JOURNAL OF MOLECULAR SIGNALING

RESEARCH ARTICLE   **Open Access**

Transformation by a nucleotide-activated P2Y receptor is mediated by activation of $G\alpha_i$, $G\alpha_q$ and Rho-dependent signaling pathways

Anurag Singh[1,2], José L Boyer[2], Channing J Der[1,2], Irene E Zohn[1,2,3,4*]

Abstract

Background: Nucleotide-actived P2Y receptors play critical roles in the growth of tumor cells by regulating cellular proliferation, differentiation and survival.

Results: Here we demonstrate that an avian P2Y purinoceptor (tP2YR) with unique pharmacological and signal transduction properties induces morphologic and growth transformation of rodent fibroblasts. tP2YR induced a transformed phenotype similar to the *mas* oncogene, a G protein-coupled receptor which causes transformation by activation of Rac-dependent pathways. tP2YR-transformed cells exhibited increased steady-state activation of Rac1 and RhoA. Like activated Rho GTPases, tP2YR cooperated with activated Raf and caused synergistic transformation of NIH3T3 cells. Our data indicate that the ability of tP2YR to cause transformation is due to its unique ability among purinergic receptors to simultaneously activate $G\alpha_q$ and $G\alpha_i$. Co-expression of constitutively activated mutants of these two $G\alpha$ subunits caused the same transformed phenotype as tP2YR and Mas. Furthermore, transformation by both tP2YR and Mas was blocked by pharmacological inhibition of $G\alpha_I$ by pertussis toxin (PTX) indicating an essential role for $G\alpha_i$ in transformation by these G-protein coupled receptors.

Conclusions: Our data suggest that coordinated activation of $G\alpha_q$ and $G\alpha_i$ may link the tP2YR and possibility the Mas oncogene with signaling pathways resulting in activation of Rho family proteins to promote cellular transformation.

Background

Extracellular nucleotides such as adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), uridine 5'-diphosphate (UDP) and uridine 5'-triphosphate (UTP) interact with purinergic receptors to modulate a broad spectrum of physiological responses [1]. Purinoceptors can be divided based on their pharmacological profiles into two major types: P1- and P2-purinoceptors [2]. Additionally, P2 receptors can be further subdivided into P2X-purinergic receptors which form ligand gated ion channels and P2Y-purinergic receptors which couple to heterotrimeric G-proteins. Most commonly, P2Y receptors activate phospholipase C (PLC) but some also inhibit or stimulate adenylyl cyclase or modulate ion channel activity [3].

While the majority of studies indicate that activation of purinergic receptors inhibits tumor growth, some recent data suggest cell context-dependent differences where in some instances purinergic receptors may contribute to tumorigenesis. For example, multiple P2 receptor subtypes have been identified in a variety of transformed cell lines and human tumors and activation of these receptors regulates apoptosis, proliferation and differentiation [4]. Consequently, depending on the combination of purinoceptors expressed in a tumor cell line and the second messenger pathways stimulated upon activation, addition or release of ATP could potentially promote or inhibit tumor growth [4-6]. For instance, expression of $P2Y_1$ receptors can inhibit and $P2Y_2$ receptor stimulate proliferation of a melanoma cell line in response to ATP [7]. In addition to its growth promoting activity in melanomas, stimulation of $P2Y_2$ receptors can stimulate cell proliferation in lung, breast, ovarian and endometrial cell lines [8-11]. In addition to

* Correspondence: izohn@cnmcresearch.org
[1]Linebergher Comprehensive Cancer Center, University of North Carolina at Chapel Hill, NC 27599, USA

a role in the promotion of cellular proliferation, a mutated $P2Y_2$ receptor has been isolated in an expression screen to identify potential transforming genes expressed in a colorectal cancer cell line [12]. The oncogenic potential of this mutated $P2Y_2$ receptor was confirmed in focus formation, soft agar and tumorigenicity assays in nude mice.

P2Y-purinoceptors can regulate a complex network of signaling pathways that may contribute to transformation. P2Y receptors can couple to $G\alpha_q$ or $G\alpha_i$ stimulating PLC either by activation of $G\alpha_q$ or release of $G\beta\gamma$ following $G\alpha_i$ activation [13]. Furthermore, increases in the activity of multiple kinases, which can promote proliferation have been reported following stimulation of P2Y receptors. These include Pyk2, protein kinase C (PKC), and the ERK, p38 and JNK mitogen-activated protein kinase cascades [13]. In addition, an RGD domain in $P2Y_2$ links this receptor to activation of $G_{12/13}$ and G_o stimulating RhoGEF/Rho and RacGEF/Rac1, respectively [14,15]. While numerous second messenger pathways can be activated by P2Y receptors, the pathways that contribute to the promotion of a transformed phenotype remain unexplored.

Other G-protein coupled receptors (GPCR) oncogenes cause tumorigenic transformation of NIH3T3 cells by activating Rho family proteins [16]. Rho proteins are members of the Ras superfamily of small GTPases that function as GDP/GTP-regulated molecular switches to modulate a variety of cellular processes including actin cytoskeletal organization, gene transcription and cell cycle progression [17,18]. Specific Rho family proteins regulate the reorganization of distinct actin cytoskeletal structures. Cdc42 stimulates the formation of filopodia, Rac1 formation of lamellipodia and membrane ruffling and RhoA formation of actin stress fibers and focal adhesions [19]. Rho family proteins themselves can cause tumorigenic transformation of rodent fibroblasts and mediate transformation by a number of oncogenes including Ras and Dbl [20]. A number of studies indicate that transformation by GPCRs is also dependent on Rho family proteins [16]. For example, Mas transformation is dependent on Rac1 function [21], whereas, G2A, Par-1, and M1 acetylcholine receptor transformation is mediated in part by activation of RhoA or a RhoA-related protein [22-24].
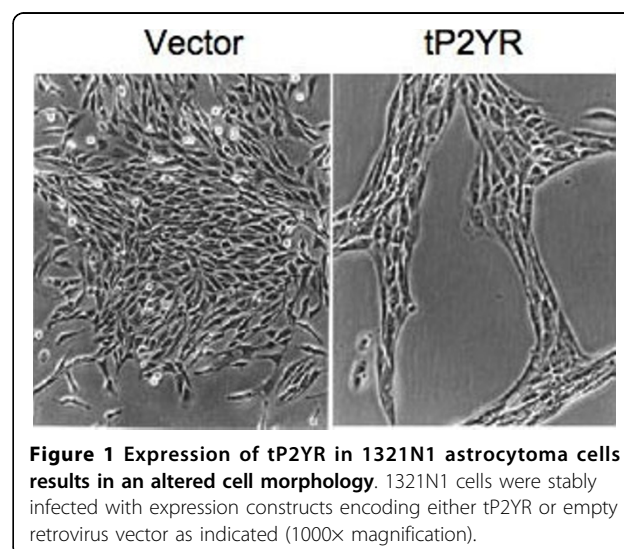
We cloned an avian P2Y purinergic receptor (tP2YR) which shows strongest amino acid sequence identity with the mammalian UTP-selective $P2Y_4$ receptor [25]. tP2YR has a unique pharmacological profile [26]. It is preferentially activated by nucleoside triphosphates and exhibits low affinity for nucleoside diphosphates. However, it displays no selectivity for the nucleotide base moiety being activated by UTP, UTP, GTP, CTP, ITP and XTP. Furthermore, in addition to stimulating PLC

through the $G\alpha_{q/11}$ class of heterotrimeric G-proteins, activation of tP2YR also results in pertussis toxin-sensitive inhibition of adenylyl cyclase indicating that this receptor also has the unique ability among purinoceptors to simultaneously couple to $G\alpha_q$ and $G\alpha_i$ [25,26]. During the course of our characterization of tP2YR, we observed that this receptor induced striking morphologic transformation of 1321N1 astrocytoma cells prompting us to determine whether the tP2YR could cause morphologic and growth transformation of NIH3T3 cells. We found that tP2YR caused activation of Rho family proteins and requires the simultaneous activation of both $G\alpha_q$ and $G\alpha_I$ for transformation. We propose that this purinergic receptor stimulates cell growth and transformation through the coordinated activation of both $G\alpha_q$ and $G\alpha_i$-dependent signaling pathways resulting in activation of Rho family proteins.

Results

Expression of the tP2YR induces altered cell morphology in 132N1 astrocytoma cells

To study the pharmacological selectivity of tP2YR, we generated a number of clonal 1321N1 human astrocytoma cell lines stably infected with a retrovirus expression vector encoding the tP2YR. Surprisingly, a number of these cell lines exhibited an unusual morphologic and growth alteration not observed with the expression of other purinergic receptors in 1321N1 cells (data not shown). While vector-infected cells were unchanged in appearance from control untransfected 1321N1 cells and formed confluent monolayers of cells, the tP2YR-expressing cells displayed an elongated morphology and showed string-like patterns and cell clusters (Figure 1). Furthermore, the analyses of multiple, independent clonal cell lines indicated that this altered morphology

Figure 1 Expression of tP2YR in 1321N1 astrocytoma cells results in an altered cell morphology. 1321N1 cells were stably infected with expression constructs encoding either tP2YR or empty retrovirus vector as indicated (1000× magnification).

correlated with elevated basal phospholipase C activity (data not shown). This ability to cause morphological transformation of the 1321N1 astrocytoma cell line was unique among the numerous receptors that we have evaluated previously, including the mammalian $P2Y_2$ receptor (Boyer and Harden, unpublished observation).

Expression of tP2YR causes transformation of NIH3T3 cells

The altered morphology of tP2YR-expressing 1321N1 cells suggested that this GPCR may exhibit transforming properties. To investigate this hypothesis further, we determined if the tP2YR could also cause transformation NIH3T3 cells, a nontransformed immortalized mouse fibroblast cell line that we and others have shown to be sensitive to morphologic and growth transformation by Rho GTPase activation [27]. This experimental paradigm has provided a sensitive assay to detect the transforming activity of other GPCR oncoproteins and has been instrumental in dissecting the signal transduction pathways responsible for their transforming activity [21-24]. Using a focus formation assay, we found that transfection of pZIP-tP2YR in to NIH3T3 cells caused the appearance of foci of transformed cells (Figure 2A). Interestingly, the appearance of these foci was distinct from the foci caused by activated Ras(61L), which are comprised of highly refractile, elongated cells and instead were similar to those caused by activated RhoA (63L), which are comprised of densely-packed, nonrefratile cells. Furthermore, tP2YR-induced foci were similar in appearance to those induced by two other transforming GPCRs that cause transformation by activation of either Rac1-dependent (Mas) or RhoA-dependent (G2A) signaling pathways [21,22].

To determine if overexpression of the tP2YR reduced the requirement of NIH3T3 cells for serum growth factors, tP2YR-expressing cells were plated at low density in growth medium supplemented with 10% calf serum. After 24 hours, the cultures were switched to growth media supplemented with 10%, 2% or 0.5% calf serum. Untransformed NIH3T3 cells require growth medium supplemented with 10% calf serum for efficient growth in culture. As expected, cells transformed by activated Ras continue to proliferate in 2% or 0.5% serum, whereas vector-transfected control cells exhibited very limited proliferation in growth medium supplemented with 2% or 0.5% serum (Figure 2B). Similar to activated Ras-expressing cells, tP2YR-expressing cells formed dense colonies even in 0.5% serum, indicating that expression of the tP2YR can reduce the growth requirement of NIH3T3 cells for serum growth factors.

To further characterize the ability of tP2YR to cause growth transformation of NIH3T3 cells, we assessed the ability of tP2YR-expressing cells to exhibit anchorage-

and serum-independent growth. For these experiments, we isolated NIH3T3 cells stably transfected with expression constructs encoding either H-Ras(61L), RhoA(63L), Mas, tP2YR, or the pZIP-NeoSV(x)1 empty vector control. Following selection in growth medium supplemented with G418, multiple antibiotic-resistant colonies (>100) were pooled together to establish stable cell lines to be used for these analyses. To assay the ability of the tP2YR expressing cells to exhibit anchorage-independent growth, each pooled cell line was suspended in soft agar. As described previously, cells expressing activated Ras or RhoA formed colonies in soft agar [28] and Figure 2C). The tP2YR-expressing cells also readily formed colonies in soft agar indicating that expression of tP2YR can cause anchorage-independent growth. Interestingly, tP2YR-expressing colonies were similar in size and morphology to those formed by Mas-transformed cells. For example, these colonies did not spread laterally in the agar as mutant Ras-expressing colonies, but instead formed clusters of cells that protruded out of the plane of the agar.

Similar to activators of Rho family proteins, tP2YR cooperates with activated Raf1 in focus formation assays

The morphological appearance of the tP2YR-induced foci suggested that tP2YR might cause transformation by activation of Rho family proteins. One well-characterized property of transformation by Rho proteins and their activators is their ability to cooperate with activated Raf-1 to cause synergistic transformation of NIH3T3 cells [28-32]. To determine if tP2YR could also cooperate with activated Raf-1, plasmids encoding activated Raf-1(340D) were co-transfected at concentrations that resulted in very low, or no, focus-formation along with either tP2YR, activated Rac1(115I) as a positive control, or the empty vector negative control. As we observed previously, co-expression of activated Rac1 (115I) with Raf-1(340D) caused a 100-fold enhancement of focus-forming activity over that seen with expression of Rac1(115I) or Raf alone [28]. Similarly, as shown in Figure 3A, co-transfection of tP2YR with activated Raf caused a 15- to 20-fold synergistic enhancement of focus-forming activity. These results indicate that like activators of Rho proteins, tP2YR can cooperate in a focus formation assay with activated Raf-1.

In addition to increasing the number of tP2YR-induced foci, co-expression with activated Raf altered the morphology of the transformed foci (Figure 3B). As described previously, co-expression of activated Raf-1 with either RhoA or G2A causes the appearance of transformed foci that are similar to those caused by activated Ras and contain elongated, highly refractile cells [21]. In contrast, co-transfection of activated Rac1 or Mas with activated Raf-1 caused the appearance of foci
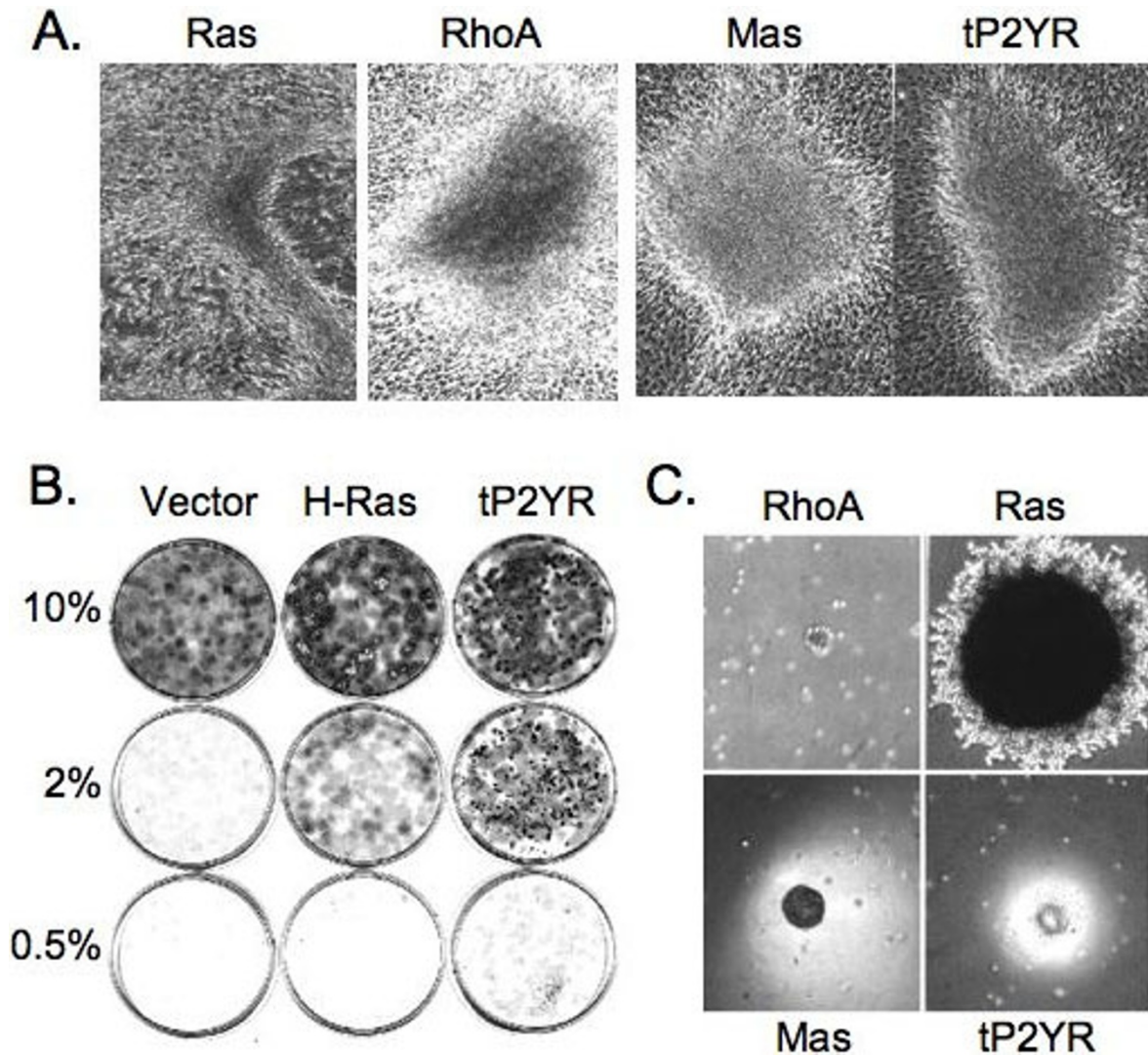
Figure 2 Expression of tP2YR causes tumorigenic transformation of NIH3T3 cells. **A**. Expression of tP2YR in NIH3T3 cells induces the formation of transformed foci. NIH3T3 cells were transfected with empty vector, H-Ras(61L) or RhoA(63L), the Mas Oncogene or tP2YR. The appearance of foci of transformed cells was evaluated 14 to 16 days after transfection (40× magnification). **B**. tP2YR-expressing NIH3T3 cells exhibit serum independent growth. NIH3T3 cells stably-transfected with the empty vector or encoding activated H-Ras(61L) or tP2YR were plated at a density of 10^3 cells per 60 mm dish in growth medium supplemented with 10% calf serum. After 24 hr, the growth medium was changed to media supplemented with 0.5%, 2% or 10% calf serum. The cultures were then maintained for 2 weeks and stained with 4% crystal violet to better visualize the cells. **C**. tP2YR-expressing NIH3T3 cells show anchorage-independent growth. NIH3T3 cells stably-transfected with pZIP-NeoSV (x)1 retrovirus vectors encoding the indicated proteins were plated in soft agar at a density of 10^3 [for Ras(61L)] or 10^4 [for RhoA(63L), Mas and tP2YR)] cells per 60 mm dish. The appearance of colonies of proliferating cells was evaluated after 3 weeks (40× magnification).

that were distinct, and contained rounded cells with a non-refractile appearance [21]. The transformed foci caused by co-expression of Raf-1 and tP2YR were indistinguishable in appearance from those caused by expression of activated Raf-1 and either activated Rac1 or Mas, suggesting that tP2YR may activate Rac1.

tP2YR causes activation of both Rac1 and RhoA
The appearance of tP2YR foci suggests that it causes transformation by activation of Rac1 or a related protein. To address this possibility, we assessed the ability of tP2YR to activate Rac1 and RhoA in pull-down assays. These assays take advantage of the increased affinity of activated Rac1 or RhoA for the isolated Rac/Rho binding domains (RBDs) of Pak and Rhotekin, effectors of Rac and RhoA, respectively. For these experiments, NIH3T3 cells were transfected with either pcDNA3 or tP2YR along with either Tiam1-C1199 (positive control for Rac1 activation) or Ect2-DH/PH/C (positive control for RhoA activation). Cell lysates were incubated with
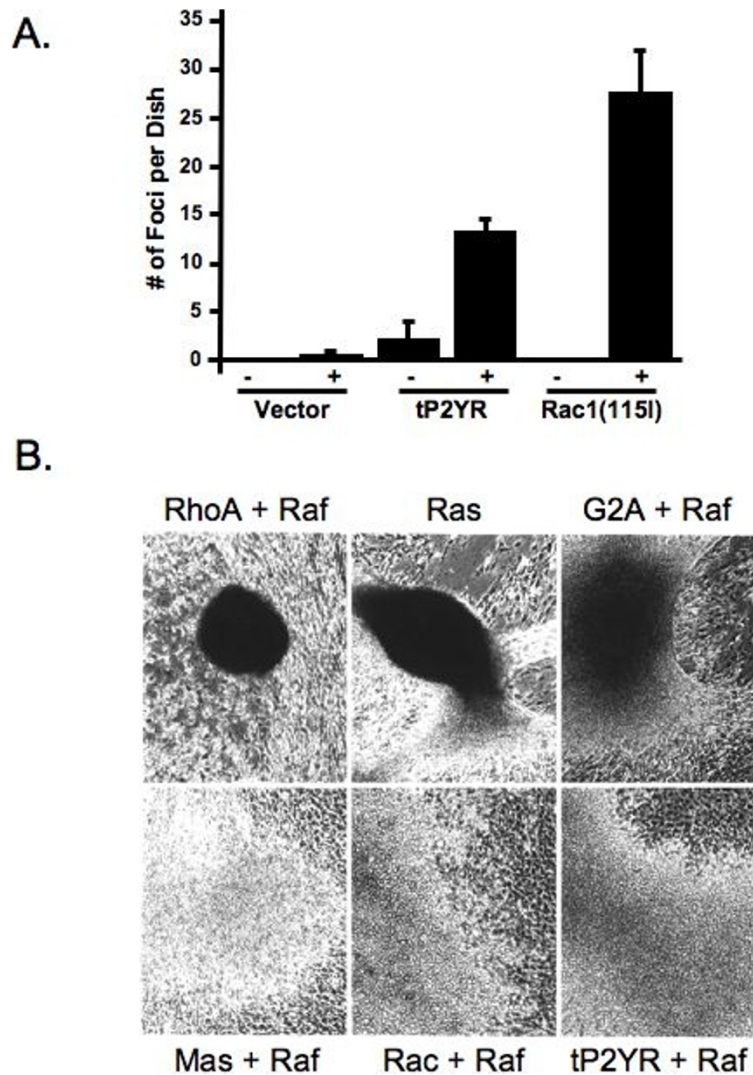
Figure 3 tP2yR synergizes with activated Raf1 to induce foci with similar characteristics to activated Rac1. A. Quantitation of the number of foci induced when tP2YR or activated Rac1(115I) were co-transfected with activated Raf-1(340D). NIH3T3 cells were co-transfected with 1 μg of pZIP-NeoSV(x)1 retrovirus expression plasmids encoding tP2YR or Rac1(115I) and either 1 μg of the empty pZIP-NeoSV(x)1 plasmid DNA (-) or encoding the weakly activated Raf-1(340D) mutant (+). The appearance of foci of transformed cells was quantitated after 14 days. Data shown are the mean ± standard error for triplicate plates and are representative of three independent assays. **B**. The appearance of transformed foci induced in NIH3T3 cells were co-transfected with activated Raf-1(340D) is similar to those induced by activated Rac(115I) and distinct from those induced by activated Rho(63L). pZIP-NeoSV(x)1 expression vectors encoding RhoA(63L), G2A, Mas, Rac1(115I) or tP2YR were co-transfected with pZIP-raf-1(340D). pZIP-H-ras(61L) was co-transfected with the empty pZIP-NeoSV(x)1 plasmid DNA. The appearance of foci of transformed cells was monitored 14 days after transfection (40× magnification).

GST-Pak-RBD to assess Rac1 activation or GST-Rhotekin-RBD to assess RhoA activation. As shown in Figure 4B and 4C, expression of tP2YR increased activation of both Rac1 and RhoA. These data indicate that tP2YR can cause activation of Rho family proteins.

The tP2YR causes transformation by coordinated activation of $G\alpha_i$ and $G\alpha_q$

We next investigated which $G\alpha$ subunit(s) may link tP2YR to Rho protein activation. P2Y-purinergic receptors can couple to either $G\alpha_q$, activating PLC or to $G\alpha_i$ inhibiting adenylyl cyclase activity. Typically purinergic receptors do not couple to both of these G-proteins. However, our previous studies indicated that this tP2YR has the unique ability to robustly activate both $G\alpha_q$ and $G\alpha_i$ [25,26]. Therefore, we sought to determine if activation of either or both of these $G\alpha$ subunits may contribute to tP2YR transforming activity by examining the ability of constitutively activated mutants of $G\alpha_i$ and $G\alpha_q$ either, alone, or in combination, to cause
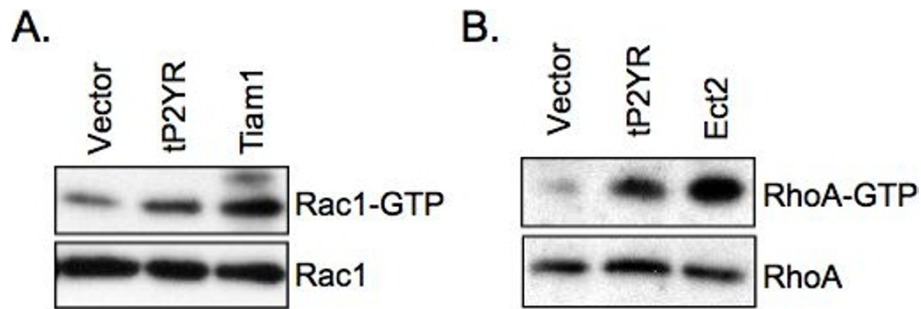
Figure 4 Expression of tP2YR results in lamellipodia formation and activation of both Rac1 and RhoA. NIH3T3 cells were transiently transfected with either empty pcDNA3 expression vector or those encoding tP2YR, Tiam1-C1199 (**A**, potent activator of Rac1) or Ect2-DH/PH/C (**B**, potent activator of RhoA). Activated GTP-bound Rac1 and RhoA were isolated in pull-down assays with GST-Pak-PBD and GST-Rhotekin-RBD, respectively.

focus-formation in NIH3T3 cells. When transfected alone, neither activated $G\alpha_i$, nor activated $G\alpha_q$ caused the appearance of transformed foci in our NIH3T3 cells (data not shown). However, co-expression of activated $G\alpha_i$ and $G\alpha_q$ caused the appearance of transformed foci that were identical in appearance to those induced by the tP2YR or Mas (Figure 5A). These results suggested that the coordinate activation of both $G\alpha_i$ and $G\alpha_q$ by the tP2YR maybe responsible for its transforming activity.

To examine the requirement for $G\alpha_i$ activation for focus formation by these GPCR oncogenes, NIH3T3 cells were transfected with expression constructs encoding either Mas, tP2YR, G2A, or Ras(61L) and the transfected cultures were maintained in the presence or absence of PTX to inactivate $G\alpha_i$. Treatment of cells with PTX caused a 90% inhibition of Mas- and tP2YR-induced focus-formation (Figure 5B). In contrast, PTX treatment did not cause any reduction in oncogenic Ras-induced focus formation, indicating that the reduction seen with tP2YR was not due to nonspecific inhibition by PTX. Interestingly, PTX also caused a 60% reduction in G2A-induced focus-formation. We previously showed that G2A causes transformation by activation of $G\alpha13$, which in turn causes activation of RhoA by stimulation of the Lsc/p115 RhoGEF [22,23]. These data suggest that GPCR oncogenes such as Mas, tP2YR and to a lesser extent G2A rely on $G\alpha_i$ activity for induction of a transformed phenotype.

Discussion

Purinergic receptors regulate a number of physiological functions and recent data indicate that purinergic signaling may also play an important role in both the promotion and inhibition of tumor growth [4,5]. Here we present evidence that an avian P2Y purinoceptor (tP2YR) with unique pharmacological and signal transduction properties induces morphologic and growth

transformation. tP2YR expressing NIH3T3 cells exhibited loss of density-dependent inhibition of growth, proliferated in the absence of attachment to the substratum, and displayed a reduced requirement for serum growth factors. Furthermore, our analyses suggest that the tP2YR causes transformation by activation of Rho proteins. The foci caused by tP2YR expression are identical in appearance to those caused by another transforming GPCR, Mas that causes transformation by activation of Rac-dependent signaling pathways [21]. Furthermore, tP2YR causes activation of both Rac1 and RhoA in an *in vitro* biochemical assay. Finally, we present data suggesting that tP2YR, as well as Mas, cause transformation by coordinate activation of $G\alpha_i$ and $G\alpha_q$.

tP2YR causes transformation by activation of Rho proteins

While our *in vitro* Rac1 and RhoA activation assays indicate that tP2YR can activate both Rho family proteins, based on the appearance of transformed foci we favor the interpretation that activation of Rac1 (or a related protein) maybe more important for the transformed phenotype induced by tP2YR. For example, the appearance of tP2YR-induced foci was identical to those induced by expression of the Mas oncogene, which causes transformation by activation of Rac1 rather than RhoA [21]. In contrast, G-proteins and GPCRs such as $G\alpha_{12/13}$ and G2A cause the appearance of Rho-like foci and require RhoA protein function for transformation [22-24,33,34]. Additionally, tP2YR cooperated with activated Raf in a focus formation assay causing the appearance of foci that resemble those by activated Raf plus Rac1 or Rac1 activators such as Mas [21]. This is in contrast to the appearance of foci induced by activated Raf plus RhoA or RhoA activators such as G2A [22]. These data suggest that while tP2YR is capable of activating both Rac1 and RhoA in an *in vitro* biochemistry assay; the morphologically predominant phenotype
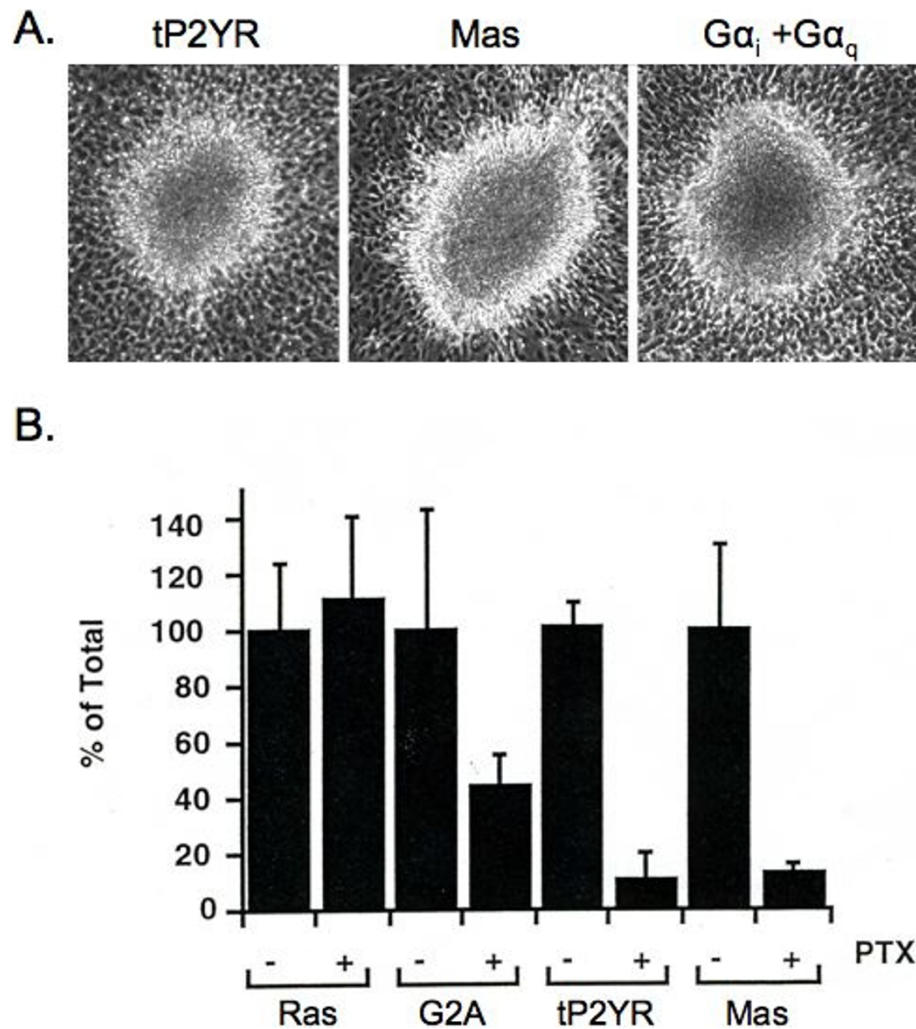
Figure 5 Transformation of NIH3T3 cells by tP2YR is caused by coordinated activation of Gα_i and Gα_q. A. Gα_i and Gα_q cooperate and cause the appearance of transformed foci similar to those caused by tP2YR. NIH3T3 cells were transfected with pZIP-NeoSV(x)1 expression vectors encoding tP2YR or Mas, or co-transfected with vectors encoding constitutively activated mutants of Gα_{I2}(QL) and Gα_q(QL). The appearance of foci of transformed cells was monitored 3 weeks after transfection (40× magnification). **B.** Transformation by tP2YR and Mas requires Gα_i protein function. NIH3T3 cells were transfected with pZIP-NeoSV(X)1 expression vectors encoding H-Ras(61L) (25 ng per 60 mm dish), G2A (2 μg per dish), tP2YR (2 μg per dish) or Mas (1 μg per dish). Cultures were then maintained in growth medium (-) or medium supplemented with 100 ng per ml PTX (+) for 14 to 16 days after which the number of foci were quantitated. Data shown are expressed as the mean of the percent of the total number of foci in the untreated dishes ± standard error and are representative of two separate assays performed in triplicate.

induced by expression of tP2YR suggests that Rac1 activation maybe more important for the transforming activity of this oncogene.

tP2YR causes transformation by the coordinated activation of both Gα_i and Gα_q

Treatment of cells with ATP can influence cell growth and proliferation in opposing ways depending on the cell type and the combination of purinoceptors expressed. In rare instances, stimulation of P2Y receptors can promote cellular proliferation and oncogenic transformation [5,6,12,13]. Since the ability of tP2YR to cause tumorigenic transformation is unique among the receptors that we have characterized, the possibility remains that this avian receptor is constitutively activated. One characteristic of the tP2YR that sets it apart from other purinoceptors is its ability to simultaneously activate both Gα_i and Gα_q [25,26]. This observation prompted us to test the hypothesis that transformation by tP2YR maybe due to the coordinated activation of both Gα_i and Gα_q. We found that while neither Gα_i nor Gα_q when expressed alone in NIH3T3 cells induced

focus formation, when co-expressed, these proteins induced the appearance of transformed foci. Furthermore, the morphology of $G\alpha_i$ and $G\alpha_q$ induced foci was identical to those induced by expression of either tP2YR or Mas indicating that similar pathways may be activated to promote transformation. While previous studies have demonstrated only a mild focus formation activity of $G\alpha_q$ in NIH3T3 cells [35], others indicate that $G\alpha_q$ expression induces apoptotic cell death [36,37], we have been able to establish stable cell lines that express $G\alpha_q$ using a relatively weak cytomegalovirus promoter (pcDNA3). Furthermore, focus formation was not observed in these stable cell lines indicating that $G\alpha_q$ expression alone is not transforming in the NIH3T3 strain used in these studies. Nor was $G\alpha_q$ transforming when co-expressed with activated Raf (data not shown). Finally, focus formation by both Mas and tP2YR requires $G\alpha_i$ activity supporting the hypothesis that it is the coordinated activation of these two $G\alpha$ subunits, which leads to growth transformation by these GPCR oncoproteins.

How coordinated activation of $G\alpha_i$ and $G\alpha_q$ by tP2YR leads to activation of Rho proteins remains unknown. The mammalian P2Y$_2$ receptor can activate both RhoA and Rac1 through interaction with integrins mediated by an RGD sequence motif located in the first extracellular loop of the receptor [13]. The interaction of the P2Y$_2$ receptor with integrins is necessary for coupling to $G\alpha_o$ leading to activation of Rac1 and $G\alpha_{12}$ and to activation of RhoA but is not required for activation of $G\alpha_q$ [14]. Since, the tP2YR does not contain an RGD sequence, it is likely that activation of Rho proteins are mediated by this alternative pathway. Previous studies have found that the activation of $G\alpha_i$ and $G\alpha_q$ by expression of multiple P2Y receptors on the same cell can cause a synergistic rise in second messenger production [13]. It is possible that this synergistic activation of second messengers can lead to Rho activation.

Conclusions
Our data suggest that overexpression of the tP2YR causes growth transformation of NIH3T3 cells via a coordinate activation of $G\alpha_i$ and $G\alpha_q$, leading to activation of Rho proteins. These studies provide novel insight into the mechanism by which GPCR signaling can promote tumorigenesis.

Methods
Molecular constructs
The tP2YR mammalian retrovirus expression construct pLXSN-*tP2YR* has been described previously [25]. pZIP-NeoSV(x)1 retrovirus expression vectors encoding wild type Mas, and the constitutively activated and transforming Rac1(61L), Rac1(115I), H-Ras(61L), RhoA(61L),

or Raf-1(340D) mutant proteins and pCDNA3 expression vectors encoding GTPase-deficient, constitutively activated mutants of $G\alpha_q$(QL), $G\alpha_i$(QL), Rac1(12V), and RhoA(12V) have also been described previously [38,39].

Cell culture and transformation assays
1321N1 human astrocytoma cells were grown in Dulbecco's minimum Eagle's medium (DMEM) supplemented with 5% fetal calf serum. Infection of 1321N1 astrocytoma cells was done as described [25]. NIH3T3 mouse fibroblasts were grown in DMEM supplemented with 10% calf serum and transfected as described previously [40]. For focus formation assays, 60 mm dishes were transfected and maintained in growth medium for three weeks. Individual foci were either photographed and/or stained with 0.4% crystal violet before quantitation. To evaluate the ability of pertussis toxin (PTX) treatment to inhibit transformation, focus formation assays were performed as above in growth medium supplemented with 100 ng/ml of PTX (Sigma). Growth medium and PTX were replaced every other day for 14-16 days. For generation of stable cell lines, transfected cultures were maintained in growth medium supplemented with 400 μg/ml G418 (Geneticin; GIBCO-BRL) and following selection, multiple G418-resistant colonies (>100) were pooled. Assays to examine growth in soft agar and low serum have been described previously [41].

Rac1 and RhoA activation assays
Assays to determine cellular Rac1-GTP and RhoA-GTP levels were performed as described previously [42,43]. Briefly, NIH3T3 cells were transiently transfected with either empty pcDNA3 expression vector or those encoding tP2YR, the C-terminal 1199 amino acids of Tiam-1, C1199 [44] or the DH/PH/C domain of Ect2 [45], using Lipofectamine Plus Reagent (Invitrogen Inc.). Cells were grown for 24 h in DMEM supplemented with 0.5% fetal calf serum. Cell lysates were incubated with GTP-dependent binding domains for Pak1 (GST-PAK-RBD) or Rhotekin (GST-Rhotekin-RBD) as we have described previously [42,43].

Author details
[1]Linebergher Comprehensive Cancer Center, University of North Carolina at Chapel Hill, NC 27599, USA. [2]Department of Pharmacology, Chapel Hill, NC 27599, USA. [3]Center for Neuroscience Research, Children's Research Institute, Children's National Medical Center, Washington, DC 20010, USA. [4]Department of Pediatrics, Pharmacology and Physiology, George Washington University School of Medicine, Washington DC 20037, USA.

Authors' contributions
AS, Carried out Rac1 and RhoA activation assays and helped to draft the manuscript. JLB made the initial observation that tP2yR caused a transformed phenotype, guided these studies and helped to draft the manuscript. CJD guided these studies and helped to draft the manuscript. CJD guided these studies and helped to draft the manuscript. IEZ performed all other experiments and drafted the manuscript.

References
1. Harden TK, Boyer JL, Nicholas RA: P2-purinergic receptors: subtype-associated signaling responses and structure. *Annu Rev Pharmacol Toxicol* 1995, **35**:541-579.
2. Burnstock G, Kennedy C: Is there a basis for distinguishing two types of P2-purinoceptor? *Gen Pharmacol* 1985, **16**:433-440.
3. Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA: International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* 2006, **58**:281-341.
4. White N, Burnstock G: P2 receptors and cancer. *Trends Pharmacol Sci* 2006, **27**:211-217.
5. Deli T, Csernoch L: Extracellular ATP and cancer: an overview with special reference to P2 purinergic receptors. *Pathol Oncol Res* 2008, **14**:219-231.
6. Pathak R, Bhatnagar S, Dubey AK: Mechanisms underlying the opposing effects of P2Y receptors on the cell cycle. *J Recept Signal Transduct Res* 2008, **28**:505-529.
7. White N, Ryten M, Clayton E, Butler P, Burnstock G: P2Y purinergic receptors regulate the growth of human melanomas. *Cancer Lett* 2005, **224**:81-91.
8. Popper LD, Batra S: Calcium mobilization and cell proliferation activated by extracellular ATP in human ovarian tumour cells. *Cell Calcium* 1993, **14**:209-218.
9. Katzur AC, Koshimizu T, Tomic M, Schultze-Mosgau A, Ortmann O, Stojilkovic SS: Expression and responsiveness of P2Y2 receptors in human endometrial cancer cell lines. *J Clin Endocrinol Metab* 1999, **84**:4085-4091.
10. Dixon CJ, Bowler WB, Fleetwood P, Ginty AF, Gallagher JA, Carron JA: Extracellular nucleotides stimulate proliferation in MCF-7 breast cancer cells via P2-purinoceptors. *Br J Cancer* 1997, **75**:34-39.
11. Schafer R, Sedehizade F, Welte T, Reiser G: ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. *Am J Physiol Lung Cell Mol Physiol* 2003, **285**:L376-385.
12. Hatanaka H, Takada S, Choi YL, Fujiwara S, Soda M, Enomoto M, Kurashina K, Watanabe H, Yamashita Y, Sugano K, Mano H: Transforming activity of purinergic receptor P2Y, G-protein coupled, 2 revealed by retroviral expression screening. *Biochem Biophys Res Commun* 2007, **356**:723-726.
13. Erb L, Liao Z, Seye CI, Weisman GA: P2 receptors: intracellular signaling. *Pflugers Arch* 2006, **452**:552-562.
14. Bagchi S, Liao Z, Gonzalez FA, Chorna NE, Seye CI, Weisman GA, Erb L: The P2Y2 nucleotide receptor interacts with alphav integrins to activate Go and induce cell migration. *J Biol Chem* 2005, **280**:39050-39057.
15. Liao Z, Seye CI, Weisman GA, Erb L: The P2Y2 nucleotide receptor requires interaction with alpha v integrins to access and activate G12. *J Cell Sci* 2007, **120**:1654-1662.
16. Whitehead IP, Zohn IE, Der CJ: Rho GTPase-dependent transformation by G protein-coupled receptors. *Oncogene* 2001, **20**:1547-1555.
17. Wennerberg K, Der CJ: Rho-family GTPases: it's not only Rac and Rho (and I like it). *J Cell Sci* 2004, **117**:1301-1312.
18. Zohn IM, Campbell SL, Khosravi-Far R, Rossman KL, Der CJ: Rho family proteins and Ras transformation: the RHOad less traveled gets congested. *Oncogene* 1998, **17**:1415-1438.
19. Jaffe AB, Hall A: Rho GTPases: biochemistry and biology. *Annu Rev Cell Dev Biol* 2005, **21**:247-269.
20. Rossman KL, Der CJ, Sondek J: GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol* 2005, **6**:167-180.
21. Zohn IE, Symons M, Chrzanowska-Wodnicka M, Westwick JK, Der CJ: Mas oncogene signaling and transformation require the small GTP-binding protein Rac. *Mol Cell Biol* 1998, **18**:1225-1235.
22. Zohn IE, Klinger M, Karp X, Kirk H, Symons M, Chrzanowska-Wodnicka M, Der CJ, Kay RJ: G2A is an oncogenic G protein-coupled receptor. *Oncogene* 2000, **19**:3866-3877.
23. Martin CB, Mahon GM, Klinger MB, Kay RJ, Symons M, Der CJ, Whitehead IP: The thrombin receptor, PAR-1, causes transformation by activation of Rho-mediated signaling pathways. *Oncogene* 2001, **20**:1953-1963.
24. Fromm C, Coso OA, Montaner S, Xu N, Gutkind JS: The small GTP-binding protein Rho links G protein-coupled receptors and Galpha12 to the serum response element and to cellular transformation. *Proc Natl Acad Sci USA* 1997, **94**:10098-10103.
25. Boyer JL, Waldo GL, Harden TK: Molecular cloning and expression of an avian G protein-coupled P2Y receptor. *Mol Pharmacol* 1997, **52**:928-934.
26. Boyer JL, Delaney SM, Villanueva D, Harden TK: A molecularly identified P2Y receptor simultaneously activates phospholipase C and inhibits adenylyl cyclase and is nonselectively activated by all nucleoside triphosphates. *Mol Pharmacol* 2000, **57**:805-810.
27. Solski PA, Abe K, Der CJ: Analyses of transforming activity of Rho family activators. *Methods Enzymol* 2000, **325**:425-441.
28. Khosravi-Far R, Solski PA, Clark GJ, Kinch MS, Der CJ: Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. *Mol Cell Biol* 1995, **15**:6443-6453.
29. Qiu RG, Abo A, McCormick F, Symons M: Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. *Mol Cell Biol* 1997, **17**:3449-3458.
30. Qiu RG, Chen J, Kirn D, McCormick F, Symons M: An essential role for Rac in Ras transformation. *Nature* 1995, **374**:457-459.
31. Khosravi-Far R, Chrzanowska-Wodnicka M, Solski PA, Eva A, Burridge K, Der CJ: Dbl and Vav mediate transformation via mitogen-activated protein kinase pathways that are distinct from those activated by oncogenic Ras. *Mol Cell Biol* 1994, **14**:6848-6857.
32. Khosravi-Far R, White MA, Westwick JK, Solski PA, Chrzanowska-Wodnicka M, Van Aelst L, Wigler MH, Der CJ: Oncogenic Ras activation of Raf/mitogen-activated protein kinase-independent pathways is sufficient to cause tumorigenic transformation. *Mol Cell Biol* 1996, **16**:3923-3933.
33. Hooley R, Yu CY, Symons M, Barber DL: G alpha 13 stimulates Na+-H+ exchange through distinct Cdc42-dependent and RhoA-dependent pathways. *J Biol Chem* 1996, **271**:6152-6158.
34. Buhl AM, Johnson NL, Dhanasekaran N, Johnson GL: G alpha 12 and G alpha 13 stimulate Rho-dependent stress fiber formation and focal adhesion assembly. *J Biol Chem* 1995, **270**:24631-24634.
35. Gupta SK, Gallego C, Lowndes JM, Pleiman CM, Sable C, Eisfelder BJ, Johnson GL: Analysis of the fibroblast transformation potential of GTPase-deficient gip2 oncogenes. *Mol Cell Biol* 1992, **12**:190-197.
36. Kalinec G, Nazarali AJ, Hermouet S, Xu N, Gutkind JS: Mutated alpha subunit of the Gq protein induces malignant transformation in NIH 3T3 cells. *Mol Cell Biol* 1992, **12**:4687-4693.
37. Althoefer H, Eversole-Cire P, Simon MI: Constitutively active Galphaq and Galpha13 trigger apoptosis through different pathways. *J Biol Chem* 1997, **272**:24380-24386.
38. Westwick JK, Lambert QT, Clark GJ, Symons M, Van Aelst L, Pestell RG, Der CJ: Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. *Mol Cell Biol* 1997, **17**:1324-1335.
39. Woon CW, Soparkar S, Heasley L, Johnson GL: Expression of a G alpha s/G alpha i chimera that constitutively activates cyclic AMP synthesis. *J Biol Chem* 1989, **264**:5687-5693.

40. Clark GJ, Cox AD, Graham SM, Der CJ: **Biological assays for Ras transformation.** *Methods Enzymol* 1995, **255**:395-412.
41. Cox AD, Der CJ: **Biological assays for cellular transformation.** *Methods Enzymol* 1994, **238**:277-294.
42. Karnoub AE, Worthylake DK, Rossman KL, Pruitt WM, Campbell SL, Sondek J, Der CJ: **Molecular basis for Rac1 recognition by guanine nucleotide exchange factors.** *Nat Struct Biol* 2001, **8**:1037-1041.
43. Mitin N, Betts L, Yohe ME, Der CJ, Sondek J, Rossman KL: **Release of autoinhibition of ASEF by APC leads to CDC42 activation and tumor suppression.** *Nat Struct Mol Biol* 2007, **14**:814-823.
44. Lambert JM, Lambert QT, Reuther GW, Malliri A, Siderovski DP, Sondek J, Collard JG, Der CJ: **Tiam1 mediates Ras activation of Rac by a PI(3)K-independent mechanism.** *Nat Cell Biol* 2002, **4**:621-625.
45. Solski PA, Wilder RS, Rossman KL, Sondek J, Cox AD, Campbell SL, Der CJ: **Requirement for C-terminal sequences in regulation of Ect2 guanine nucleotide exchange specificity and transformation.** *J Biol Chem* 2004, **279**:25226-25233.