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**Met-driven invasive growth involves transcriptional regulation of Arhgap12.**

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Running title: Arhgap12 encodes a novel Rac1-GAP downstream HGF

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**Abstract**

Invasive growth is a complex biological program triggered by Hepatocyte Growth Factor (HGF) through its tyrosine kinase receptor encoded by the Met proto-oncogene. The program involves -besides proliferation- cell dissociation, motility and invasiveness, controlled by intracellular signals impinging on PI3K and on the small G-proteins of the Rac/Rho family. The mechanism(s) unbalancing Rac/Rho activation are still not completely clarified. Here we describe a functional link between HGF and Arhgap12, a gene encoding a previously uncharacterized protein of the RhoGAP family. We identified Arhgap12 as a transcriptional target of HGF, through a novel gene trapping strategy. We found that Arhgap12 mRNA and protein are robustly suppressed by HGF treatment, but not by serum. Arhgap12 displayed GAP activity towards Rac1 and, upon overexpression, impaired cell scattering, invasion and adhesion to fibronectin in response to HGF. Consistently, Arhgap12 silencing by RNA interference selectively increased the scatter and adhesion responses. These data show that HGF-driven invasive growth involves transcriptional regulation of a Rac1-specific GAP.

## Introduction

Invasive growth is a highly integrated biological program that instructs cells to dissociate from their neighbors, migrate through the extracellular matrix, colonize new sites, proliferate and differentiate. The program is orchestrated in time and space by specific extracellular ligands of the HGF family (Trusolino and Comoglio 2002). Invasive growth is physiologically required for tissue and organ morphogenesis during embryonic development, and it is aberrantly activated in the adult during cancer progression, conferring invasive and metastatic ability to neoplastic cells (Comoglio and Trusolino 2002; Birchmeier et al. 2003). HGF binds to and activates a specific transmembrane tyrosine-kinase receptor, encoded by the MET proto-oncogene (Ma et al. 2003). Met activation results in tyrosine phosphorylation of the c-terminal tail and recruitment of a wide spectrum of adaptors and signal transducers, including GRB2, STAT3, PLC-gamma and PI3K (Ponzetto et al. 1994; Boccaccio et al. 1998; Furge et al. 2000; Bertotti and Comoglio 2003). Extensive studies linked the various biological processes controlled by Met to specific signaling pathways: (i) the GRB2-RAS-ERK pathway is the main driver of cell proliferation (Ponzetto et al. 1996); (ii) STAT3 activation promotes branching morphogenesis (Boccaccio et al. 1998); (iii) phospholipase C-gamma is required for acquisition of cell polarity (Gual et al. 2000); (iv) PI3K drives survival through the Akt pathway (Zeng et al. 2002), and cell adhesion and motility through small GTP-binding proteins of the Rho family (Royal and Park 1995; Ridley et al. 1995; Parker 1995).

The Rho-family GTPases are key players in actin cytoskeleton remodeling and subsequent regulation of cell migration and adhesion (Hall 1998). It is also known that Rho and Rac are involved in the establishment of cadherin-mediated cell-cell interactions (Braga et al. 1997), and that Rac promotes invasion (Wheeler et al. 2006).

The balance in the activities of Rho and Rac is tightly regulated and modulates cell adhesiveness (Zandy et al. 2007). Several GEFs and GAPs specifically activating and inactivating either Rho or Rac have been identified and characterized (Bar-Sagi and Hall 2000).

Despite the knowledge accumulated, it is still unclear how Rho and Rac orchestrate the invasive growth response to HGF. By a functional screening aimed at identifying transcriptional targets of HGF, we identified the mRNA for a previously uncharacterized member of the RhoGAP family, Arhgap12. Functional studies showed that this GAP protein specifically inactivates Rac1 and plays a role in regulation of the invasive growth response to HGF.

## Results

*Identification and validation of Arhgap12 as an HGF transcriptional target.*

It is known that HGF-stimulated scattering of epithelial cells requires *de novo* transcription and protein synthesis (Rosen et al. 1990). To identify genes potentially involved in HGF-driven invasive growth, we conceived a novel screening based on gene trapping. Our gene trap system exploits random integration of vectors carrying the PGN selectable reporter not preceded by a promoter, to generate reporter cell clones displaying the transcriptional activity of virtually any gene in the genome. PGN allows positive pharmacological selection by puromycin treatment, negative selection by metronidazole (MN) treatment and expression monitoring by flow cytometry (further details are provided in Supplementary Information and Supplementary Figure 1). Retroviral and lentiviral traps were used on MLP-29 mouse embryo liver cells (Medico et al. 1996) to identify genes transcriptionally regulated by HGF, either induced or suppressed, according to two selection schemes illustrated in Figure 1. The treatment cycles were repeated once to improve efficiency. We obtained 179 clones surviving the selection, of which more than 40% resulted to be responsive to HGF, which confirmed the high efficiency of the system. The range of regulation spanned from 6-fold induction to 2.2-fold suppression, with many of the traps responding within a 1.5-fold range, indicating that selection does not require extreme expression differences.

Among the various clones, we selected for detailed analysis the one named SH24, which, unlike most others, changed its fluorescence only in response to HGF and not to serum, as assessed by FACS analysis (Figure 2A). As the PGN half-life is longer than 24 hours (not shown), the observed 40% reduction of fluorescence at 48h is indicative of a strong transcriptional suppression. Using the newly developed “repeat-anchored PCR” procedure (see Supplementary Information and Supplementary Figure 2), we

mapped the SH24 trap integration site in the first exon of the Arhgap12 gene. Arhgap12 encodes for a putative member of the RhoGAP family of proteins, which negatively regulate small GTP-binding proteins of the Rho family by promoting their intrinsic GTPase activity (Peck et al. 2002).

We carried out quantitative Real-Time PCR on MLP-29 cells stimulated with HGF or serum and validated Arhgap12 specific response to HGF, peaking at 6 hours of stimulation with an 8-fold suppression (Figure 2B). Real-Time PCR analysis was also conducted on human cancer cell lines (A549, DU145, ARO and MDA-MB-435), showing a similar response but with different kinetics (Figure 2C). Interestingly, Arhgap12 suppression by HGF occurred in MDA-MB-435 only upon exogenous expression of Integrin Beta 4, known to act as a Met-signaling amplifier required for full biological response to HGF (Trusolino et al. 2001). To verify if Arhgap12 is specifically downregulated by HGF also at the protein level, we produced a polyclonal antibody to a GST-fusion containing an aminoacid sequence specific for Arhgap12 and conserved across species (human, mouse, dog). Western blot analysis confirmed protein downregulation by HGF (Figure 2D). Experiments with signaling inhibitors highlighted a broad and redundant spectrum of signaling pathways upstream Arhgap-12, as no single pathway inhibitor could abrogate its transcriptional regulation (not shown).

*Arhgap12 is a negative regulator of Rac1*

Typically, RhoGAP proteins share the ability to stimulate GTP hydrolysis by one or more specific members of the Rho family (Moon and Zheng 2003). In the case of Arhgap12, GAP activity was inferred by the presence of a typical GAP domain, though with no experimental evidence confirming its activity or determining its substrate specificity. Therefore we constructed and purified a fusion between the Arhgap12 GAP domain and GST (Supplementary Figure 3), and measured its GAP activity *in vitro*

towards the main representatives of the Rho family: RhoA, Rac1 and Cdc42. As shown in Figure 3, the GAP domain of Arhgap12 stimulated GTP hydrolysis by Rac1 but not by RhoA. A lower activity towards Cdc42 was also detected. Indeed, other GAP proteins have been found to exert GAP activity *in vitro* towards both Rac1 and Cdc42, probably as a result of their similarity (Cicchetti et al. 1995). Nevertheless, since the activity of Arhgap12 resulted to be substantially higher on Rac1, we concluded that Arhgap12 is a GTPase activating protein for Rac1 *in vitro*.

To verify if other Rac1-GAPs are transcriptionally regulated by HGF, we extracted microarray expression data for ten Rac1-GAPs from a time-course HGF stimulation experiment performed on MLP-29 cells (our unpublished results). Only two other Rac1 GAPs (Arhgap5 and Arhgap29) displayed a moderate transcriptional suppression by HGF (2- to 4-fold), confirming that Arhgap12 suppression by HGF is selective (supplementary Figure 4).

*Arhgap12 affects cell scattering, adhesion and invasiveness.*

To study the involvement of Arhgap12 in the response of epithelial cells to HGF, we constructed a lentiviral vector for overexpression of Arhgap12 fused with GFP at its C-terminal (see Materials and Methods). For stable gene downregulation by RNA interference, the pLPG-Arhagap12-sh vector was constructed using a lentiviral backbone. As a control, cells were transduced either by the empty lentiviral vector, or by a lentiviral vector expressing GFP alone or a scrambled shRNA. The vectors were used to infect MDCK canine kidney cells, a well-characterized model for studying the role of small GTPases and their regulators in HGF-driven invasive growth. In particular, Rac1 was found to be essential for key steps in the scattering response of MDCK cells to HGF, namely membrane ruffling, cytoskeleton remodeling, lamellipodia formation and colony spreading (Ridley et al. 1995; Royal et al. 2000).

Western blot analysis with anti-Arhgap12 antibody confirmed the efficacy of both vectors (Figure 4A). The fusion protein of 120kDa, corresponding to the sum of the molecular masses of Arhgap12 and GFP was highly abundant in Arhgap12-GFP transduced cells, while the endogenous protein of 97 kDa was robustly downregulated in shRNA-transduced cells, and expressed in transduced cells at slightly -but not significantly- higher levels, as quantified on three independent blots (Scramble/WT = 1.12+/-0.08; Arhgap12-GFP/WT=1.25+/-0.13). Flow cytometric analysis showed that almost all Arhgap12-GFP transduced cells expressed the recombinant protein at medium-to-high levels (not shown). Fluorescence microscopy on Arhgap12-GFP transduced cells showed that the fusion protein is recruited to the plasma membrane in various cell types (Figure 4), consistently with the presence of the PH domain and with its recent isolation as a novel component of adherens junctions (Matsuda et al. 2008).

The biological properties of Arhgap12 over- or under-expressing cells were assessed in a number of biological assays, both in the presence or absence of HGF, to highlight a possible functional role of this protein in the various aspects of invasive growth. The major read–out of HGF activity is the induction of cell scattering, for which we observed a reduced response to HGF of Arhgap12-GFP expressing cells, mirrored by an increased response in shRNA-transduced cells (Figure 5A). Time-lapse recording allowed us to identify the kinetic of cells scattering: while Arhgap12 overexpressing cells initiated scattering later than controls, cells in which Arhgap12 was functionally ablated were more prompt and efficient in the response (Supplementary Figure 5). We reasoned that Arhgap12 downregulation by HGF may play a role in the early phases of the HGF response, and exploited an assay of cell adhesion to fibronectin treating the cells with HGF for 1 hour to obtain a short-term quantitative readout of the invasive growth program (Figure 5B). Indeed, cell adhesion was strongly impaired in the Arhgap12-overexpressing cells. Conversely, depletion of Arhgap12 enhanced cell

adhesion to fibronectin. The effect of Arhgap12 up- or downregulation was similar in HGF-treated and untreated cells, likely due to a basal Rac1 activity in MDCK cells cultivated in 10% serum, a condition known to promote cell-matrix adhesion via Rac1 (Guo et al. 2006).

We addressed later phases of the invasive growth program using an assay for cell invasiveness through matrigel-coated transwells. In such assays cell invasion was achieved upon addition of HGF for 24 hours. We found that in HGF-treated cells, invasion was stimulated to a similar extent both in wild-type and Arhgap12-silenced cells. Conversely, Arhgap12 overexpression inhibited the ability of cells to invade in response to HGF (Figure 5C). The lack of effect of shRNA-mediated Arhgap12 silencing in this assay is probably due to the fact that already at six hours of HGF stimulation the endogenous Arhgap12 protein strongly suppressed also in wild-type cells (Fig. 2D). It is therefore likely that the potential proinvasive effect of Arhgap12 artificial downregulation has a very limiter temporal window, i.e. the very first hours. However, before six hours, the quicker Rac1 activation in the shRNA-transduced cells is probably counteracted by the still undegraded matrix barrier.

Finally, we found that Arhgap12 overexpression or silencing did not grossly impair the proliferative response to HGF (not shown), which ruled out the possibility of a non-specific effect due to altered cell viability. Arhgap12 localization and its effects on cell adhesion, scattering and migration were confirmed in additional normal and neoplastic cell lines, namely MLP-29, HeLa, DU145 and MCF10A (Supplementary Figure 6).

The sum of these results demonstrates that suppression of Arhgap12 transcript and protein by HGF plays a relevant role in the invasive-growth program elicited by HGF.

## Discussion

By a novel gene trapping technique, we identified Arhgap12 as a specific transcriptional target of HGF in normal and cancer cells. Arhgap12 is selectively suppressed at transcriptional level by HGF but not by serum, the latter promoting a robust transcriptional response leading to cell proliferation but not to invasive growth (Iyer et al. 1999; Medico et al. 2001). Little was known about Arhgap12, other than its genomic structure and ubiquitous expression (Zhang et al. 2002). Arhgap12 is part of a subfamily including two other members, ArhGAP9 and 15 (Furukawa et al. 2001; Seoh et al. 2003). Protein sequence analysis shows that Arhgap12 encodes a GAP domain and three other functional motifs: (i) a PH domain, known to be involved in protein-lipid interaction, (ii) two WW domains and (iii) one SH3 domain potentially involved in protein-protein interactions. We show here that, *in vitro*, Arhgap12 inactivates Rac1 by increasing the rate of GTP hydrolysis. In addition, we found that when constitutively expressed, Arhgap12 negatively regulates cell adhesion, scattering and invasion, all processes requiring Rac1 activity (Hall 1998; Guo et al. 2006). RNA interference-mediated Arhgap12 suppression confirmed that it effectively inhibits Rac1-mediated processes also when endogenously expressed. Downregulation of Arhgap12 by HGF is rather selective, as nine other Rac1 GAPs surveyed by our microarray analysis either did not respond at all or were only slightly affected (Arhgap5 and Arhgap29). It is known that Rac1 promotes specific cellular functions essential for the invasive growth program, such as actin cytoskeleton reorganization and polymerization (Bosse et al. 2007), interaction with the extracellular matrix via focal adhesion complexes (Guo et al. 2006), and random and directional cell migration (Pankov et al. 2005; Keely et al. 1997). Rac1 also promotes the assembly of adherens junctions, specialized sites of cell-cell contact (Braga et al. 1997). Indeed, increased cell-cell adhesion driven by the constitutively active V12Rac mutant was found to inhibit HGF-induced cell scattering

(Hordijk et al. 1997), as well as motility and invasiveness of Ras-transformed MDCK cells (Sander et al. 1998). Thus, in epithelial cells, HGF-driven activation of Rac1 has to be tightly controlled in time and space to properly balance two potentially opposing processes, migration and cell-cell adhesion. Several factors have been found to influence the balance between adhesive and migratory responses to Rac1, such as composition of the extracellular matrix (Sander et al. 1998), activation of other Rho family proteins (Sander et al. 1999), and subcellular localization. In the migrating cell, PI3K-dependent activation of Rac1 takes place at the leading edge, where it induces membrane extensions, in part, through the WAVE–Arp2/3 complex (Takenawa and Miki 2001). Recruitment of Rac1 to the membrane and increased cell migration are also stimulated by elevated membrane phosphatidylserine content (Finkielstein et al. 2006). In this view, the PH, WW and SH3 domains of Arhgap12 can actually mediate its recruitment at specific membrane sites and signaling complexes, so that the overall result of its activity is inhibition of Rac1 in restricted areas of the cell membrane critical for cell-matrix adhesion, migration and invasion. Interestingly, Arhgap-12 overexpression, while inhibiting cell-matrix adhesion and scattering, did not reduce cell-cell adhesion (Figures 4-5). It has indeed been recently demonstrated that Arhgap12 is a component of adherens junctions (Matsuda et al. 2008). It was known that HGF regulates cell migration by activating Rac1 via a rapid cascade of biochemical events (Itoh et al. 2002; Kurokawa et al. 2004). Here we show that the long-term (and long lasting) invasive growth response to HGF is mediated by Rac1 activation via transcriptional suppression of Arhgap12.

## Materials and Methods

*Reagents, antibodies and Cell Cultures*

Cell lines were cultured with Dulbecco's Modified Eagle Medium (DMEM; Gibco) medium supplemented with 10% FBS (Sigma) in a humidified atmosphere of 5% $CO_2$. Stimulations were performed with recombinant HGF (40 Units/ml; (Naldini et al. 1995)). Human plasmatic FN was from Sigma. GFP antibody was from Roche (Roche anti-GFP 1814460). Actin antibody was from Santa Cruz. Puromycin and MN were from Sigma.

*Cell trapping and pharmacological selections.*

pP-TRACT was transfected using the Lipofectin Reagent (Gibco BRL, Grand Island, NY), according to the manufacturer's protocol. pP-TRACT was linearized before transfection with DraI restriction enzyme to avoid plasmid breaking within the trap cassette, and extensive intracellular replication of the trap through the SV40ori. L-TRACTS and R-TRACTS viruses were used at a low multiplicity: in each experiment, $10^7$ cells were infected with $10^6$ CFU of L/R-TRACT to avoid multiple integrations in the same cell. For trap selection, cells were treated with different doses of puromycin (Sigma, St. Louis, MO) going from 1μg/ml to 20μg/ml for 2-4 days. MN (Sigma, St. Louis, MO) was used at a concentration of 10mM for at least 4 days. Stimulation with recombinant HGF was combined with puromycin treatment for the selection of traps in HGF-induced genes, and with MN treatment for the selection of traps in HGF-suppressed genes.

*Flow cytometry and biological assays*

For flow cytometry, cells were detached by trypsinization, diluted in cold DMEM (Gibco) with 10% FBS, carefully mixed to disrupt cell aggregates and allowed to sediment for 1 min to eliminate residual clumps. Flow cytometry was conducted on Becton Dickinson (San Jose, CA) FACS Calibur. To improve sensitivity in PGN detection, we carried out analyses by comparing for each cell the fluorescence in the green channel (FL1) with fluorescence in the red channel (FL3), which indicated individual autofluorescence. For quantitative analysis of PGN expression, average red fluorescence was subtracted from average green fluorescence to obtain an estimate of specific PGN fluorescence. The same approach has been used to quantify Arhgap12-GFP expression in infected cell lines. For scatter assay**,** MDCK cells expressing Arhgap12 at different levels were cultured in 10% FBS allowing them to reach 40% confluence forming packed colonies, and subsequently treated with HGF for various times and photographed every 30 minutes under transmitted light with a Pathway system (BD Biosciences). For time-lapse microscopy, phase contrast micrographs were taken every 15 minutes in a Multi-Dimensional Workstation (Leica). For invasion assay, $10^5$ cells were seeded on the upper side of a Transwell chamber on a porous polycarbonate membrane (8.0 μm pore size, Falcon BD) coated with artificial basement membrane (Matrigel, 20μg per filter; Becton Dickinson Labware); the lower chamber of the Transwell was filled with DMEM containing 2% FBS in the absence or presence of HGF. After 24h of incubation, cells attached on the upper side of the filter were mechanically removed while cells that migrated to the lower side of the filter were fixed with glutheraldeyde 11%, stained with crystal violet, photographed (10 microscopic fields/sample) and quantified using the Metamorph software (Universal Imaging, USA). For cell adhesion assays, Microtiter plates (96-well; Nunc, Naperville, IL) were coated

with FN (2.5–20 µg/ml) in PBS. Proteins were allowed to bind overnight at 4°C before washing the wells with PBS and blocking for 2 h at 37°C with 2% heat-denatured BSA in PBS. Cells pretreated or not with HGF (for 1 hour) were harvested and added to the wells ($10^4$ cells/0.1 ml). After incubation for 45 min at 37°C, the wells were gently washed with PBS. Adherent cells were fixed in 11% glutaraldehyde in PBS, stained with 0.1% crystal violet in 20% methanol, photographed and quantified using the Metamorph software (Universal Imaging, USA). Data presented are the means ± SD of quadruplicate wells from six experiments. For fluorescence microscopy, cells were plated onto 24-well plates (Costar) containing 1.4-$cm^2$ glass coverslips. After incubation, cells were fixed in a freshly prepared solution of 3% paraformaldehyde and 2% sucrose in PBS (pH 7.6) for 5 min at room temperature. Images were captured by a Multi-Dimensional Workstation (Leica).

*RNA extraction and Real-Time PCR*

RNA was extracted using Trizol Kit (Ambion), according to the manufacturer's protocol. Quantitative Real-Time PCR with Sybr Green assay (Applera) was used to measure the relative amount of the cDNA of interest respect to the amount of an housekeeping gene (PGK) in different samples (unstimulated and stimulated cells). Human and mouse primers were designed with Primer Express software (Applied Biosystems). and are: hARHGAP12: sense 5'-TGGTTTGGATATTGATGGGATATACA-3';

antisense 5'-CATTCAAGTCCAATTTCTCATCATG-3';

mARHGAP12: sense 5'-TCGGTCAGAGTGGGAATTGC-3';

antisense 5'-TATTGGTTCTTGCAGCCGC-3';

hPGK: sense 5'-CTTATGAGCCACCTAGGCCG-3';

antisense 5'-CATCCTTGCCCAGCAGAGAT-3';

mPGK: sense 5’-CAGATTGTTTGGAATGGTCCTG-3’;

antisense 5’-CCCCTAGAAGTGGCTTTCACC-3’.

PCR reactions were carried out using a 7900HT Sequence Detection System (Applera), according to standard protocols.

*Construction and Expression of the Arhgap12-GFP Fusion and of Arhgap12 shRNA in lentiviral vectors.*

We engineered a lentiviral vector for the expression of GFP fusion proteins driven by the PGK promoter. To this aim, we replaced the GFP coding sequence in the lentiviral plasmid pRRLsin.PPT.hPGK.PGK-GFP.Wpre (Follenzi et al. 2000) with a new GFP coding sequence lacking the translation start ATG and preceded by a 3x (Gly-Gly-Gly-Gly-Ser) linker and a multicloning site. We named the plasmid pLP-X-GFP, which stands for Lentiviral vector, PGK promoter, X (ORF)-GFP. To generate the pLP-Arhgap12-GFP plasmid, the mouse Arhgap12 coding sequence was obtained by RT-PCR from MLP-29 cells using the sense primer 5'-CCATCGATATGGCCGAGAGAAGTGGAAA-3' (containing the ClaI site), and the antisense primer 5'-CTAGCTAGCTCCAAAGACAGTGCTTAATTCC-3' (designed to amplify the Arhgap12 coding sequence excluding the 3' termination codon and containing the restriction site NheI) and ligated into the pLP-X-GFP multicloning site, in frame with GFP. The construct was sequence-verified. Two control lentiviruses were also used, one expressing GFP and another without any transgene (empty). The Arhgap12-specific shRNA vector was generated using the following oligonucleotides containing the specific 19-mer targeting sequence (in bold); forward primer: 5’-GATCCCCGAACAGAACTGCTAATTCATTCAAGAGATGAATTAGCAGTTCTGTTCTTTTTGGAAA-3’ and reverse primer: 5’-AGCTTTTCCAAAAAGAACAGAACTGCTAATTCATCTCTTGAATGAATTAGCA

GTTCTGTTCGGG-3'. The oligonucleotides were annealed by boiling 1min at 80°C and slow cooling at RT, and ligated in pSuper (Brummelkamp et al. 2002), downstream from the H1 promoter. Lentiviral Arhgap12 shRNA was generated by subcloning the H1-RNA-promoter-Arhgap12 shRNA cassette from pSUPER in EcoRV-XhoI sites of the above-mentioned vector pCCL.sin.PPT.hPGK.GFP.Wpre. The construct was named pLPG-Arhagap12-sh (Lentiviral PGK-GFP-H1-Arhagap12 short hairpin). The construct was sequence-verified, and a scrambled lentiviral shRNA vector was generated as a control. Vector stocks were produced by transient transfection of 293T cells and purified by ultra-centrifugation as described (Dull et al. 1998). Transduction experiments were performed by adding a 1:2 dilution of the concentrated virus onto $3X10^4$ cells in 96-well plates (Costar) in the presence of polybrene (8 µg/ml). Transduction efficiency was checked by flow cytometry for GFP expression.

*Production of Arhgap12 antibody and Western Blot*

A sequence from base 220 to base 789 of the Arhgap12 mRNA (GenBank ID: NM_018287) was PCR-amplified using sense primer: 5'-CGGGATCCACGCGCAAAGCTCTCAT-3' and antisense primer: 5'-CGGAATTCTTACGGGCTCCCAGGAA-3'. The PCR fragment was cloned in pGEX-4T-1, a vector for bacterial expression of Glutathione-S-Transferase (GST)-fusion proteins (Amersham Biosciences). Rabbit polyclonal antibodies against the (GST)-fusion protein, purified from BL21 bacterial strain as described (Grisendi et al. 2001), were produced by Eurogentec (Belgium). For western blot, cells were washed twice with cold PBS, lysed with a buffer containing 50 mmol/L HEPES (pH 7.4), 5 mmol/L EDTA, 2 mmol/L EGTA, 150 mmol/L NaCl, 10% glycerol, and 1% Triton X-100, in the presence of protease and phosphatase inhibitors. The extracts were clarified at 12,000 rpm for 15 minutes. Proteins were quantified with the Bicinchoninic Acid

Protein Assay Reagent kit (Pierce, Rockford, IL), resolved on SDS-PAGE and subsequently transferred onto Hybond-C filters (Amersham). Filters were blocked with 10% BSA for 1 h at 45°C and probed with antibodies diluted in TBS (Tris-buffered saline)-5% BSA, for 16 h at 4°C. After extensive washing, the blots were stained with peroxidase-conjugated secondary antiserum followed by enhanced chemiluminescence reaction (ECL; Amersham).

*In vitro GAP assay*

The GAP domain of Arhgap12 (residues 582-838), was amplified by PCR using the sense primer 5'-GGAATTCCCAGATTCACCAGGGGTAG-3' and the antisense primer 5'-CCGCTCGAGTCAACGTCCAAAGACA-3'. The PCR fragment was cloned in the above-mentioned pGEX-4T-1 vector, and the (GST)-fusion protein purified as above (see Supplementary Figure 3). The GAP assay was performed according to standard procedures (Ridley et al. 1993). Briefly, 5μg of recombinant Rho, Rac and Cdc42 (200 nmoles) were preloaded with [$\gamma$-$^{32}$P]GTP (5 μCi, 5000 Ci/mmol; Amersham Biosciences). The [$\gamma$-$^{32}$P]GTP-loaded GTPases were incubated with 17.8 or 178 nmoles of either GST-GAP domain or GST, aliquots were taken at different time points and subjected to filter binding assay. The reactions mixture were filtered through nitrocellulose filters (Millipore), and washed with 5 ml of ice-cold Washing Buffer. The radioactivity retained on the filters was then quantified by scintillation counting.

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**Titles and legends to figures**

**Figure 1. Flowchart representation of the procedure for selecting cells carrying traps in in HGF-induced or HGF-suppressed genes**. After infection, cells are exposed to a low dose of puromycin for 3 days, to obtain populations of cells carrying traps in genes expressed at various levels. Several hundred thousand integrations were generated for the screening. To isolate traps in genes induced or suppressed by HGF, the populations were sequentially stimulated with HGF, in combination with puromycin treatment for selection of induced genes, or MN treatment for selection of suppressed genes. The treatment cycles were repeated once to improve the selection efficiency.

**Figure 2. Identification and validation of Arhgap12 as a transcriptional target of HGF in murine and human cell lines.** (A) Specific response of the SH24 trap clone to HGF. The histogram shows the changes in PGN fluorescence induced by HGF and serum (as percent of green fluorescence respect to untreated cells) on the SH24 trapped clone measured by FACS. The error bars report standard deviations of triplicates. (B) Real-Time PCR validation of Arhgap12 transcriptional regulation by HGF in MLP-29 cells. Changes of Arhgap12 transcript levels have been measured at different times of stimulation with HGF, as indicated, or with serum (FBS). Arhgap12 levels are normalized against the PGK housekeeping gene, measured on the same samples. The error bars report standard deviations of triplicates. (C) Real-Time PCR validation of Arhgap12 transcriptional regulation by HGF in human cancer cell lines. Changes of human ARHGAP12 transcript levels, normalized against the PGK housekeeping gene, have been measured at different times of stimulation of MDA-MB-435 (with or without B4 Integrin), A549, DU145 and ARO cells with HGF, as indicated. The error bars report standard deviations of triplicates. (D) Western blot analysis of Arhgap12

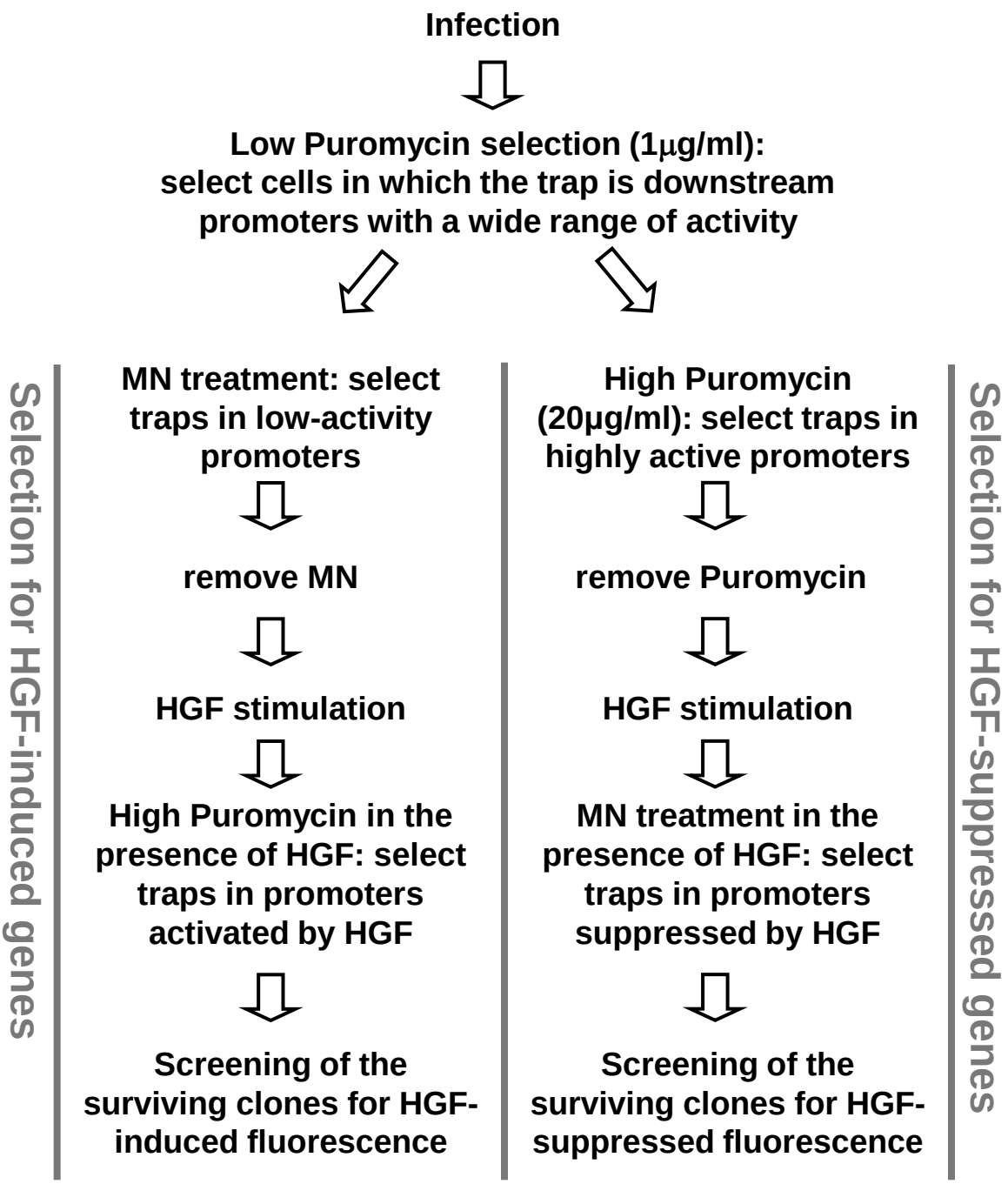
expression in MLP-29 cells, before and after treatment with HGF or with FBS at various times, as indicated. Actin staining is also reported as a control of total loaded proteins.

**Figure 3. Arhgap12 has GAP activity towards Rac1 *in vitro.*** Time-dependent kinetic of the GAP activity of the Arhgap12 GAP domain (GST-Arhgap12) on RhoA (A), Rac1 (B) and Cdc42 (C), as indicated. Rho, Rac1 and Cdc42 (200 nmoles) were loaded with [$\gamma$-$^{32}$P]-GTP and incubated for 5 or 15 minutes with either GST or GST-Arhgap12. The percentage of the GTP-bound fraction of each GTPase in the presence of GST alone or GST-Arhgap12 is shown on the y axis of the graph. The figure shows the means and standard deviations of three independent experiments.

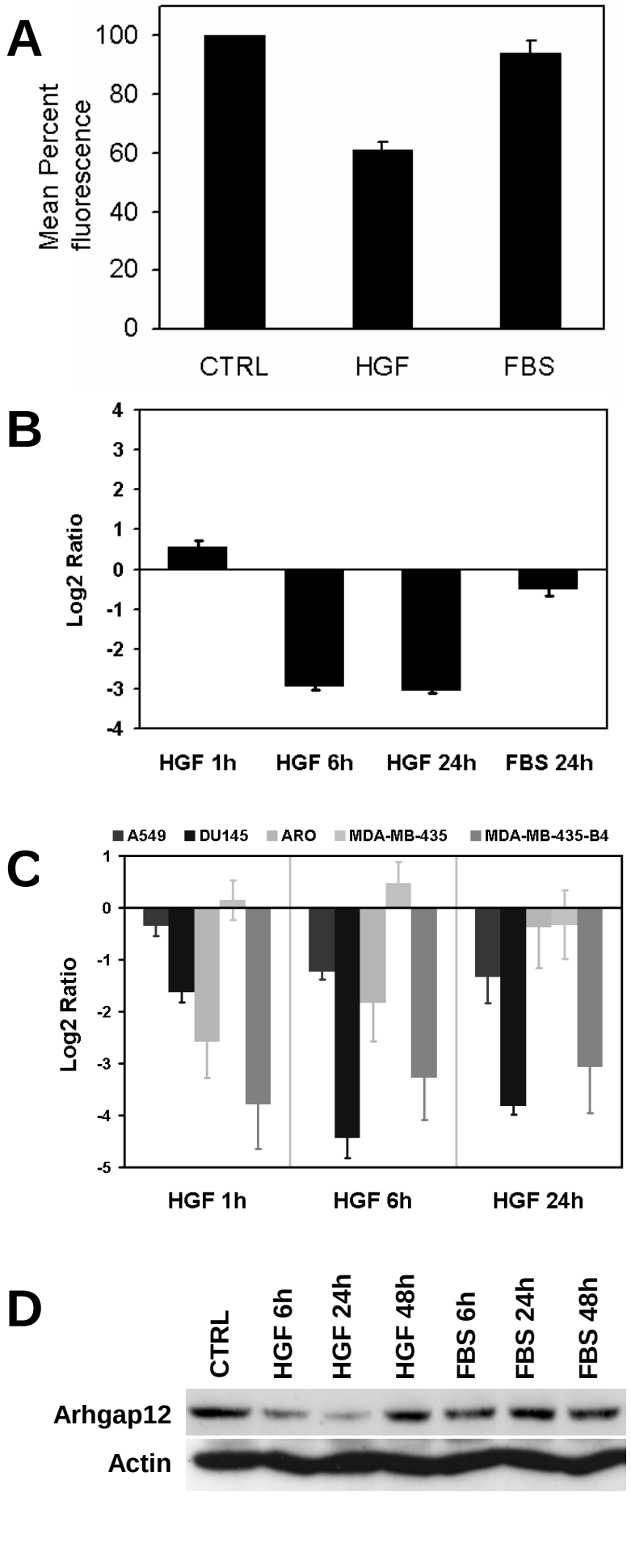
**Figure 4. Functional characterization of Arhgap12 by gain- and loss-of-function in epithelial cells.** (A) Western Blot analysis with an anti-Arhgap12 antibody of MDCK cells transduced with lentiviral vectors, either empty (mock), or encoding a scrambled shRNA, the Arhgap12-shRNA or the Arhgap12-GFP fusion, as indicated. The top and bottom arrows indicate the Arhgap12-GFP fusion and the wild type Arhgap12 (120 and 97 kDa, respectively). (B) Detection of Arhgap12-GFP in infected MDCK cells by fluorescence microscopy, showing membrane localization. The white arrows indicate membrane regions in which Arhgap12-GFP is particularly concentrated

**Figure 5. Arhgap12 negatively regulates HGF-driven cell scattering, adhesion and invasion.** (A) Arhgap12 affects cell scattering. Micrographs of MDCK cells either transduced with Arhgap12-GFP, or with Arhgap12 shRNA or with scrambled shRNA (CTRL), taken at 0, 5 and 15 hours of stimulation with HGF or with control medium (NS), as indicated. (B) Adhesion assay on fibronectin of MDCK cells either transduced

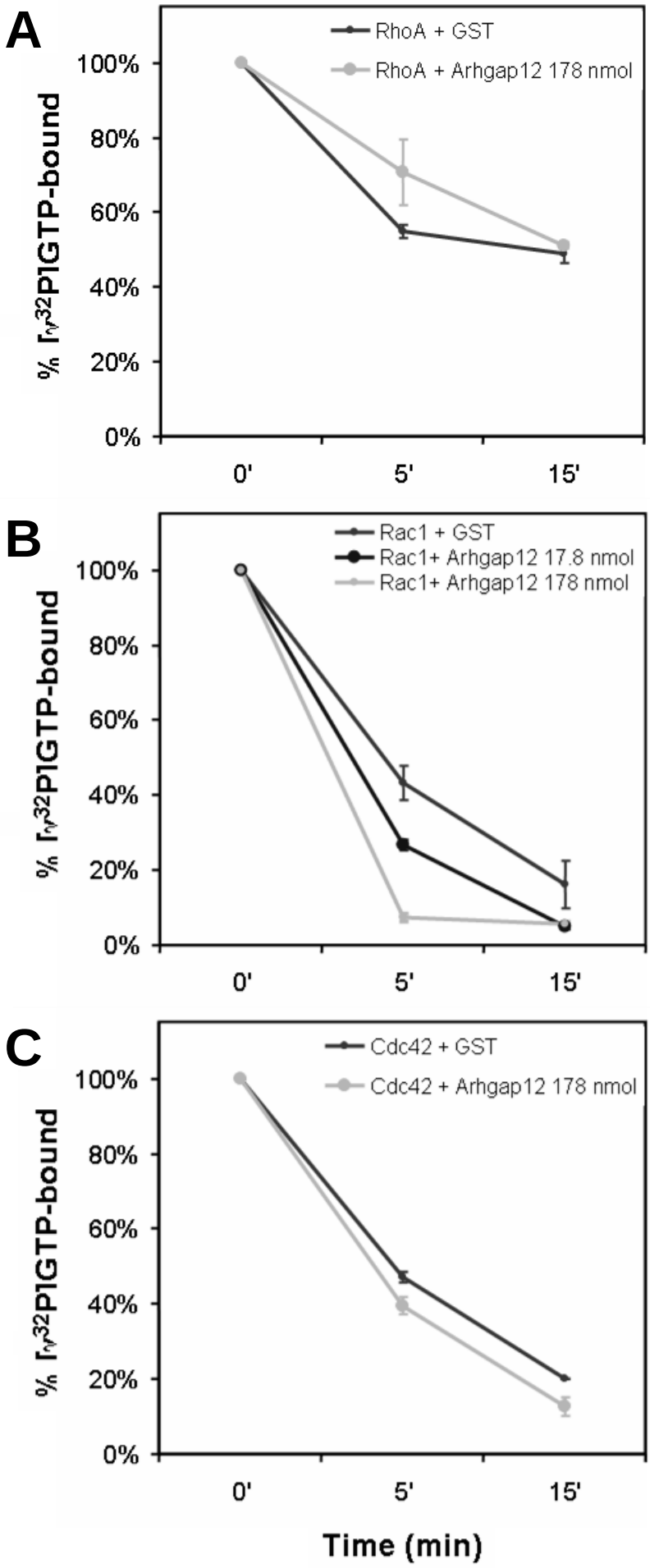
with Arhgap12-GFP, or with Arhgap12 shRNA or with scrambled shRNA (CTRL), pretreated for 1 hour with HGF or control medium (NS). The figure shows the means and standard deviations of three independent experiments. Arbitrary Units indicate the surface occupied by cells as quantified using the Metamorph software. (C) Invasion assay on matrigel-coated Transwells, performed on MDCK cells either transduced with Arhgap12-GFP, or with Arhgap12 shRNA or with scrambled shRNA (CTRL) in the absence (NS) or presence of HGF for 24 hours, as indicated. The figure shows the means and standard deviations of three independent experiments. Arbitrary Units indicate the surface occupied by cells as quantified using the Metamorph software.




*Gentile et al., Figure 1*

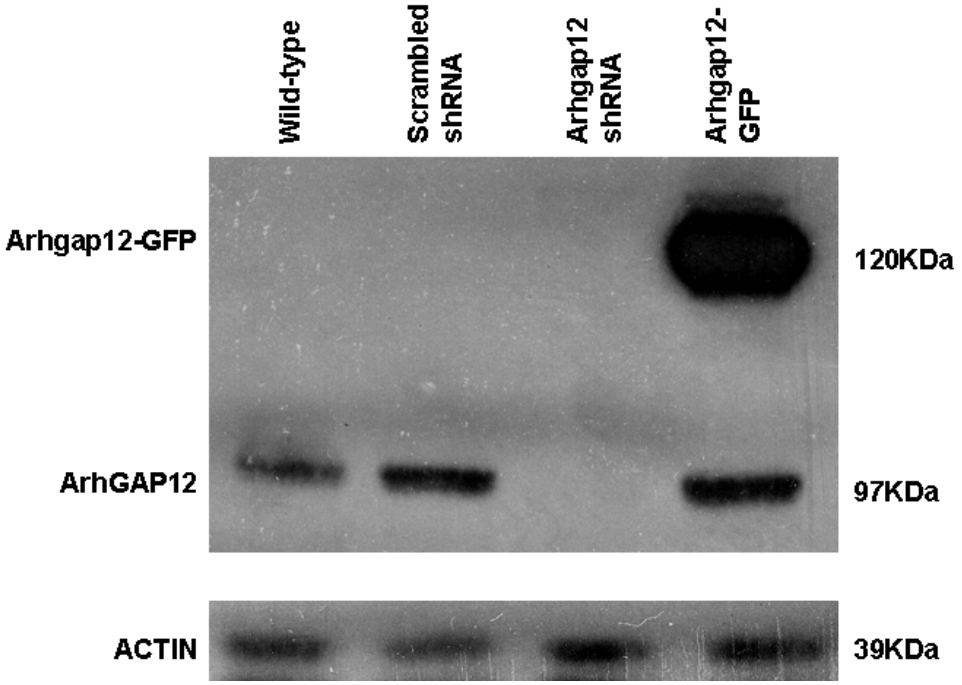



*Gentile et al., Figure 2*




*Gentile et al., Figure 3*

A

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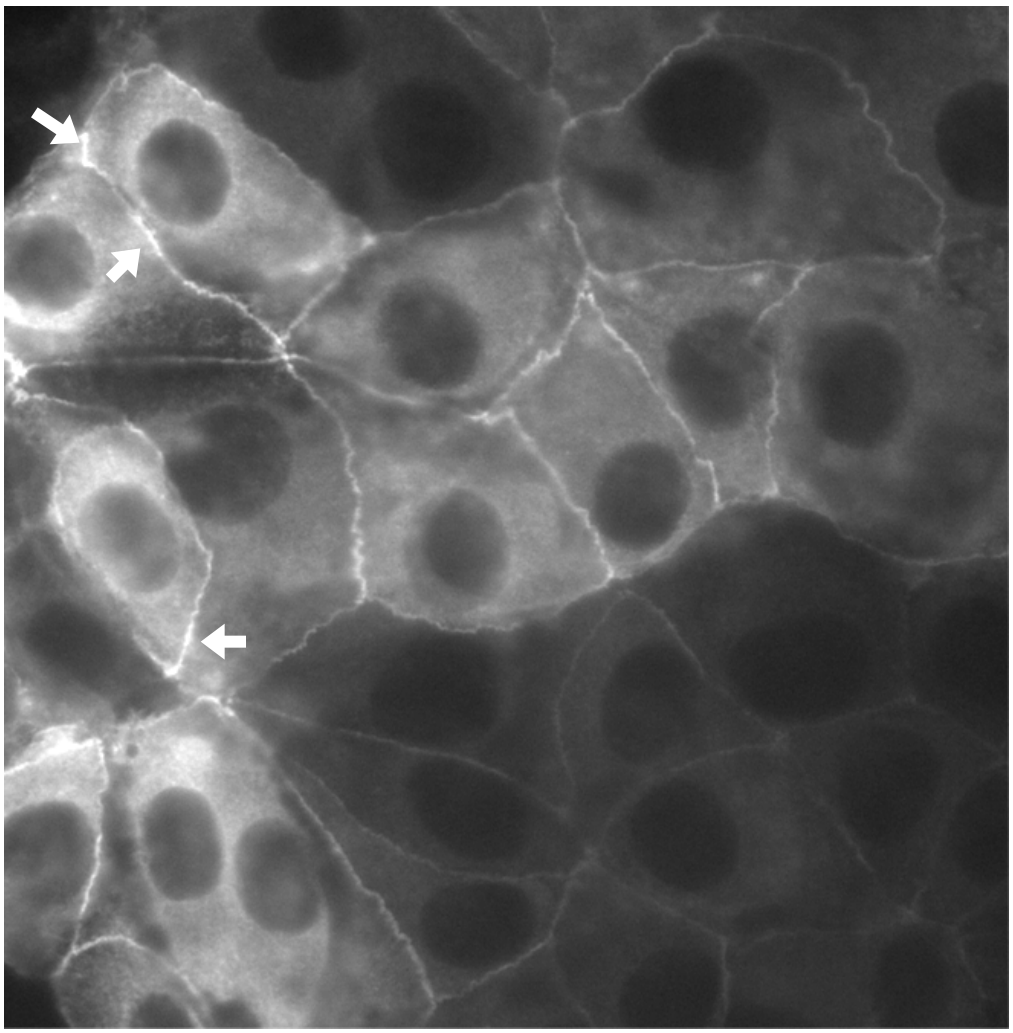
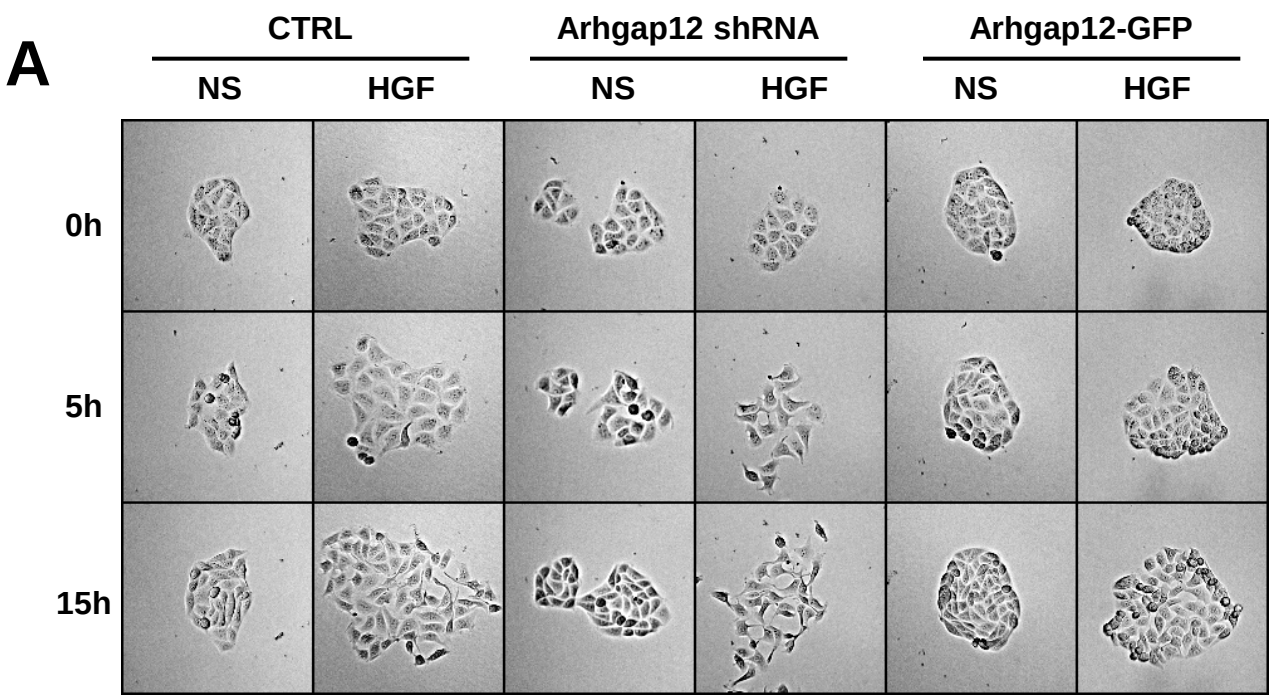
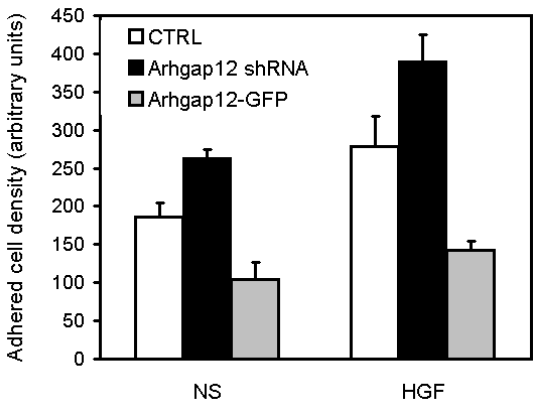
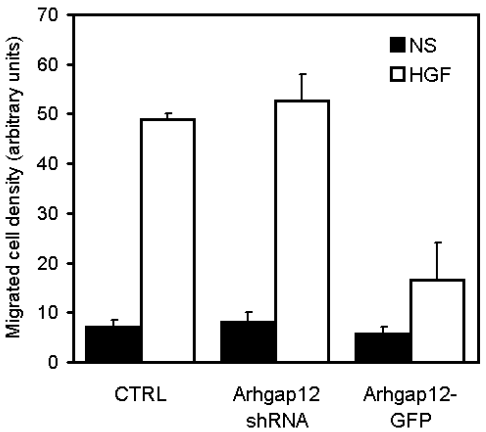


*Gentile et al., Figure 4*




*Gentile et al., Figure 5*