/ml EGF and 10 µg/ml insulin. The Tiam1-specific siRNA oligo GCGAAGGAGCAGGTTTTCT (Malliri et al. 2004) was transfected into the MDA-MB-361 cells using the Dharmafect-1 reagent (Dharmacon). A scrambled sequence was used as nonspecific control siRNA oligo (si*CONTROL* nontargeting siRNA, Dharmacon). Six hours after transfection, the transfection mix was replaced by culture medium, which was refreshed again 24 h after transfection and apoptosis was quantified 72 h after transfection. Both floating and adherent cells were lysed together and apoptosis was analyzed using the Cell Death Detection ELISA kit (Roche) according to manufactures instructions.

Results

Tiam1 expression in the mammary gland is increased in tumor tissue

We started our analyses by determining Tiam1 expression in the mammary glands and mammary tumors of transgenic and nontransgenic female mice. Tiam1 is expressed in the normal mammary gland as shown by Western blot analysis of primary cells isolated from 16-week-old wild type mice (Fig. 1a, left lane). At this age, we found increased Tiam1 expression levels in the precancerous mammary gland of MMTV-c-*neu* transgenic mice (Fig. 1a, right lane). Western blot analysis revealed Tiam1 expression in both MMTV-c-*myc* and MMTV-c-*neu* tumors (Fig. 1b). Consistent with these data, others also found that Tiam1 was increased in MMTV-c-*neu* tumors compared to normal tissue at the mRNA level (Landis et al. 2005). Tiam1 expression in tumors was confirmed by immunohistochemical staining of mammary tumors isolated from $Tiam1^{+/+}$ mice (Fig. 1c). These data demonstrate that Tiam1 is expressed in the mammary gland and that its expression is increased in Neu-induced and Myc-induced mammary tumors.

Normal mammary gland development in Tiam1-deficient mice

Tiam1 knockout mice are viable and do not show any obvious aberrant phenotype (Malliri et al. 2002b). Also the mammary glands of $Tiam1^{-/-}$ mice are functionally normal as $Tiam1^{-/-}$ females are able to suckle their offspring. To exclude that tumorigenesis is influenced by morphological differences in mammary gland development, we compared

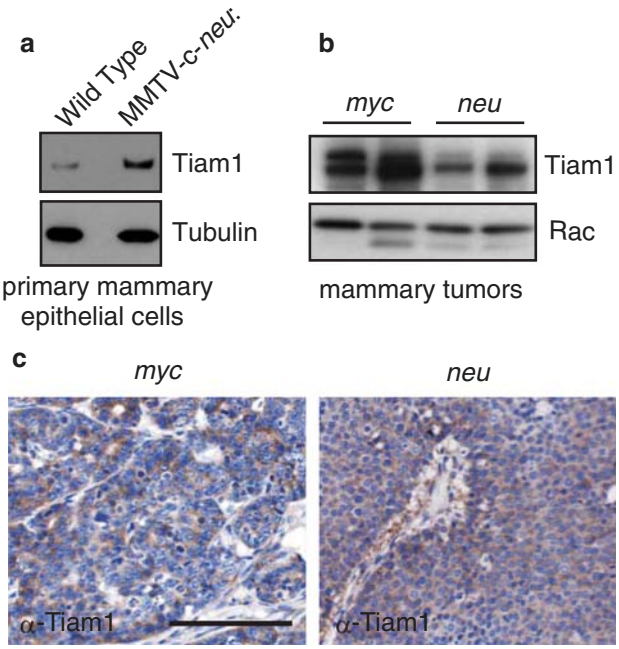

Fig. 1 Tiam1 expression in mouse mammary tissue and tumors. **a** Tiam1 is detected (C16 antibody) by Western blotting in primary epithelial cells isolated from precancerous mammary glands of 16-week-old mice. Tiam1 expression is increased in the mammary gland of MMTV-c-*neu* mice compared to wild type mice. The same blot was probed for the detection of α-tubulin for loading control. **b** Western blot analysis of Tiam1 protein levels in tumor samples derived from MMTV-c-*myc* and MMTV-c-*neu* mice (C16 antibody). Two SDS lysates of snap-frozen tumors isolated from different mice are shown for each transgenic line. The same blot was probed with a Rac antibody and used as a loading control. **c** Expression of Tiam1 in histological tumor slides. Mammary carcinomas from MMTV-c-*myc*;*Tiam1*$^{+/+}$ and MMTV-c-*neu*;*Tiam1*$^{+/+}$ are shown probed for Tiam1 (anti-DH antibody). The *scale bar* indicates 100 μm

in detail the appearance of the inguinal mammary glands of virgin *Tiam1*$^{-/-}$ female mice with that of *Tiam1*$^{+/+}$ mice in nontransgenic and transgenic animals at different ages (Fig. 2). The appearance of *Tiam1*$^{-/-}$ mammary glands was identical to that of wild type glands in adult mice (not shown). However, we found a delay in the early development of the mammary gland in *Tiam1*$^{-/-}$ when compared to wild type mice. The outgrowth of the inguinal mammary glands is determined by how far the glands extend beyond the inguinal lymph node (to the right side in Fig. 2). The mammary ductal system was significantly less far proliferated within the adipose stroma in 4-week-old and 6-week-old *Tiam1*$^{-/-}$ mice when compared to age-matched wild type mice (Fig. 2 left panel). At 8 weeks, the appearance of the mammary gland in Tiam1$^{+/+}$ and Tiam1$^{-/-}$ mice was indistinguishable, indicating that development was delayed but not impaired (Fig. 2 left panel). This delay in early mammary gland elongation during puberty in *Tiam1*$^{-/-}$ mice was also observed for both transgenic strains in a *Tiam1*$^{-/-}$ background as exemplified in 6-week-old mice (Fig. 2 right panel). By analyzing mammary glands at different time points, we found that the delay in outgrowth of the mammary gland was most apparent at 4 weeks of age, declined within the subsequent weeks and by 8 weeks of age we could not discriminate anymore between wild type and Tiam1 knockout mammary glands. As the first tumors in MMTV-oncogene mice became detectable only by 17 weeks of age, we conclude that it is unlikely that the delay in early normal mammary gland development affects mammary tumorigenesis in the mouse mammary tumor models used.

Tiam1 is involved in mammary tumorigenesis induced by Neu and not by Myc

To address whether Tiam1 is involved in specific oncogenic pathways, we examined mammary tumorigenesis in MMTV-c-*neu* and MMTV-c-*myc* female mice in *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ backgrounds. Weekly palpation of the mice revealed that Tiam1-deficiency strongly delayed the appearance of tumors in MMTV-c-*neu* transgenic mice, whereas the latency of mammary tumors induced by Myc was not affected by the loss of Tiam1 (Fig. 3). MMTV-c-*myc* mice developed mammary tumors with the same latency in *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ mice (Fig. 3a). Although all except one of the MMTV-c-*neu* mice developed palpable tumors over an observation period of 1 year (Table 1), the latency was significantly longer in the Tiam1-deficient mice ($P < 0.001$) (Fig. 3b). At the time when tumors were detected in 100% of the MMTV-c-*neu*;*Tiam1*$^{+/+}$ mice (age 29 weeks), detectable tumors were found in only 19% of the MMTV-c-*neu*;*Tiam1*$^{-/-}$ mice. This delay in the rate of tumor initiation is also reflected in the T_{50} that denotes the age at which 50% of the populations possess at least one palpable tumor. The T_{50} was 23.5 weeks for MMTV-c-*neu*;*Tiam1*$^{+/+}$ mice and 32.5 weeks for MMTV-c-*neu*;*Tiam1*$^{-/-}$ mice. Together, these data indicate that, while Tiam1 is not required for the induction of mammary tumors by Myc, it does play a critical role in the initiation of mammary tumors induced by Neu. More specifically, Tiam1-deficiency extends the latency of Neu-induced mammary tumor initiation.

Tiam1-deficiency does not influence the type of tumor differentiation in MMTV-c-*neu* and MMTV-c-*myc* mice

We analyzed a possible relation between the involvement of Tiam1 and the tumor characteristics. The MMTV-c-*myc* and c-*neu* tumors were defined as adenocarcinomas based on histopathological analysis of hematoxylin and eosin (H&E) stainings (Fig. 4a). To further analyze the differentiation status of the tumors in our analysis, we performed additional stainings for Keratins. The MMTV-c-*myc* and

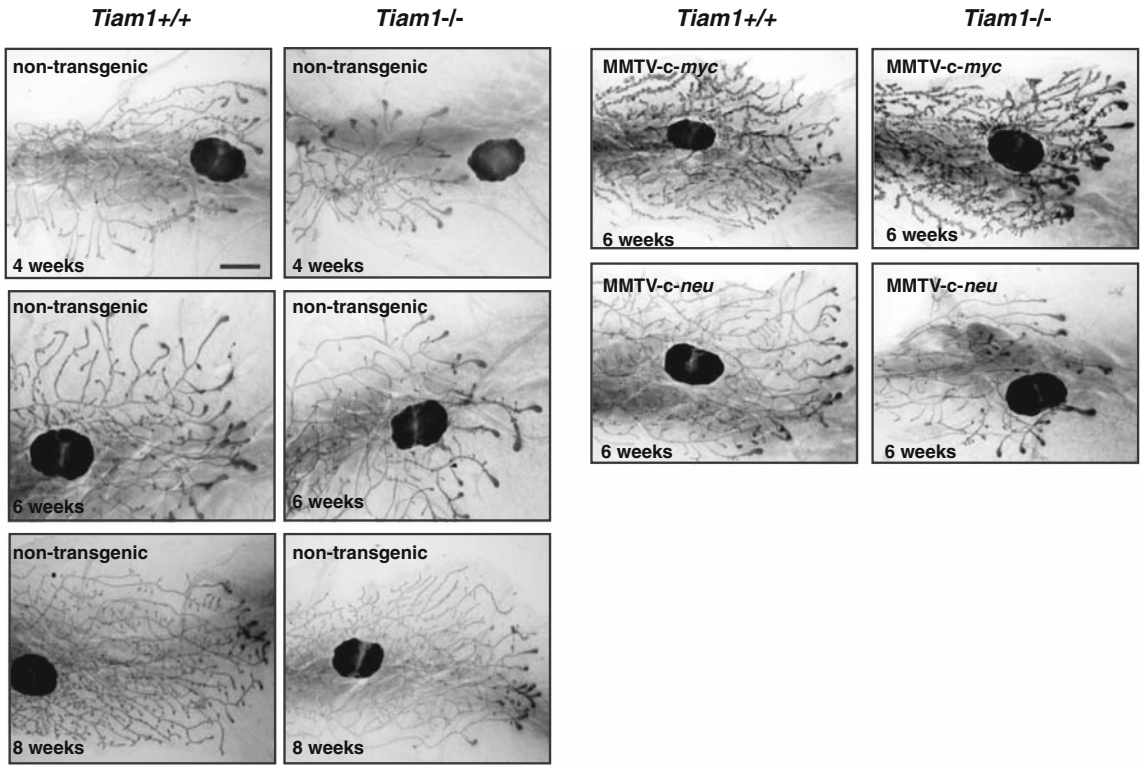

Fig. 2 Analysis of mammary gland development. Inguinal mammary glands from 4-, 6-, and 8-week-old *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ virgin female mice were compared within the nontransgenic mice (*left panel*) and from 6-week-old *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ mice in the MMTV-c-*myc* and MMTV-c-*neu* transgenics (*right panel*). All mammary glands are positioned in the same orientation so that the outgrowth of the mammary glands is presented from left to right. The distance towards or beyond the inguinal lymph node is a measure for the outgrowth of the mammary gland. Representative images show the delay in elongation of the system of mammary ducts in the *Tiam1*$^{-/-}$ mice compared to *Tiam1*$^{+/+}$ mice in the different strains. Mammary whole mounts were stained with carmine red. The *scale bar* indicates 1.5 mm

c-*Neu* tumors were all positive for Keratin 8 in both *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ mice (Fig. 4b, A–D), indicating that they all had a glandular character, although this was less evidently suggested by the H&E staining in the case of the MMTV-c-*neu* tumors. Keratin 1 is normally not expressed in the mammary gland and is used as a marker for epidermal differentiation in mammary tumors. All tumors were negative for Keratin 1 (Fig. 4b, E–H), while the adjacent skin tissue served as an intrinsic positive control (not shown). Although the Keratin 1 staining suggested that none of the tumors contained epidermal characteristics, MMTV-c-*myc* tumors showed a positive Keratin 14 staining indicating squamous metaplasia (Fig. 4b, I, J) in both *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ mice. However, the MMTV-c-*neu* tumors were completely negative for Keratin 14 (Fig. 4b, K, L). From these immunohistological analyses, we conclude that the presence or lack of Tiam1 has no effect on the differentiation type of the MMTV-c-*myc* and MMTV-c-*neu* mammary tumors. The MMTV-c-*neu* tumors show homogeneously a glandular differentiation (Keratin 8-positive), whereas MMTV-c-*myc* tumors consist of different components with glandular (Keratin 8-positive) and squamous (Keratin 14 positive) differentiation. This is in agreement with the notion that MMTV-c-*neu* mice uniformly develop mammary tumors (Bargmann et al. 1986; Muller et al. 1988), whereas MMTV-c-*myc* mice develop mammary tumors in a stochastic fashion. As reported earlier, c-*myc* is necessary but not sufficient for tumorigenesis, indicating that additional events are required for mammary tumor development in these mice (Li et al. 2000; Stewart et al. 1984; Tsukamoto et al. 1988). Taken together, MMTV-c-*neu* tumors can be discriminated from MMTV-c-*myc* tumors by the fact that they show glandular differentiation only and no squamous characteristics. Furthermore, the presence or absence of Tiam1 does not influence the histological type of tumors raised by Myc or Neu expression.

Tiam1-deficiency affects initiation but not growth of *neu*-induced mammary tumors

Western blot analysis of mammary tumors in *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ mice showed that disruption of Tiam1 expression did not alter the expression levels of the Neu protein (Fig. 5a). This excludes the possibility that the delay in initiation of Neu-induced mammary tumors in *Tiam1*$^{-/-}$ mice was caused by an inadequate expression of the *neu*

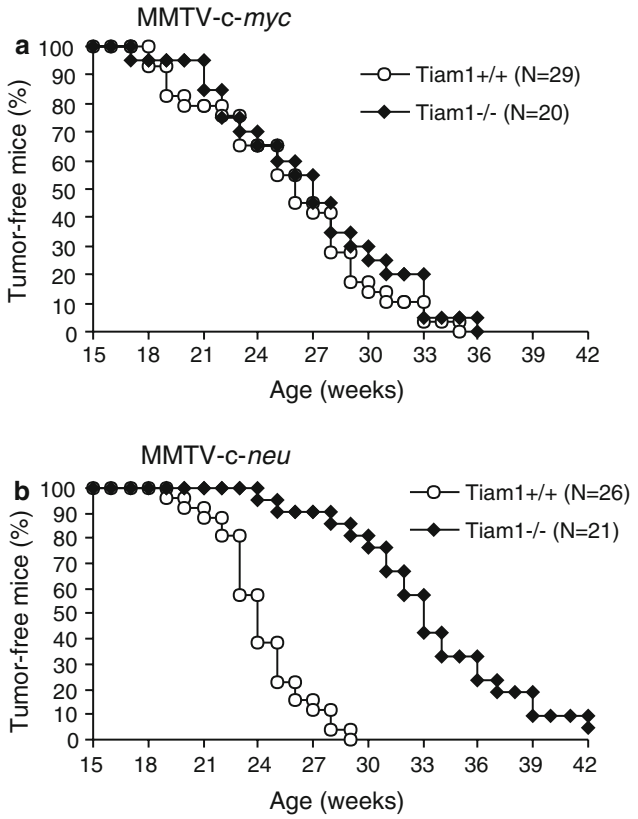

Fig. 3 Kinetics of oncogene-induced mammary tumor initiation in *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ mice. The age when a palpable mammary tumor first appears represents the latency of tumor initiation. **a** Tumor initiation in MMTV-*myc*;*Tiam1*$^{-/-}$ ($n = 20$) compared to MMTV-*myc*;*Tiam1*$^{+/+}$ ($n = 29$) is not different ($P = 0.41$, Student's t test). **b** The longer latency in MMTV-*neu*;*Tiam1*$^{-/-}$ ($n = 21$) compared to MMTV-*neu*;*Tiam1*$^{+/+}$ ($n = 26$) is statistically significant ($P < 0.001$, Student's t test). N is the number of mice analyzed

Table 1 Incidence of mammary tumors in transgenic mice

	Tiam1$^{+/+}$	Tiam1$^{-/-}$
MMTV-c-*myc*	29/29	20/20
MMTV-c-*neu*	26/26	20/21

The presented numbers are the ratios of females displaying mammary tumors to the total number of females observed over a period of 52 weeks

transgene in the mammary glands of these mice. The latency of MMTV-c-*neu*-induced tumors in the *Tiam1*$^{\pm}$ group was intermediate between that in the *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ groups (Fig. 5b) suggesting a dose-dependent effect of Tiam1 on the initiation of Neu-induced mammary tumors. We found earlier a similar dose-dependent effect of Tiam1 on Ras-induced skin tumors (Malliri et al. 2002b).

The decreased susceptibility to Neu-induced tumors in Tiam1-deficient mice was also reflected by a decreased number of tumors per mouse (Fig. 5c). Mice were euthanized for dissection when they harbored at least one mammary tumor that reached a size of 10 mm. The median number of tumors with a size >4 mm per mouse is four in *Tiam1*$^{+/+}$ mice, three tumors per mouse in *Tiam1*$^{\pm}$ and two tumors per mouse in *Tiam1*$^{-/-}$ mice, again suggesting a dose-dependent effect of Tiam1 on tumor initiation (Fig. 5c). Tumor growth was determined by the time between detection of the first palpable tumor and necropsy, i.e., when the tumor had grown out to a size that reached 10 mm. We found that the average growth of individual MMTV-c-*neu* tumors was not significantly different in *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ backgrounds (Fig. 5d). This indicates that once a tumor is initiated in the *Tiam1*$^{-/-}$ mice, it is able to grow as fast as in the *Tiam1*$^{+/+}$ mice. Tiam1-deficiency also did not affect mammary tumor growth in the MMTV-c-*myc* model in which tumor initiation was not affected (not shown). Together, these data indicate that the delayed latency and the fewer tumors in *Tiam1*$^{-/-}$ mice compared to *Tiam1*$^{+/+}$ mice are a consequence of a specific function of Tiam1 in Neu-induced tumor initiation rather than an involvement of Tiam1 in the growth of the mammary tumors.

Tiam1 provides survival signaling in Neu-induced mammary tumor cells

Prevention of apoptosis is a necessary step during the process of tumor initiation. The increased susceptibility for apoptosis was found to be the underlying mechanism of decreased skin tumor incidence in *Tiam1*$^{-/-}$ mice (Malliri et al. 2002b). As in Ras-induced skin tumors, it is possible that Tiam1-deficiency results in a reduced number of tumors in the MMTV-c-*neu* mouse model by increasing the susceptibility to apoptosis of the targeted mammary epithelial cells. Attempts to analyze apoptosis in vivo in established tumors did not discriminate Neu-induced tumors between Tiam1$^{+/+}$ and Tiam1$^{-/-}$ mice. Therefore, we analyzed the dependency of the survival of Neu-expressing breast cancer cells on Tiam1 in vitro. As a model, we used the human breast cancer cells MDA-MB-361, which are characterized by high Neu expression. Western blot analysis showed that MDA-MB-361 cells express Tiam1 that can be downregulated by Tiam1-specific siRNA (Fig. 6a). The susceptibility to apoptosis was measured using a cell death detection kit that quantitatively determines cytoplasmic histone-associated DNA fragments by ELISA (enzyme-linked immunosorbent assay). Interestingly, the number of apoptotic cells is increased upon downregulation of Tiam1 when compared to cells that were transfected with a nonspecific control siRNA (Fig. 6b), indicating that Tiam1 also controls apoptosis in Neu-expressing tumor cells.

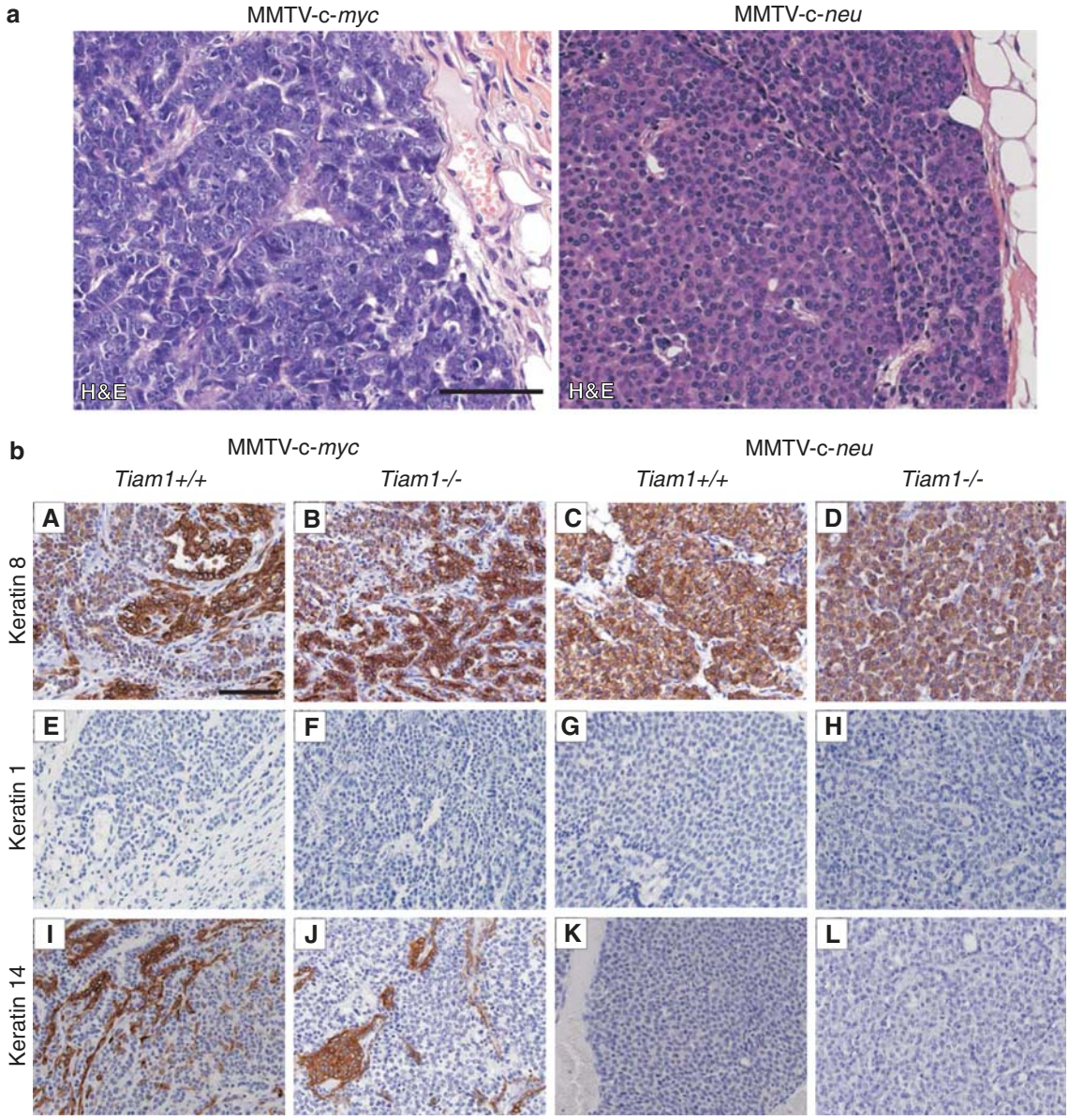

Fig. 4 Tiam1 does not affect differentiation of mammary tumors in MMTV-c-*myc* and MMTV-c-*neu* mice. **a** Sections of mammary tumors from the indicated transgenic mice, stained with hematoxylin and eosin (H&E). The *scale bar* indicates 100 μm. **b** Immunohistochemical stainings of MMTV-c-*myc* and MMTV-c-*neu* tumors in *Tiam*$^{+/+}$ and *Tiam1*$^{-/-}$ mice. All tumors are positive for Keratin 8, which is specific for glandular characteristics. All tumors are negative for the epidermal marker Keratin 1. Keratin 14 staining shows squamous differentiation in MMTV-c-*myc* tumors, while MMTV-c-*neu* tumors are negative for Keratin 14. The *scale bar* indicates 100 μm

Tiam1-deficiency affects metastatic potential of Neu-induced mammary tumors

We also analyzed the MMTV-c-*neu* tumor-bearing mice for the presence of metastases by histopathological analysis. We found metastases mainly in the lungs and occasionally in the heart. This is consistent with earlier observations that MMTV-c-*neu*-induced mammary tumors metastasize to the lung with high frequency (Guy et al. 1992; Siegel et al. 2003; Taverna et al. 2005). Most of the observed lung lesions were intravascular metastases representing tumor cells that remain fully contained within a pulmonary vessel without extravasations as shown by an H&E staining of lung tissue (Fig. 6c). The percentage of mice with lung micrometastases was found to be significantly lower in MMTV-c-*neu*;*Tiam1*$^{-/-}$ mice compared to MMTV-c-*neu*;*Tiam1*$^{+/+}$ mice. We detected lung metastases in 50% of the MMTV-c-*neu*;*Tiam1*$^{+/+}$ mice and only in 18% of the MMTV-c-*neu*;*Tiam1*$^{-/-}$ mice (Fig. 6d). Tiam1 has been shown to be involved in strengthening of E-cadherin-based cell–cell adhesions, which influences metastatic capacities of tumor cells (Malliri et al. 2004). However, E-cadherin

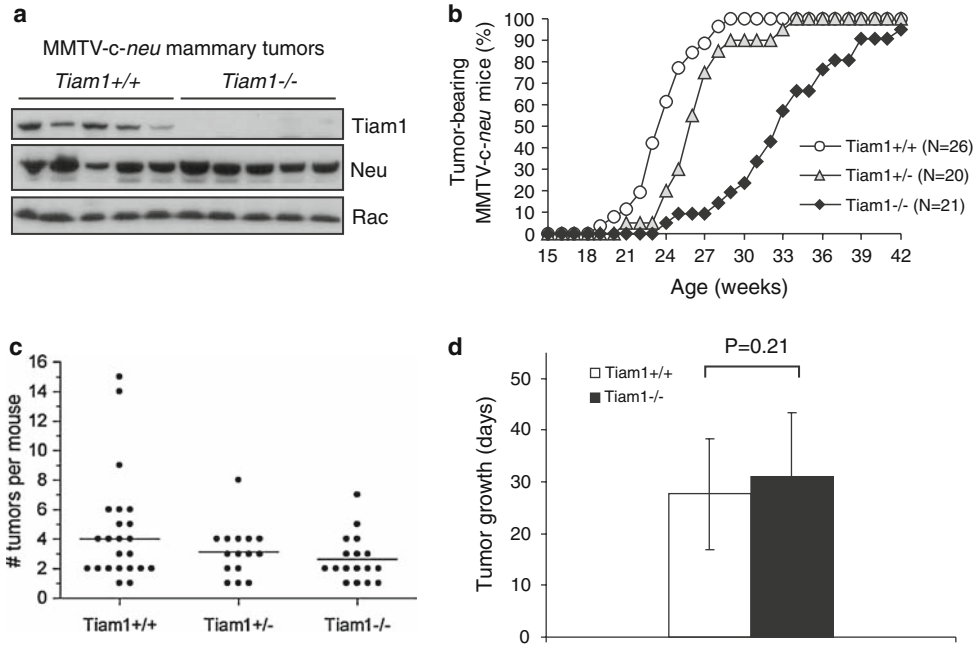

Fig. 5 Tiam1 affects number of *neu*-induced mammary tumors. **a** Western blot analyses. SDS lysates of snap frozen tumors isolated from *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ MMTV-c-*neu* mice were blotted and probed for detection of endogenous Tiam1 and transgenic Neu. The same blot was probed for Rac1 and used as loading control. **b** Kinetics of the tumor susceptibility in MMTV-*neu*;*Tiam1*$^{\pm}$ mice ($N = 20$) is intermediate between MMTV-*neu*;*Tiam1*$^{+/+}$ ($N = 26$) and MMTV-*neu*;*Tiam1*$^{-/-}$ ($N = 21$) mice. The percentage of mice with a palpable mammary tumor is presented in time. *N* is the number of mice analyzed. **c** Plot of the total number of tumors with a size >4 mm per mouse in MMTV-c-*neu*;*Tiam1*$^{-/-}$, MMTV-c-*neu*;*Tiam1*$^{\pm}$ and MMTV-c-*neu*;*Tiam1*$^{+/+}$ mice at the time of necropsy. The median number of tumors per mouse is indicated as a *horizontal line* and is 4 in *Tiam1*$^{+/+}$ mice ($N = 23$; range 1–15 tumors per mouse), 3 in *Tiam1*$^{\pm}$ mice (N = 15; range 1–8 tumors per mouse) and only 2 in *Tiam1*$^{-/-}$ mice ($N = 17$; range 1–7 tumors per mouse). *N* is the number of mice analyzed. **d** Tumor growth is similar in *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ mice. The average time for a tumor to grow out from just palpable to a diameter that reached 10 mm in the *Tiam1*$^{-/-}$ mice (N = 14) compared to *Tiam1*$^{-/-}$ mice (N = 23) is not significantly changed ($P = 0.21$, Student's *t* test). *N* is the number of mice analyzed

staining of MMTV-c-*neu* mammary tumors showed fields of E-cadherin-negative cells within a majority of E-cadherin-positive tumor cells in both *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ mice (Fig. 6e), suggesting that Tiam1-deficiency did not affect the metastatic capacity of Neu-induced mammary tumors by affecting the E-cadherin-mediated cell–cell adhesions. Presumably, the lower incidence of metastases in the *Tiam1*$^{-/-}$ mice is a direct consequence of the lower number of mammary tumors produced per mouse in *Tiam1*$^{-/-}$ mice.

Discussion

We crossed *Tiam1*$^{-/-}$ mice with MMTV-c-*myc* and MMTV-c-*neu* transgenic mice to study the consequences of Tiam1-deficiency in Myc-induced and Neu-induced mammary tumorigenesis. Our analyses revealed that Tiam1 is required for oncogenic signaling induced by Neu but not by Myc. More specifically, we found that Tiam1-deficiency delays the initiation of Neu-induced mammary tumors but does not affect the growth of these tumors. Initiation and growth of Myc-induced mammary tumors was independent of the expression of Tiam1.

Besides the dramatic delay in the onset of the first detectable Neu-induced mammary tumors, also the total number of tumors per mouse was lower in *Tiam1*$^{-/-}$ mice than in *Tiam1*$^{+/+}$ animals. This is consistent with the findings in skin and intestinal tumors, where the latency of tumor onset and the number of tumors per mouse were dramatically decreased in a Tiam1-deficient background (Malliri et al. 2002b, 2006). In the skin tumor model, we found that *Tiam1*$^{-/-}$ mice produced less Ras-induced tumors, because keratinocytes in the basal layer of the epidermis are more susceptible to apoptosis (Malliri et al. 2002b). Recently, we found that the Tiam1/Rac-mediated survival pathway in keratinocytes acts through ROS-mediated activation of the ERK pathway (Rygiel et al. 2008). Also in APC Min mice, the decreased initiation of intestinal tumors by aberrant β-catenin signaling in Tiam1-deficient mice compared to wild type mice was attributed to increased apoptosis susceptibility (Malliri et al. 2006). Consistent with this, Tiam1 expression levels correlate with apoptosis susceptibility in human colon tumor cells (Minard et al. 2006).

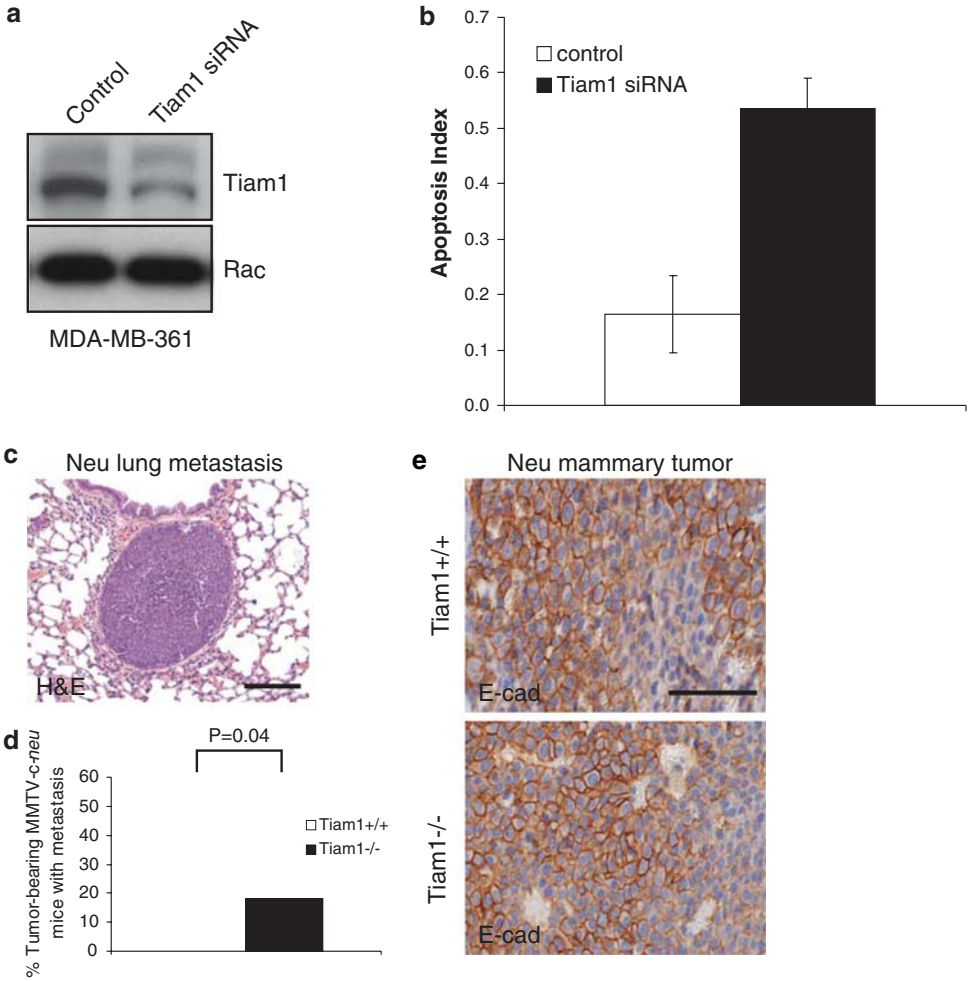

Fig. 6 **a**, **b** Apoptosis downstream of Neu signaling is Tiam1-dependent. **a** Western blot analysis showing Tiam1 downregulation in MDA–MB-361 breast cancer cells 3 days after transfection of Tiam1-specific siRNA. As a control, a nonspecific scrambled siRNA sequence was used. The same blot was probed with a Rac antibody and used as a loading control. **b** Tiam1 downregulation increased apoptosis as determined by the measurement of the amount of cytoplasmic histone-associated DNA fragments. Apoptosis was measured 3 days after siRNA transfection. *Bars* show the average of three independent experiments. **c–e** Metastasis of Neu-induced tumors was lower in $Tiam^{-/-}$ than in $Tiam^{+/+}$ mice. **c** Representative image of an H&E-stained slide showing a typical nonextravasating metastatic embolus of mammary adenocarcinoma cells in the lung of a MMTV-c-*neu* mice. The *scale bar* indicates 100 μm. **d** Mammary tumor-bearing $Tiam1^{+/+}$ mice show pulmonary metastases in 50% of the animals ($N = 22$), while metastatic emboli in the lungs were found for only 18% of the $Tiam^{-/-}$ mice ($N = 17$). N is the number of mice analyzed. **e** E-cadherin expression in mammary tumors in MMTV-c-*neu*;$Tiam1^{+/+}$ and MMTV-c-*neu*;$Tiam1^{-/-}$ mice. The *scale bar* indicates 100 μm

It is likely that Tiam1-deficiency in the MMTV-c-*neu* model affects tumor initiation by increasing the susceptibility to apoptosis of the targeted mammary epithelial cells. We have attempted to study apoptosis sensitivity in vivo, but we could not find significant differences in apoptosis between established Neu-induced tumors produced in $Tiam1^{+/+}$ and $Tiam1^{-/-}$ mice (data not shown). Analysis of apoptosis in tumor samples of MMTV-c-*neu* tumors by TUNEL and Caspase 3 stainings appeared difficult because of the heterogeneity of these tumors. Moreover, apoptosis resistance is most likely essential during the initiating events of tumorigenicity, which is difficult to study in vivo. Once tumors have been formed, differences in apoptosis sensitivity in $Tiam1^{+/+}$ and $Tiam1^{-/-}$ tumors are presumably not detectable anymore, as Tiam1-independent events have rescued the tumor-initiating cells from apoptosis. We performed therefore in vitro studies using MDA-MB-361 breast cancer cells with high Neu expression and found that the survival signaling of these tumor cells is dependent on the presence of Tiam1. Similarly, as found in DMBA-induced skin tumors and β-catenin-induced intestinal tumors, the presence of Tiam1 seems to be required to prevent apoptosis during initiation of mammary tumors by the Neu oncogene. Tiam1-mediated Rac-signaling might prevent apoptosis by activating various well-known survival signaling pathways including the NFkappaB and ERK

pathways (Joneson and Bar-Sagi 1999; Rygiel et al. 2008; Zahir et al. 2003).

Although less mammary tumors were produced in *Tiam1*$^{-/-}$ mice, the growth of the tumors in *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ mice was the same once they were formed. In contrast, we showed in skin and intestinal tumors a function of Tiam1 in both initiation and growth of these tumors (Malliri et al. 2002b, 2006). Interestingly, in DMBA (Ras)-induced skin tumors, decreased growth of tumors in *Tiam1*$^{-/-}$ mice was found only when proliferation of tumors was promoted by TPA treatment. Skin tumors that were generated by treatment with DMBA only grew equally well in the presence or absence of Tiam1 (Malliri et al. 2002b), indicating that TPA-induced but not DMBA-induced proliferation depends on Tiam1-mediated Rac activation. TPA is able to induce cyclin D1 expression, a regulator of cell proliferation (Yan and Wenner 2001), suggesting that Tiam1 is required for TPA-induced proliferation by influencing cyclin D1 levels. Moreover, both the Ras and Neu oncogenes are absolutely dependent on cyclin D1 expression for mammary tumor formation in MMTV-*ras* and MMTV-c-*neu* transgenic mice (Yu et al. 2001). Cyclin D1-deficient mice are resistant to Ras-induced and Neu-induced mammary tumors, while they remain fully sensitive to other oncogenic pathways (Yu et al. 2001). However, the fact that the proliferation of Neu-induced tumors is independent of Tiam1 suggests that Tiam1 does not regulate cyclin D1 levels in mammary tumors. Indeed, tumor lysates from MMTV-c-*neu*;*Tiam1*$^{+/+}$ and MMTV-c-*neu*;*Tiam1*$^{-/-}$ mice revealed a large variation in cyclin D1 levels independent of the presence of Tiam1 (data not shown). As Neu predominantly signals through Ras and the growth of DMBA-only treated skin tumors is independent of Tiam1, it is unlikely that Tiam1–Rac signaling contributes to Ras-controlled proliferation of tumors. Interestingly, it has been shown that Neu-mediated protection from apoptosis is dependent on its association with the Par polarity complex, while Neu-mediated proliferation is not (Aranda et al. 2006). Tiam1 associates with the Par polarity complex and is able to activate this complex (Mertens et al. 2006), providing a possible mechanism by which Tiam1 could interfere in initiation but not growth of Neu-induced mammary tumors.

A higher number of metastases was found in the MMTV-c-*neu*;*Tiam1*$^{+/+}$ mice when compared to MMTV-c-*neu*;*Tiam1*$^{-/-}$ mice, suggesting that Tiam1 promotes metastasis of breast tumors. Studies in human tumors also show a positive correlation between Tiam1 expression and progression and invasiveness of mammary, colon and prostate tumors (Adam et al. 2001; Engers et al. 2006; Liu et al. 2007; Minard et al. 2005, 2006). This is in contrast to the findings in skin and intestinal tumors, where progression was associated with loss of Tiam1 (Malliri et al. 2002b, 2006). The latter could be explained by a function of Tiam1 in the formation and maintenance of intercellular adhesions (Engers et al. 2001; Hordijk et al. 1997; Mertens et al. 2005; Uhlenbrock et al. 2004). In the MMTV-c-*neu* mice, the metastases appear in pulmonary blood vessels as tight tumor emboli that are thought to arise from circulating cell clumps that get stocked in the veins of the lungs. As in human inflammatory breast cancers (IBC), such circulating tumor emboli might benefit from strong E-cadherin-mediated cell–cell interactions favoring passive dissemination in distinct organs (Kleer et al. 2001; Tomlinson et al. 2001). However, we could not find significant differences in E-cadherin expression between Neu-induced tumors in *Tiam1*$^{+/+}$ and *Tiam1*$^{-/-}$ mice that could support such a mechanism. Alternatively, the higher number of metastases found in the MMTV-c-*neu*;*Tiam1*$^{+/+}$ mice could be the result of the increased number of tumors found in these mice.

In conclusion, the effects of Tiam1-mediated Rac signaling on tumorigenesis appear oncogene-dependent and tumor cell type-dependent and either positively or negatively correlate with tumor progression. As Tiam1-mediated Rac activation controls different signaling pathways that may influence initiation, growth and progression of tumors, the cellular outcome of altered Tiam1 expression may depend on a balance between factors that promote or inhibit the formation and progression of tumors.

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Conflict of interest statement There is no conflict of interest regarding this manuscript.

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