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CUCURBITACIN I INHIBITS RAC1 ACTIVATION IN BREAST CANCER CELLS BY A ROS-MEDIATED MECHANISM AND INDEPENDENTLY OF JAK2 AND P-REX1

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Running title: Cucurbitacin I inhibits Rac1 in breast cancer cells.

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Abbreviations:

GEF: guanine nucleotide exchange factors

NAC: N-Acetyl L-cysteine

HRG: heregulin β1

DCFDA: 2,7-dichlorofluorescein diacetate

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ABSTRACT

The small GTPase Rac1 has been widely implicated in mammary tumorigenesis and metastasis. Previous studies established that stimulation of ErbB receptors in breast cancer cells activates Rac1 and enhances motility via the Rac-GEF P-Rex1. As the Jak2/Stat3 pathway has been shown to be functionally associated with ErbB receptors, we asked if this pathway could mediate P-Rex1/Rac1 activation in response to ErbB ligands. Here we found that the anticancer agent cucurbitacin I, a Jak2 inhibitor, reduced the activation of Rac1 and motility in response to the ErbB3 ligand heregulin in breast cancer cells. However, Rac1 activation was not affected by Jak2 or Stat3 RNAi, suggesting that the effect of cucurbitacin I occurs through a Jak2-independent mechanism. Cucurbitacin I also failed to affect the activation of P-Rex1 by heregulin. Subsequent analysis revealed that cucurbitacin I strongly activates RhoA and the Rho effector ROCK in breast cancer cells and induces the formation of stress fibers. Interestingly, disruption of the RhoA-ROCK pathway prevented the inhibitory effect of cucurbitacin I on Rac1 activation by heregulin. Lastly, we found that RhoA activation by cucurbitacin I is mediated by reactive oxygen species (ROS). The ROS scavenger N-Acetyl L-cysteine (NAC) and the mitochondrial anti-oxidant Mito-TEMPO rescued the inhibitory effect of cucurbitacin I on Rac1 activation. In conclusion, these results indicate that ErbB-driven Rac1 activation in breast cancer cells proceeds independently of the Jak2 pathway. Moreover, they established that the inhibitory effect of cucurbitacin I on Rac1 activity involves the alteration of the balance between Rho and Rac.

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INTRODUCTION

ErbB receptors represent a family of tyrosine-kinases that has been widely implicated in cancer progression. These receptors comprise four members (ErbB1/EGFR, ErbB2, ErbB3, and ErbB4) that differentially couple to a complex network of signaling adaptors and effectors, and they trigger a range of cellular responses including proliferation, survival, and motility (Citri and Yarden, 2006; Lemmon and Schlessinger). Hyperactivation of ErbB receptor signaling has been extensively associated with the progression of human cancer, including breast, prostate, and lung cancer. Overexpression of ErbB2 receptors is often associated with aggressive tumor behavior and poor prognosis, and hyperactivation of EGFR and ErbB3 are frequent events in breast cancer. Dysregulation of ErbB receptor signaling may be the consequence of enhanced production of ErbB ligands, including EGF, TGFα (EGFR ligands) and heregulins (ErbB3/ErbB4 ligands), or gain-of-function mutations in downstream effectors, such as in PI3Kα (Bublil and Yarden, 2007; Montero et al., 2008; Wilson et al., 2009).

Rho GTPases are well-established effectors of ErbB receptors. These small GTP-binding proteins cycle between active (GTP-bound) and inactive (GDP-bound) states and mediate actin cytoskeleton reorganization and cell motility (Jaffe and Hall, 2005; Rossman et al., 2005; Vega and Ridley, 2008). Rac1, a member of the Rho family, has been widely implicated in breast cancer cell migration and proliferation in response to ErbB ligands (Wang et al., 2006; Wertheimer et al.). A strong activation of Rac is observed upon stimulation with EGF or heregulin β1 (HRG) in breast cancer cells, followed by a characteristic formation of membrane ruffles and lamellipodia and enhanced motile behavior (Yang et al., 2006; Yang et al., 2005). Moreover, Rac1 has been implicated in ErbB2-mediated tumorigenesis in mice (Muraoka et al.,

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2003; Sosa et al.). The activity of Rac1 is primarily regulated by Guanine nucleotide Exchange Factors (Rac-GEFs) responsible for GTP loading, and GTPase-activating proteins (GAPs) that accelerate GTP hydrolysis thus leading to Rac inactivation (Jaffe and Hall, 2005; Rossman et al., 2005; Vega and Ridley, 2008). Whereas overexpression of Rac-GAPs in breast cancer cells essentially abolishes cell motility, ectopic expression of Rac-GEFs confers a motile phenotype (Wertheimer et al.; Yang et al., 2006; Yang et al., 2005). Studies in recent years support a role for Rac-GEFs in the development and progression of breast cancer. Rac GEFs Vav3, Trio, and Tiam1 are overexpressed in human breast tumors, and P-Rex1 and P-Rex2 have been recently implicated in breast cancer progression and metastasis (Barrio-Real and Kazanietz, 2012; Citterio et al., 2012; Fine et al., 2009; Montero et al.; Sosa et al.; Wertheimer et al.). P-Rex1 is highly expressed in human breast tumors of luminal origin, particularly in ErbB2 and ER positive tumors, and mediates Rac1 activation and migration in response to HRG and EGF. P-Rex1/Rac1 activation by the ErbB3 ligand HRG is mediated by transactivation of EGFR and CXCR4 receptors (Sosa et al.).

In this study we explored the potential involvement of the tyrosine-kinase Jak2 in Rac1 activation by ErbB ligands. The rationale behind this study is several-fold. First, several laboratories reported that stimulation of ErbB receptors activates Jak2. For example, EGF phosphorylates and activates Jak2 in ovarian cancer cells (Colomiere et al., 2009) as well as it stimulates the formation of a Jak2-STAT signaling complex that confers a motile response to esophageal keratinocytes (Andl et al., 2004). A physical association between EGFR and Jak2 has been demonstrated (Yang et al., 2008), and Jak2 can phosphorylate EGFR (Yamauchi et al., 1997). Second, Jak2 mediates ErbB receptor responses, as demonstrated by the ability of Jak2 inhibitors to attenuate EGF signaling (Andl et al., 2004; Coaxum et al., 2009; Liu and Kern,

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2002; Lo et al., 2008). Third, the Jak effector STAT3 is activated by EGFR stimulation, either by direct ligand activation or via transactivation mechanisms, and STAT3 activation contributes to proliferative responses. Moreover, Jak/STAT3 constitutive activation coexists with ErbB receptor hyperactivation in cancer (Lo et al., 2008). Lastly, Jak2 has been shown to play critical roles in mammary gland development and the progression of breast cancer (Watson, 2001). In order to address the potential involvement of this pathway in ErbB- mediated activation of Rac1 we took advantage of the Jak2/Stat3 inhibitor cucurbitacin I, a natural product with antitumorigenic and anti-inflammatory properties (Chen et al., 2005; Iwanski et al.; Lee et al.). Here we show that cucurbitacin I inhibits breast cancer cell motility and Rac1 activation by ErbB receptors. However, mechanistic analysis revealed that unexpectedly, the effect of cucurbitacin I is independent of Jak2/Stat3 but rather involves the disruption of the balance between RhoA and Rac1.

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MATERIALS AND METHODS

Materials. Heregulin β1 was purchased from R&D (Minneapolis). EGF was obtained from BD Biosciences (San Jose, CA). Cucurbitacin I and the ROCK inhibitor Y-27632 were purchased from EMD/Calbiochem (Gibbstown, NJ). The toxin C3 transferase was purchased from Cytoskeleton (Denver, CO). The dye 2,7-dichlorofluorescein diacetate (DCFDA) and N-acetyl L-cysteine (NAC) were purchased from Sigma (St. Louis, MO). Mito-TEMPO was obtained from Enzo Life Sciences (Farmingdale, NY).

Cell lines. Human breast cancer cell lines (T-47D, MCF7, BT-474 and HCC1419) were obtained from ATCC and cultured in DMEM medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Western blot analysis. Cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP40, 5 mM β-glycerophosphate, 1 mM DTT, and protease inhibitors, and extracts subjected to SDS-polyacrylamide gel electrophoresis. For Western Blot, the following antibodies were used: anti-Rac1 clone 23A8 (Upstate Biotechnology); anti-RhoA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-EGFR, anti-phospho-EGFR (Tyr845), anti-ErbB2, anti-phospho-ErbB2 (Tyr1248), anti-ErbB3, anti-phospho-ErbB3 (Tyr1289), anti-Akt, anti-phospho-Akt (Ser473), anti-Erk1/2, anti-phospho-Erk1/2 (Thr202/Tyr204), anti-Jak1, anti-Jak2, anti-phospho-MYPT1(Thr853) (Cell Signaling Technology, Beverly, MA); anti-vinculin and anti-P-Rex1 (Sigma). Bands were visualized by Enhanced Chemiluminescence

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(ECL). Images were captured using a FUJIFILM LAS-3000 system and the LAS-2000 software. Densitometric analysis of the bands was carried out using the NIH ImageJ software.

RNAi. For transient depletion of Jak2 we used two different RNAi sequences, ON-TARGET Plus RNAi duplexes from Dharmacon (Catalog # J-003146-13-0005) and Silencer Validated RNAi from Ambion (Catalog # 609). For RhoA depletion, we used siRNA sc-29471 from Santa Cruz Biotechnology. For STAT3 and JAK1 we used ON-TARGETplus SMARTpool from Dharmacon (Catalog # L-003544-00-0005 and L-003145-00-0005, respectively). ON-TARGETplus Non-Targeting pool (Catalog # D-001810-0-05) was used as control. siRNAs were transfected with Lipofectamine RNAi/MAX (Invitrogen). After 24 h cells were serum-starved and used for the indicated experiments 48 h later.

Rac1-GTP and Rho-GTP pull-down assays. Cells growing at low confluence were serum-starved for 48 h and stimulated with either HRG or EGF. Rac1-GTP pull-down assays were carried out essentially as previously described (Wang and Kazanietz, 2002). Briefly, cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP40, 5 mM β-glycerophosphate, 1 mM DTT, protease inhibitors, and 10 μg/ml of GST-PBD (the Rac/Cdc42 binding domain of human p21 activated kinase 1 Pak1). Lysates were cleared by centrifugation (10 min at 4ºC, 13,000 x *g*) and incubated with glutathione-Sepharose 4B beads (GE Healthcare) for 45 min at 4ºC. After centrifugation, the beads were washed twice with the pull-down buffer and run on SDS-PAGE gels. Rac1 was detected by Western blot using an anti-Rac1 antibody (clone 23A8, Upstate Biotechnology, 1:1000 dilution).

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RhoA-GTP levels were determined with a pull-down assay using the rhotekin binding domain, as previously described (Xiao et al., 2009). Briefly, cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, and 10 mM $MgCl_2$. The supernatant was collected after centrifugation (10 min at 4ºC, 13,000 x *g*), and incubated with GST-rhotekin binding domain (20 μg) for 60 min at 4°C. Beads were washed with 50 mM Tris-HCl, pH 7.2, containing 1% Triton X-100, 150 mM NaCl, and 10 mM $MgCl_2$, and run on SDS-PAGE gels. RhoA was detected by Western blot using an anti-RhoA antibody (Santa Cruz Biotechnology, 1:1000 dilution).

Cell migration. Cells were serum-starved for 24 h, harvested with 1 mM EDTA, and suspended in 0.1% BSA/DMEM. Cells (3 $x10^4$ cells/well) were seeded in the upper compartment of a Boyden chamber (NeuroProbe). A 12 μm pore polycarbonate filter (NeuroProbe) coated overnight with type IV collagen in cold PBS was used to separate the upper and lower compartments. In the lower chamber, 0.1% BSA/DMEM with or without HRG (20 ng/ml) was used. After 6 h incubation at 37ºC, non-migrating cells on the upper side of the membrane were wiped off the surface, and migrating cells on the lower side of the membrane were fixed, stained with DIFF Quik Stain Set (Dade Behring), and counted by contrast microscopy in 5 independent fields.

Phalloidin staining. After 48 h of serum starvation, cells cultured in coverslides were pretreated with various inhibitors for 30 min and then stimulated with HRG (20 ng/ml, 5 min). Cells were washed twice with PBS, fixed in 4% formaldehyde in PBS for 10 min, and then permeabilized using 0.1% Triton X-100 in PBS for 30 min. Cells were stained with rhodamine-phalloidin

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(Molecule Probes-Invitrogen) in PBS containing 1% BSA (30 min, room temperature), and then counterstained with 4′,6′-diamidino-2-phenylindole (1 μg/ml, 20 min). Cells were visualized with a Nikon TE2000-U fluorescence microscope.

P-Rex1 immunocytochemistry. Cells in coverslides were serum starved for 48 h, stimulated with HRG (20 ng/ml, 5 min), and fixed with 4% formaldehyde in PBS. For staining we used an anti-P-Rex1 antibody (Sigma, 1:250 dilution, 90 min incubation), followed by a Cy3-conjugated goat anti-rabbit secondary antibody (Jackson Laboratory, 1:1500, 90 min incubation). Slides were mounted using Vectashield and visualized by fluorescence microscopy.

Viability assay. Cells (1×10^5) were seeded in 96-well plates in triplicate. Twenty-four h later cells were serum-starved for 48 h, and then incubated with different concentrations of cucurbitacin I (0.03-3 μM, 6 h). Cells were then washed with PBS, fixed in methanol, and stained with Crystal Violet (3.5 mg/ml in 19% ethanol/1% methanol). After solubilization in 2% SDS, optical density was measured at 570 nm.

Determination of intracellular reactive oxygen species (ROS) production. Production of intracellular ROS was determined with the fluorescent dye DCFDA. Cells were pre-incubated with DCFDA (5 mM) for 30 min and subject to treatment with cucurbitacin I (0.1 μM, 1 h). The stained cells were analyzed with a FACSCalibur flow cytometer, and data processed with the CellQuest software (BD Biosciences).

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RESULTS

Cucurbitacin I inhibits Rac1 activation and cell motility in breast cancer cells

We have previously shown that ErbB receptors signal via Rac1 to promote a motile response in breast cancer cells. Stimulation of ErbB3 receptors with its ligand HRG activates Rac1 through transactivation of EGFR, as pharmacological inhibition or RNAi depletion of EGFR significantly reduces Rac1-dependent motility (Yang et al., 2006). As there is considerable evidence that EGFR-mediated responses involve the Jak2/Stat3 pathway in various cancer cell models (Andl et al., 2004; Coaxum et al., 2009; Colomiere et al., 2009; Liu and Kern, 2002; Lo et al., 2008; Yamauchi et al., 1997; Yang et al., 2008), we decided to examine whether Jak2 was implicated in Rac1 activation by HRG. To address this issue, we used the Jak2 inhibitor cucurbitacin I, which is known to inhibit Jak2/Stat3 with an IC_{50} in the high nanomolar range (Chen et al., 2005; Iwanski et al.; Lee et al.) (Blaskovich et al., 2003). Four different breast cancer cell lines (T-47D, MCF-7, BT-474, and HCC-1419) were treated with HRG, and Rac1-GTP levels determined using a pull-down assay. HRG caused a marked activation of Rac1 in breast cancer cells, as expected from our previous studies (Sosa et al., 2010; Yang et al., 2006). Notably, Rac1 activation was inhibited by cucurbitacin I in a concentration-dependent manner (Fig. 1A, *upper panels*). Determination of IC_{50} values using densitometry revealed that they were all in the high nanomolar range (IC_{50} T-47D = 0.3 μM; IC_{50} MCF-7 = 0.3 μM, IC_{50} BT-474 = 0.77 μM; IC_{50} HCC-1419 = 0.6 μM) (Fig. 1A, *lower panels*). We next examined if cucurbitacin I could also affect the activation of Rac1 by EGF in breast cancer cells. As shown in Fig. 1C, cucurbitacin I also impaired EGF-induced Rac1 activation in MCF-7 and BT-474 cells, with

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IC_{50}'s of 0.2 μM and 0.1 μM, respectively (Fig. 1D). In all cell lines examined, cucurbitacin I did not affect the activation of Erk and Akt by the ErbB ligands.

HRG is a strong inducer of cell motility in breast cancer cells. In a previous study we determined that upon Rac1 RNAi depletion or inhibition by expressing the Rac-GAP β2-chimaerin, HRG-induced migration in breast cancer cells is essentially abolished (Yang et al., 2006; Yang et al., 2005). To determine the effect of cucurbitacin I on breast cancer cell motility we utilized a Boyden chamber. In order to avoid cytotoxic effects that may be observed particularly after long-term incubation with cucurbitacin I we optimized the assay to use a relatively short incubation time (6 h) with this compound. Under this experimental condition, we found that cucurbitacin I inhibited MCF-7 cell migration in a dose-dependent manner with an IC_{50} = 0.03 μM (Fig. 2A and 2B). Under the same experimental conditions the cytotoxic effect of cucurbitacin I became noticeable only at concentrations ≥ 0.3 μM (Fig. 2C). Based on these initial results, we speculated that Jak2 could be implicated in ErbB-receptor induced activation of Rac1 and cell motility.

Inhibition or Rac1 activation and migration by cucurbitacin I is independent of Jak2

Analysis of Jak2 expression in breast cancer cells by Western blot revealed that this tyrosine-kinase was highly expressed in MCF-7 cells, however expression was somehow lower in T-47D and HCC-1419 cells and essentially undetected in BT-474 cells (Fig. 3A). This suggested the possibility that the effect of cucurbitacin I on Rac1 activation may be mediated by a Jak2-independent mechanism. To test this hypothesis, we silenced Jak2 expression using RNAi in those cells in which Jak2 could be detected. A non-target RNAi sequence was used as a negative control. A significant Jak2 depletion (>90%) was observed upon transfection of Jak2

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RNAi duplexes into T-47D, MCF-7 or HCC-1419 breast cancer cells. Activation of Rac1 by HRG was essentially the same in control and Jak2-depleted breast cancer cells (Fig. 3B and 3C). Moreover, when we examined HRG induced MCF-7 cell motility using a Boyden chamber we found that silencing Jak2 had essentially no effect (Fig. 3D and 3E). Likewise, Rac1 activation by HRG was not affected upon depletion of the Jak2 effector STAT3 (Fig. 3F and 3G) or by depletion of Jak1 (Supplemental Figure 1). Taken together, these results indicate that the blockade of Rac1 activation by cucurbitacin I does not involve the inhibition of the Jak2/STAT3 pathway, and therefore is rather mediated through other mechanisms.

Cucurbitacin I does not affect the activation of P-Rex1 in breast cancer cells

In a previous study we identified P-Rex1, a PI3K- and Gβγ-dependent Rac-GEF, as an essential mediator of Rac1 activation by ErbB ligands. P-Rex1 is highly expressed in human breast cancer cells and tumors, primarily in those of luminal origin. Depletion of P-Rex1 markedly impairs Rac1 activation and motility of breast cancer cells in response to ErbB ligands (Montero et al.; Sosa et al.). As P-Rex1 translocates to the plasma membrane in response to HRG and EGF, where it activates Rac1 (Sosa et al.), we examined whether cucurbitacin I could affect this intracellular redistribution. Immunocytochemical analysis in MCF-7 cells revealed that *endogenous* P-Rex1 is mainly localized in the cytoplasm and that it relocalizes to the cell periphery in response to HRG, as we previously reported (Sosa et al.). P-Rex1 peripheral translocation was not affected by cucurbitacin I (Fig. 4A), arguing that P-Rex1 or inputs that activate P-Rex1 are not responsible for the inhibitory effect of this compound on Rac1 activation. Consistent with this result, activation of ErbB2/ErbB3 receptors and transactivation of EGFR by HRG were not affected by cucurbitacin I (Fig. 4B).

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Cucurbitacin I activates RhoA in breast cancer cells

As Rho GTPases play important roles in cytoskeleton reorganization, we next carried out phallodin staining studies in MCF-7 cells. We have previously shown that in breast cancer cells HRG induced the formation of membrane ruffles and lamellipodia via P-Rex1 and Rac1 (Sosa et al.; Yang et al., 2006). We hypothesized that the inhibitory effect of cucurbitacin I on Rac1 activation and motility may be reflected in an impaired formation of ruffles in response to ErbB ligands. To our surprise, we noticed that treatment of serum-starved MCF-7 cells with cucurbitacin I caused a marked reorganization of the cytoskeleton. Indeed this compound induced the formation of stress fibers. When we treated parental MCF-7 cells with HRG, we observed the characteristic formation of ruffles, as expected. However, cucurbitacin I-treated cells retained the stress fibers upon HRG treatment and were unable to form ruffles (Fig. 5A)

As the formation of stress fibers is a hallmark of Rho activation, we speculated that cucurbitacin I activates Rho in breast cancer cells. Using a rhotekin pull-down assay (Xiao et al., 2009), we observed that treatment of MCF-7 cells with cucurbitacin I led to a marked elevation in RhoA-GTP levels. Moreover, cucurbitacin I caused a strong activation of the RhoA effector ROCK (Fig. 5B and Fig. 5C), as determined by increased phosphorylation of the ROCK substrate MYPT1 using a specific phospho-antibody against Thr850-MYPT1 (Xiao et al., 2009). These results indicate that cucurbitacin I activates RhoA and Rho-mediated responses.

Activation of RhoA is required for the inhibition of Rac1 by cucurbitacin I

There is extensive evidence for a tightly regulated balance between Rac and Rho signaling. Studies have shown a functional antagonism where activation of one of these two small G-proteins inhibits actin organization governed by the other (Nimnual et al., 2003;

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Wildenberg et al., 2006). We speculated that RhoA activation by cucurbitacin I may alter Rac responses. To address this issue, we first used RNAi to knock-down RhoA. MCF-7 cells were transfected with RhoA SMARTPool RNAi, which led to > 90% depletion 48 h after transfection, as revealed by Western blot. Interestingly, when RhoA was silenced, cucurbitacin I failed to inhibit Rac1 activation by HRG (Fig. 6A and 6B). Therefore, the inhibitory effect of cucurbitacin I on Rac1 activation is linked to its ability to activate RhoA. To validate this conclusion, we used the Rho inhibitor C3 toxin (Aktories et al., 2004). In agreement with the RhoA RNAi depletion experiments, treatment of MCF-7 cells with C3 toxin rescued the inhibitory effect of cucurbitacin I on HRG-induced Rac1 activation. Whereas cucurbitacin I inhibits Rac1 activation by 51% in control MCF-7 cells, this effect was essentially lost (8% inhibition) in C3 toxin-treated cells (Fig. 6C and 6D). Furthermore, pretreatment of MCF-7 cells with the Y-27632, an inhibitor of the RhoA effector ROCK (Rho kinase) was able to rescue the activation of Rac1 by HRG (Fig. 6E and 6F).

Next, we examined whether inhibition of Rho/ROCK could restore the ability of HRG to induce ruffle formation in cucurbitacin I-treated cells. As shown in Fig. 7 treatment of MCF-7 cells with either C3 toxin or Y-27632 inhibited the formation of actin stress fibers by cucurbitacin I, therefore confirming the involvement of the Rho/ROCK pathway in the effect of this compound. Notably, the inhibitory effect of cucurbitacin I on ruffle formation in response to HRG was overcome by pretreatment with either C3 toxin or the ROCK inhibitor Y-27632. Altogether, these results suggest that the inhibitory effect of cucurbitacin I on Rac is secondary to its ability to activate RhoA and ROCK.

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Activation of RhoA by cucurbitacin I is mediated by ROS

Next, we investigated the underlying mechanisms leading to the modulation of small GTPases by cucurbitacin I. It has been recently reported that cucurbitacin I induces the formation of ROS, particularly from a mitochondrial compartment (Zhang et al., 2012b), and ROS have been implicated in the activation of Rho and Rho effectors (Jin et al., 2004). We therefore speculated that ROS could be involved in the activation of RhoA/ROCK and RhoA-mediated responses by cucurbitacin I. To examine whether cucurbitacin I elicits the formation of intracellular ROS in breast cancer cells we used the fluorogenic dye DCFDA. Treatment of MCF-7 cells with cucurbitacin I induces the generation of ROS, as determined by fluorescence microscopy (Fig. 8A, *left panel*) and flow cytometric analysis (Fig. 8A, *middle and right panels*) (see also Supplemental Figure 2). The induction of ROS by cucurbitacin I was inhibited by pretreatment with the ROS scavenger N-acetyl L-cysteine (NAC).

Next, we determined the effect of NAC on the activation of RhoA by cucurbitacin I. Elevation of RhoA-GTP levels by cucurbitacin I can be inhibited by NAC. As shown in Figs. 8B and 8C, pre-treatment of MCF-7 cells with the ROS scavenger essentially impaired the activation of RhoA by cucurbitacin I, and in addition it efficiently blocked the phosphorylation of the ROCK substrate MYPT1. Furthermore, we observed that NAC prevented the formation of stress fibers by cucurbitacin I (Fig. 8D). Therefore, activation of RhoA and RhoA downstream signaling by cucurbitacin I is dependent upon the generation of intracellular ROS.

NAC rescues the inhibitory effect of cucurbitacin I on Rac1 activation and motility

As RhoA activation is necessary for cucurbitacin I to exert its inhibitory effect on Rac1 activity, we hypothesized that this response should be prevented by NAC. Indeed, we found that

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cucurbitacin I failed to impair HRG-induced activation of Rac1 in MCF-7 cells in the presence of the ROS scavenger (Fig. 9A and 9B). To further confirm these findings, we determined MCF-7 cell migration. Using a Boyden chamber assay, we found that the inhibitory effect of cucurbitacin I on HRG-induced cell motility could be prevented by pretreatment with NAC (Fig. 9C). A quantitative analysis of multiple migration experiments revealed that NAC fully rescued the induction of cell motility in response to the growth factor. Thus, ROS play an essential role in mediating the effects of cucurbitacin I on small GTPases.

Finally, to examine if mitochondrial ROS could be involved in the cucurbitacin I effect, we used the specific mitochondrial-targeting antioxidant Mito-TEMPO. As shown in Fig. 9E and 9F, the impairment of HRG-induced activation of Rac1 in MCF-7 could be prevented by Mito-TEMPO. Moreover, the inhibitory effect of cucurbitacin I on HRG-induced migration was essentially blocked by the mitochondrial antioxidant (Fig. 9G and 9H).

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DISCUSSION

There is ample evidence for the involvement of Rho small GTPases in human cancer (Jaffe and Hall, 2005; Mardilovich et al.; Rossman et al., 2005; Vega and Ridley, 2008). Different members of the Rho family have been implicated in cellular transformation and metastasis as effectors of tyrosine-kinase receptors (Garcia-Mata and Burridge, 2007; Jaffe and Hall, 2002; Jaffe and Hall, 2005). Given the fact that Rac1 is required for actin cytoskeleton to reorganize and confer a motile phenotype, this small G-protein plays an important role in controlling metastatic dissemination of cancer cells. Hyperactivation of Rac1 occurs as a consequence of excessive inputs from receptors and signaling effectors that activate Rac-GEFs, including Ras and PI3K, and is therefore a major factor in metastasis (Khosravi-Far et al., 1995; Patel et al., 2007; Sosa et al.; Wertheimer et al.). In breast cancer cells, either inhibition or silencing of Rac1 drastically reduces the formation of membrane ruffles and the formation of lamellipodia in response to stimulation of ErbB receptors, with the subsequent impairment of cell motility (Sosa et al.; Yang et al., 2006).

The mechanisms by which ErbB receptors signal to Rac1 still remain partially understood. The recent identification of the PI3K-dependent Rac-GEF P-Rex1 as a main mediator of ErbB receptor-driven breast cancer cell motility and its overexpression in a large fraction of mammary tumors highlight the relevance of the Rac pathway in the progression of the disease (Sosa et al.). The multiple cross-talks among different membrane receptors, both tyrosine-kinases and GPCRs, suggests a high complexity in the mechanisms leading to Rac activation in response to oncogenic inputs. An example of such intricate interactions is the requirement of EGFR and the GPCR CXCR4 for the activation of Rac1 by the ErbB3 ligand

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HRG (Sosa et al.). A main goal in this study was to establish whether Jak2, a tyrosine-kinase reported to interact physically and functionally with ErbB receptors (Andl et al., 2004; Coaxum et al., 2009; Colomiere et al., 2009; Liu and Kern, 2002; Lo et al., 2008; Yamauchi et al., 1997; Yang et al., 2008), is part of the network of receptors that ultimately signal to P-Rex1 and Rac1. The inhibitory effect of the Jak2 inhibitor cucurbitacin I on Rac1 activation led us to reason that Jak2 was implicated in the effect of HRG; however, the lack of effect of Jak2 RNAi on Rac1 activation and motility ruled out our initial assumption. From our studies, we can therefore conclude that P-Rex1/Rac1 activation by ErbB receptors is independent from Jak2 as well as from STAT3.

The discrepancy between the effects of the pharmacological Jak2 inhibitor and Jak2 RNAi on Rac1 activation prompted us to investigate this issue in more detail. The unanticipated finding that cucurbitacin I activates RhoA in breast cancer cells led us to speculate that the inhibitory effect of this compound on Rac1 activation could be related to a dysbalance between the activity of Rac and Rho. Cucurbitacin I and its derivatives are natural terpenoids isolated from plants that have been extensively studied due to their anticancer and anti-inflammatory activities (Chen et al., 2005; Iwanski et al.; Lee et al.), and more recently identified as potent anti-metastatic agents in breast cancer (Zhang et al., 2012a) Cucurbitacin B treatment of cancer cells, including breast cancer cells, leads to cell growth arrest and/or cell death (Dakeng et al.; Tannin-Spitz et al., 2007; Wakimoto et al., 2008). In fact, numerous reports show that cucurbitacin derivatives induce cell cycle arrest, mainly in G2/M. Exposure of MCF-7 and MDA-MB-231 breast cancer cells to cucurbitacin B inhibits the expression of cyclin B1 and G2/M cyclin-dependent kinases (Tannin-Spitz et al., 2007). In addition, this compound causes a marked up-regulation of the cell cycle inhibitor $p21^{cip1}$ in several cancer cell lines (Lee et al.;

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Tannin-Spitz et al., 2007; Thoennissen et al., 2009). It is noteworthy that Rac1 regulates G1/S and G2/M transitions, and inhibition of Rac function leads to accumulation of cells in G1 and G2 phases of the cell cycle (Klein et al., 2007; Moore et al., 1997; Yang et al., 2005). It could be speculated that inhibition of Rac1 may contribute, at least in part, to the cell cycle arrest induced by cucurbitacin. It should be noted that cucurbitacin induces several morphological changes in cells, and in some cases these effects could be explained by the dysregulation of the cytoskeleton homeostasis. For example, cucurbitacins F cause F-actin aggregation in various human cancer cell lines, including breast cancer cells (Lee et al.; Maloney et al., 2008; Wakimoto et al., 2008). In a recent study, Knecht *et al.* reported that cucurbitacin I potently inhibits the migration of MDCK kidney epithelial cells and melanoma cells through a mechanism that does not involve the direct stabilization of actin filaments. Although experiments in that study failed to identify the potential targets or mechanisms that lead to the inhibitory effect on cell motility, these authors speculate that cucurbitacin I more likely exerts this effect independently of Jak/Stats and possibly targets other proteins involved in signaling and the regulation of actin dynamics (Knecht et al.).

We noticed in our study that cucurbitacin I causes a marked induction in the formation of stress fibers in MCF-7 cells, which is consistent with its ability to activate RhoA. Stress fibers must disassembly in order to allow cancer cells to acquire a motile phenotype via Rac. Although the direct cellular target(s) of cucurbitacin I responsible for RhoA/ROCK activation and actin cytoskeleton reorganization still need(s) to be identified, our studies underscore the alteration in the balance of Rho and Rac as a major causative factor for the ability of this compound to impair breast cancer cell motility. Notably, activation of RhoA signaling and stress fiber formation by cucurbitacin I was dependent upon the generation of intracellular ROS. Recent studies in a

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number of cancer cellular models showed that cucurbitacin I and B induces the production of ROS (Yasuda et al., 2010; Zhang et al., 2012b; Zhang et al., 2011), as we observed in breast cancer cells. We found that the specific ROS mitochondrial antioxidant Mito-TEMPO prevented the effect of cucurbitacin I on Rac inhibition, arguing that mitochondrial-derived ROS have a prominent role in this effect. This is an agreement with Zhang *et al.* (2012b), who found that cucurbitacin I enhances the production of mitochondrial-derived ROS. One likely possibility is that ROS generated by cucurbitacin I act directly on RhoA to cause its activation. Previous studies reported the involvement of ROS in the activation of Rho/ROCK signaling (Jin et al., 2004; Moon et al., 2010). Interestingly, a mechanism involving the direct activation of RhoA by ROS has been identified both *in vitro* and in cells. Mutation of key cysteine residues in a redox-sensitive motif present in RhoA abolishes ROS-mediated activation of this small G-protein as well as it impairs its ability to promote the formation of stress fibers (Aghajanian et al., 2009; Heo et al., 2006). Possible effects of cucurbitacin I on exchange factors that promote GTP loading onto RhoA or other upstream mechanisms that contribute to Rho activation cannot be ruled out. It would be interesting to determine if cucurbitacin I also affects the activity of other members of the Rho family, such as RhoB or RhoC, which have dissimilar effects on actin cytoskeleton (Vega and Ridley, 2008). It may be also possible that cucurbitacin I affects the activity of Rho GDP Dissociation Inhibitors (RhoGDIs), proteins that inhibit the dissociation of GDP from the GTPase and therefore prevent the binding of GTP. Post-translational modifications in RhoGDIs are important to regulate the Rho/Rac switch, and moreover, Rho GDIs can be regulated by EGFR and other tyrosine-kinases (DerMardirossian et al., 2004; Garcia-Mata et al.). Thus, cucurbitacin I may be targeting Rho GDIs or proteins that directly or indirectly regulate Rho GDI activity. The relative contribution of these mechanisms to

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RhoA/ROCK activation in breast cancer cells remains to be determined. It is also interesting that in a recent study Boykin *et al.* reported a cucurbitacin analogue (cucurbitacin IIa) that fails to inhibit Jak2/Stat3 but still displays prominent effects on actin cytoskeleton reorganization and causes a phenotype characteristic of RhoA activation (Boykin et al., 2011). This suggest that the effects on Jak2/Stat3 and cell morphology can be dissociated and possibly utilize different mechanisms.

In summary, we raised two major conclusions from our study. First, we ruled out the involvement of Jak2/STAT3 in the activation of P-Rex1/Rac1 in breast cancer cells by ErbB ligands, and second we underscored a novel activity of the Jak2 inhibitor cucurbitacin I as a regulator of RhoA and Rac1. Our study emphasizes the complexities in the signaling mechanisms that regulate ErbB receptor-driven motility in breast cancer cells and highlight the relevance of natural products as modulators of signaling molecules that are key for cancer cell transformation and metastatic dissemination.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Lopez-Haber and Kazanietz.

Conducted experiments: Lopez-Haber.

Performed data analysis: Lopez-Haber and Kazanietz.

Wrote or contributed to the writing of the manuscript: Lopez-Haber and Kazanietz.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Cucurbitacin I inhibits Rac1 activation in breast cancer cells. *Panel A*, breast cancer cells lines were serum-starved for 48 h and then treated with HRG (10 ng/ml, 5 min) in the presence of increasing concentrations of cucurbitacin I (0.03-1 μM). Rac1-GTP levels were determined using a pull-down assay. *Panel B*, densitometric values of Rac1-GTP levels (normalized to total Rac1) are presented as mean ± S.D. (n=3). *Panel C*, effect of cucurbitacin I on the activation of Rac1 by EGF (100 ng/ml, 2 min). *Panel D*, densitometric analysis of data from *Panel C*.

Figure 2. Cucurbitacin I inhibits migration of MCF-7 cells. *Panel A*. Cell motility in response to HRG (20 ng/ml, 6 h) was determined in the presence of cucurbitacin I (0.01-0.1 μM) in serum-starved MCF-7 cells using a Boyden chamber. Migrating cells on the membrane were fixed and stained. Representative images are shown. *Panel B*. Quantification of migrating cells by contrast microscopy in 5 independent fields. Results are presented as fold-increase relative to control cells in the absence of HRG, and expressed as mean ± S.D. of triplicate measurements. Two additional experiments gave similar results. *Panel C*. Analysis of cytotoxicity by cucurbitacin I. MCF-7 cell viability was determined using Crystal Violet staining after incubation with different concentrations of cucurbitacin I for 6 h.

Figure 3. Jak2 RNAi depletion does not affect Rac1 activation and motility of breast cancer cells. *Panel A*. Expression of Jak2 in breast cancer cells was determined by Western blot. *Panel B*. MCF-7 cells were transfected with RNAi duplexes for Jak2 or non-target control (*NTC*), as

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detailed in Materials and Methods. After 24 h cells were serum-starved for 48 h and then treated with HRG (10 ng/ml, 5 min). Rac1-GTP levels were determined using a pull-down assay. *Panel C.* Densitometric values of Rac1-GTP levels (normalized to total Rac1) are presented as mean ± S.D. (n=3). *Panel D.* Cell motility in response to HRG (20 ng/ml, 6 h) was determined in MCF-7 cells subject to Jak2 RNAi using a Boyden chamber. Migrating cells on the membrane were fixed and stained. Representative images are shown. *Panel E.* Quantification of migrating cells in 5 independent fields was done by microscopy. Results are presented as fold-increase relative to control cells in the absence of HRG, and expressed as mean ± S.D. of triplicate measurements. Two additional experiments gave similar results. *Panel F.* MCF-7 cells were transfected with RNAi duplexes for STAT3 or non-target control (*NTC*). After 24 h cells were serum-starved for 48 h and then treated with HRG (10 ng/ml, 5 min). Rac1-GTP levels were determined using a pull-down assay. *Panel G.* Densitometric values of Rac1-GTP levels (normalized to total Rac1) are presented as mean ± S.D. (n=3).

Figure 4. *Panel A.* Cucurbitacin I does not affect HRG-induced translocation of P-Rex1 or activation of ErbB receptors. MCF-7 cells were serum starved for 48 h, stimulated with HRG (20 ng/ml, 5 min), fixed, stained using an anti-P-Rex1 antibody, and visualized by fluorescence microscopy. Similar results were observed in > 50 individual cells in 2 separate experiments. *Panel B.* MCF-7 cells were serum-starved for 48 h and then treated with HRG (10 ng/ml, 5 min) in the presence or absence of cucurbitacin I (0.1 μM). Phosphorylation of ErbB receptors was determined in cell extracts by Western blot.

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Figure 5. Cucurbitacin I activates RhoA/ROCK and induces the formation of stress fibers in breast cancer cells. *Panel A.* MCF-7 cells were serum starved for 48 h, treated with cucurbitacin I (0.1 μM, 30 min), fixed, stained with rhodamine-phalloidin, and visualized by fluorescence microscopy. Similar results were observed in > 50 individual cells in 2 separate experiments. *Panel B.* MCF-7 cells were serum starved for 48 h, and treated with cucurbitacin I (0.1 μM, 30 min). RhoA-GTP levels were determined with a pull-down assay. MYPT1-Thr853 phosphorylation was determined in cell extracts by Western blot. *Panel C.* Densitometric analysis of RhoA activation and MYPT1-Thr853 phosphorylation (n=3).

Figure 6. The inhibitory effect of cucurbitacin I on Rac1 activation depends on its ability to activate RhoA. *Panel A.* MCF-7 cells were transfected with RNAi duplexes for RhoA or non-target control (*NTC*). After 24 h cells were serum-starved for 48 h and then treated with HRG (10 ng/ml, 5 min). Rac1-GTP levels were determined using a pull-down assay. *Panel B.* Densitometric analysis of multiple RhoA silencing experiments. Rac1-GTP levels (normalized to total Rac1) are presented as mean ± S.D. (n=3). **, $p < 0.01$. *Panel C.* Effect of C3 toxin (2 μg/ml, 24 h) on Rac1 activation by HRG. *Panel D.* Densitometric analysis of multiple C3 toxin experiments (n=3). *, $p < 0.05$. *Panel E.* Effect of the ROCK inhibitor Y-27632 (10 μM, 1h). *Panel F.* Densitometric analysis of multiple Y-27632 experiments (n=3). *, $p < 0.05$.

Figure 7. Effect of Rho and ROCK inhibition on ruffle formation. MCF-7 cells were pre-incubated with either C3 toxin (2 μg/ml, 24 h) or Y-27632 (10 μM, 1h), and then treated with cucurbitacin I (0.1 μM, 30 min) or vehicle. Cells were then stimulated with HRG (20 ng/ml, 10

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min), fixed, stained with rhodamine-phalloidin, and visualized by fluorescence microscopy. Similar results were observed in > 50 individual cells in 2 separate experiments.

Figure 8. Cucurbitacin I activates RhoA and ROCK through the generation of intracellular ROS. *Panel A.* Serum-starved MCF-7 cells were pre-incubated with DCFDA (5 mM) for 30 min and then treated for 1 h with cucurbitacin I (0.1 μM) in the presence or absence of the ROS scavenger NAC (10 mM). *Left*, representative microphotographs of MCF-7 cells subject to the various treatments; *middle*, generation of ROS by flow cytometry; *right*, quantification of mean fluorescence intensity as determined by flow cytometry. *BF*, bright field; *F*, fluorescence; *Cu*, cucurbitacin I. *Panel B.* Serum-starved MCF-7 cells were treated with cucurbitacin I (0.1 μM, 30 min) in the presence or absence of NAC (10 mM). RhoA-GTP levels were determined with a pull-down assay. MYPT1-Thr853 phosphorylation was determined in cell extracts by Western blot. *Panel C.* Densitometric analysis of RhoA activation and MYPT1-Thr853 phosphorylation (n=3). *Panel D.* Rhodamine-phalloidin staining of MCF-7 cells subject to the treatments indicated in the figure.

Figure 9. The ROS scavenger NAC prevents the inhibition of Rac1 by cucurbitacin I in MCF-7 cells. *Panel A.* Serum-starved MCF-7 cells were pre-treated with cucurbitacin I (0.1 μM, 30 min) in the presence or absence of NAC (10 mM, 30 min), and then stimulated with HRG (10 ng/ml, 5 min). Rac1-GTP levels were determined using a pull-down assay. *Panel B.* Densitometric analysis. Rac1-GTP levels (normalized to total Rac1) are presented as mean ± S.D. (n=3). **, $p < 0.01$. *Panel C.* Cell motility in response to HRG (20 ng/ml, 6 h) was determined in the presence of cucurbitacin I (0.1 μM), with or without NAC (10 mM), using a Boyden chamber.

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Representative images are shown. *Panel D.* Quantification of migrating cells by contrast microscopy in 5 independent fields. Results are presented as fold-increase relative to control cells in the absence of HRG, and expressed as mean ± S.D. of triplicate measurements. A second additional experiment gave similar results. *Panels E, F, G,* and *H*. Experiments were carried out as in Panels A, B, C, and D, but using the mitochondrial anti-oxidant Mito-TEMPO (100 μM, 1 h).

Figure 1

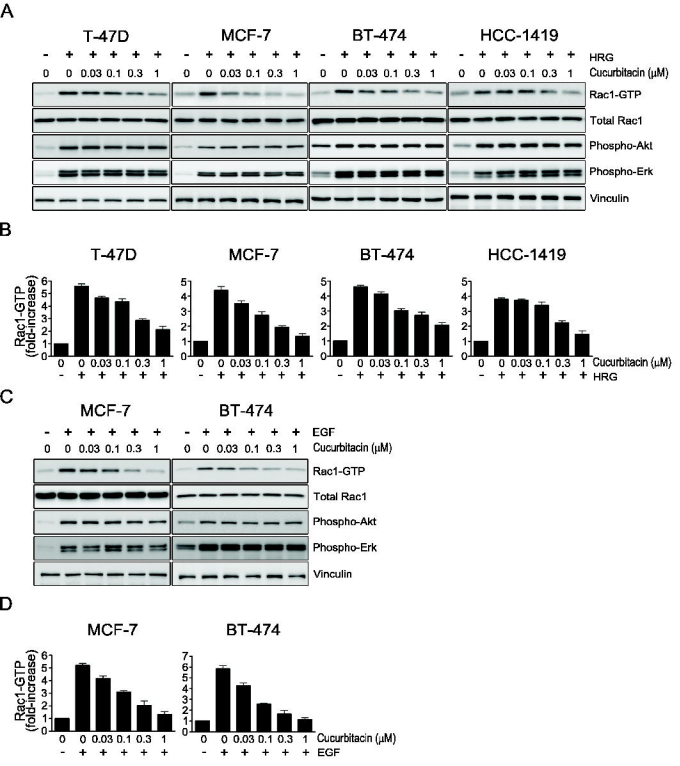

A
T-47D
MCF-7
BT-474
HCC-1419
HRG
Cucurbitacin (μM)
Rac1-GTP
Total Rac1
Phospho-Akt
Phospho-Erk
Vinculin
B
Rac1-GTP (fold-increase)
Cucurbitacin (μM)
HRG
C
EGF
Cucurbitacin (μM)
D
Rac1-GTP (fold-increase)
Cucurbitacin (μM)
EGF

Figure 2

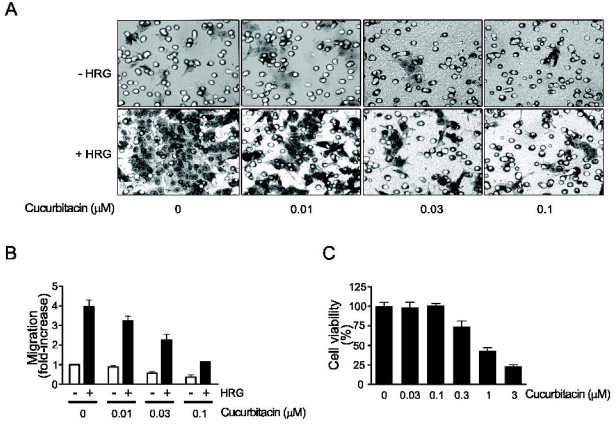

A
- HRG
+ HRG
Cucurbitacin (μM)
0
0.01
0.03
0.1
B
Migration (fold-increase)
5
4
3
2
1
0
- + - + - + - +
HRG
0
0.01
0.03
0.1
Cucurbitacin (μM)
C
Cell viability (%)
125
100
75
50
25
0
0
0.03
0.1
0.3
1
3
Cucurbitacin (μM)

Figure 3

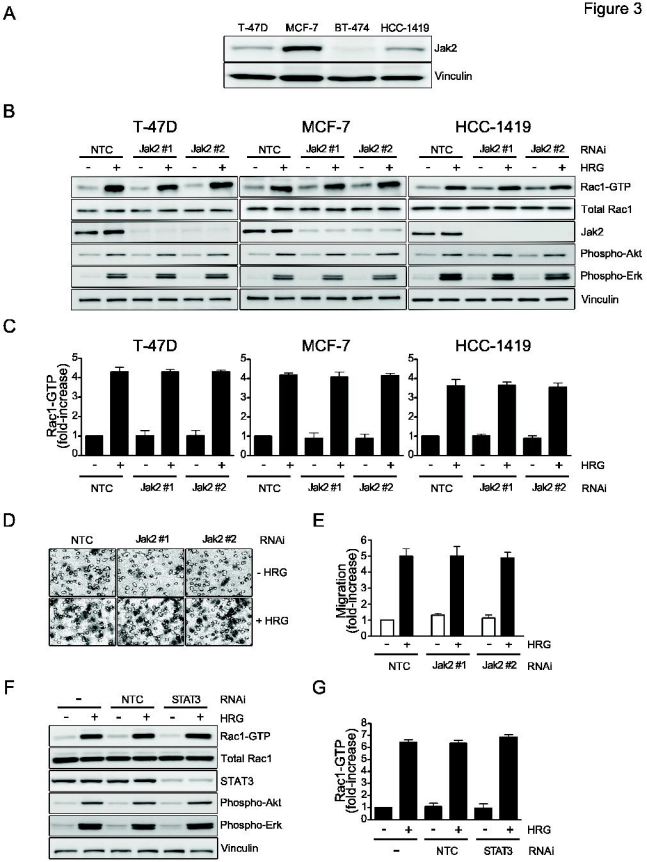

A
T-47D
MCF-7
BT-474
HCC-1419
Jak2
Vinculin
B
T-47D
MCF-7
HCC-1419
NTC
Jak2 #1
Jak2 #2
RNAi
HRG
Rac1-GTP
Total Rac1
Jak2
Phospho-Akt
Phospho-Erk
Vinculin
C
Rac1-GTP (fold-increase)
D
- HRG
+ HRG
E
Migration (fold-increase)
F
STAT3
G

Figure 4

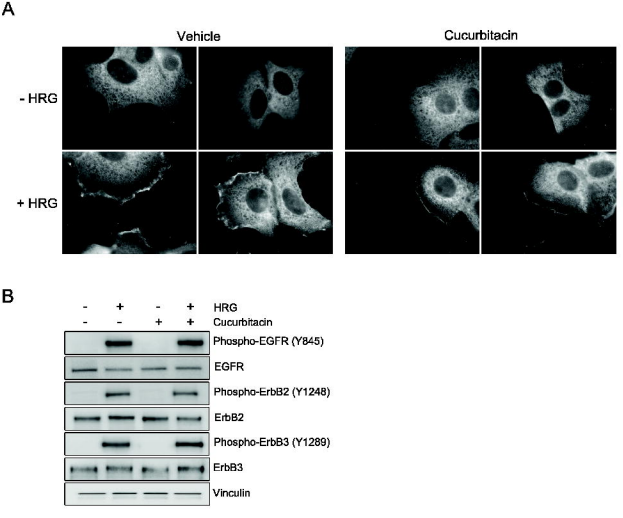

A
Vehicle
Cucurbitacin
- HRG
+ HRG
B
- + - + HRG
- - + + Cucurbitacin
Phospho-EGFR (Y845)
EGFR
Phospho-ErbB2 (Y1248)
ErbB2
Phospho-ErbB3 (Y1289)
ErbB3
Vinculin

Figure 5

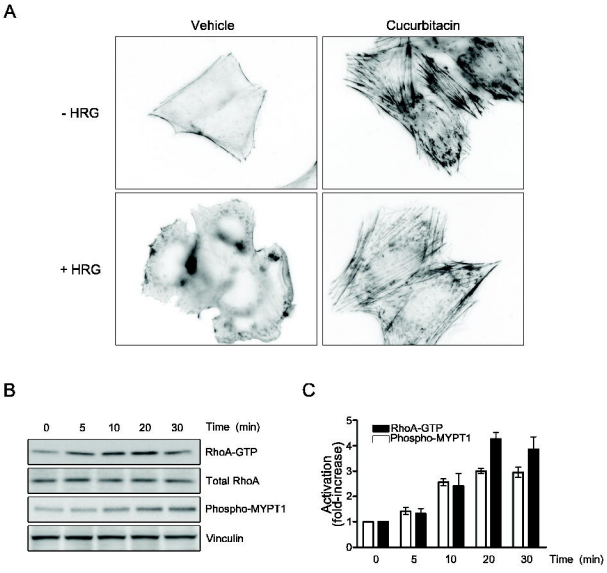

A
Vehicle
Cucurbitacin
- HRG
+ HRG
B
0
5
10
20
30
Time (min)
RhoA-GTP
Total RhoA
Phospho-MYPT1
Vinculin
C
RhoA-GTP
Phospho-MYPT1
Activation (fold-increase)
0
1
2
3
4
5
0
5
10
20
30
Time (min)

Figure 6

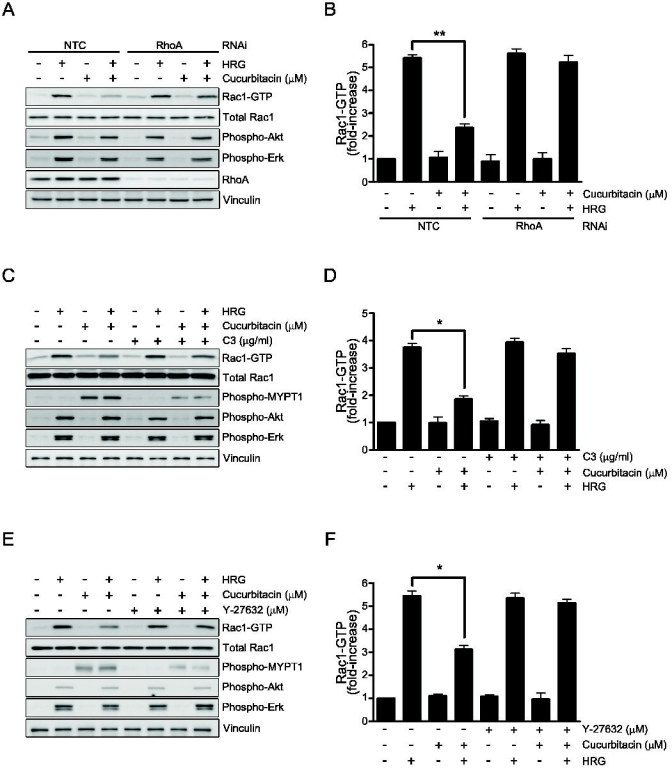

A
NTC
RhoA
RNAi
HRG
Cucurbitacin (μM)
Rac1-GTP
Total Rac1
Phospho-Akt
Phospho-Erk
RhoA
Vinculin
B
Rac1-GTP (fold-increase)
**
Cucurbitacin (μM)
HRG
NTC
RhoA
RNAi
C
HRG
Cucurbitacin (μM)
C3 (μg/ml)
Rac1-GTP
Total Rac1
Phospho-MYPT1
Phospho-Akt
Phospho-Erk
Vinculin
D
Rac1-GTP (fold-increase)
*
C3 (μg/ml)
Cucurbitacin (μM)
HRG
E
HRG
Cucurbitacin (μM)
Y-27632 (μM)
Rac1-GTP
Total Rac1
Phospho-MYPT1
Phospho-Akt
Phospho-Erk
Vinculin
F
Rac1-GTP (fold-increase)
*
Y-27632 (μM)
Cucurbitacin (μM)
HRG

Figure 7

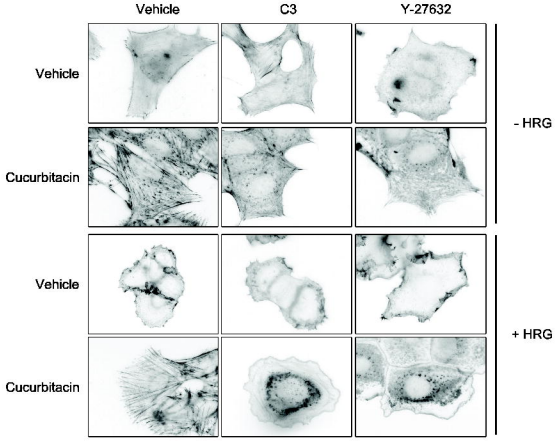

Vehicle
C3
Y-27632
Vehicle
Cucurbitacin
- HRG
Vehicle
Cucurbitacin
+ HRG

Figure 8

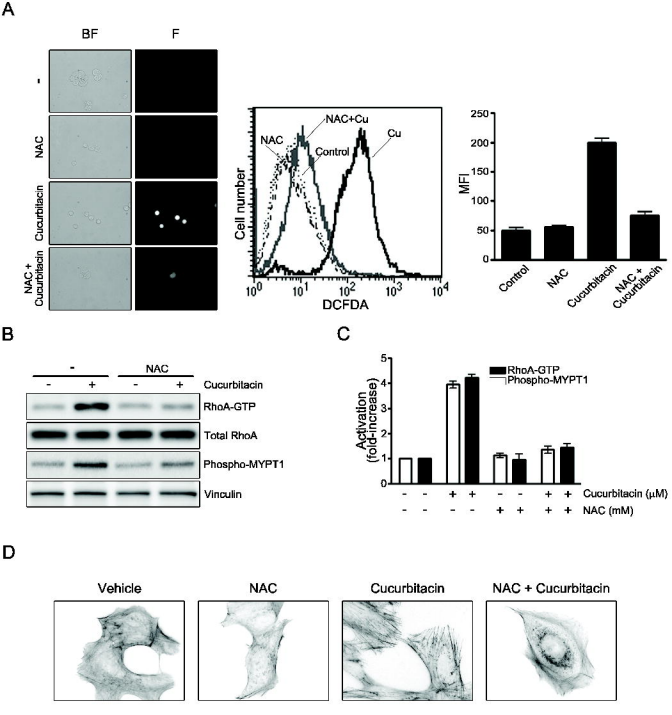
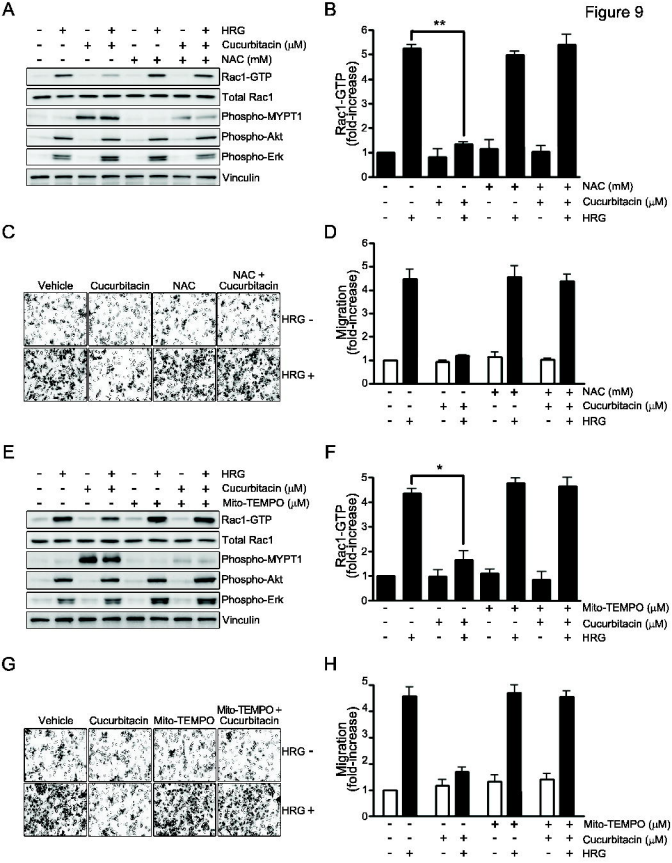

A
BF
F
-
NAC
Cucurbitacin
NAC + Cucurbitacin
NAC
NAC+Cu
Control
Cu
Cell number
DCFDA
MFI
Control
NAC
Cucurbitacin
NAC + Cucurbitacin
B
-
NAC
Cucurbitacin
RhoA-GTP
Total RhoA
Phospho-MYPT1
Vinculin
C
RhoA-GTP
Phospho-MYPT1
Activation (fold-increase)
Cucurbitacin (μM)
NAC (mM)
D
Vehicle
NAC
Cucurbitacin
NAC + Cucurbitacin

Figure 9


A
HRG
Cucurbitacin (μM)
NAC (mM)
Rac1-GTP
Total Rac1
Phospho-MYPT1
Phospho-Akt
Phospho-Erk
Vinculin
B
Rac1-GTP (fold-increase)
**
NAC (mM)
Cucurbitacin (μM)
HRG
C
Vehicle
Cucurbitacin
NAC
NAC + Cucurbitacin
HRG -
HRG +
D
Migration (fold-increase)
NAC (mM)
Cucurbitacin (μM)
HRG
E
HRG
Cucurbitacin (μM)
Mito-TEMPO (μM)
Rac1-GTP
Total Rac1
Phospho-MYPT1
Phospho-Akt
Phospho-Erk
Vinculin
F
Rac1-GTP (fold-increase)
*
Mito-TEMPO (μM)
Cucurbitacin (μM)
HRG
G
Vehicle
Cucurbitacin
Mito-TEMPO
Mito-TEMPO + Cucurbitacin
HRG -
HRG +
H
Migration (fold-increase)
Mito-TEMPO (μM)
Cucurbitacin (μM)
HRG