Published in final edited form as:

Biochem Cell Biol. 2019 October ; 97(5): 638–646. doi:10.1139/bcb-2018-0367.

Effect of Caveolin-1 upon Stat3-tyr705 levels in breast and lung carcinoma cells

Mulu Geletu^{a,b,c}, Zaid Taha^d, Rozanne Arulanandam^d, Reva Mohan^a, Hikmat H. Assi^{e,*}, Maria G. Castro^e, Ivan Robert Nabif, Patrick T. Gunning^{b,c}, Leda Raptis^a

^aDepartment of Biomedical and Molecular Sciences, Pathology and Molecular Medicine, and Queen's University Cancer Research Institute, Queen's University, Kingston, Ontario, Canada K7L 3N6.

^bDepartment of Chemical and Physical Sciences, University of Toronto Mississauga, 3359 Mississauga Rd N., Mississauga, Ontario L5L 1C6, Canada

^cDepartment of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ontario, Canada

^dOttawa Hospital Research Institute – Cancer Therapeutics, 501 Smyth Road, Ottawa, ON, K1H 8L6

^eDepartment of Neurosurgery and Department of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, MI 48019, USA.

^fDepartment of Cellular and Physiological Sciences, Life Sciences Institute, University of British Columbia, Vancouver, BC, Canada V6T 1Z3

Abstract

We recently demonstrated that Cav1 is a negative regulator of Stat3 activity in mouse fibroblasts and human lung carcinoma SHP77 cells. We now examined whether the cellular context may affect their levels as well as the relationship between them, by assessing Cav1 and Stat3-tyr705 amounts in different lines.

In MDA-MB-231, A549 and HaCat cells Cav1 levels were high and Stat3-tyr705 levels were low, consistent with the notion of a *negative* effect of the endogenous Cav1 upon Stat3-tyr705 levels in these lines. In addition, manipulation of Cav1 levels revealed a *negative* effect in MCF7 and mouse fibroblast cells, while Cav1 upregulation induced apoptosis in MCF7 cells. In contrast however, line MRC9 had high Cav1 and high Stat3-tyr705 levels, indicating that the high Cav1 is insufficient to reduce Stat3-tyr705 levels in this line. MCF7 and LuCi6 cells had very low Cav1 and Stat3-tyr705 levels, indicating that the low Stat3-tyr705 can be independent from Cav1 levels altogether.

Our results reveal a further level of complexity in the relationship between Cav1 and Stat3-tyr705 than previously thought. In addition, we demonstrate that, in a feedback loop, Stat3 inhibition upregulates Cav1 in HeLa cells but not in other lines tested.

Corresponding author: Dr. Mulu Geletu, **Address:** Department of Chemical and Physical Sciences, University of Toronto Mississauga, 3359 Mississauga Rd N., Mississauga, Ontario L5L 1C6, Canada, **Phone:** 905-569-4588, mulu.geletu@utoronto.ca.

*Present Address: Cytomx Therapeutics, 151 Oyster point Blvd. South San Francisco, CA 94080

Introduction

The Signal Transducer and Activator of Transcription-3 (Stat3) is activated by receptor and non-receptor tyrosine kinases. Upon activation, Stat3 is phosphorylated at a critical tyrosine residue (tyr-705). Stat3-tyr705 subsequently dimerizes and migrates to the nucleus where it activates transcription of genes that play a role in cellular survival and proliferation (Yu et al. 2014). We and others also demonstrated that engagement of E-cadherin (Arulanandam et al. 2009), N-cadherin or cadherin-11 (Geletu et al. 2013a), as induced by cell confluence or aggregation, triggers a striking increase in Stat3, tyr705 phosphorylation, through Rac1/Cdc42, IL6 and Jak (Geletu et al. 2013b). Stat3 has been shown to play an important role in tumorigenesis, and in a mutationally activated form (Stat3C) Stat3 can transform cultured fibroblasts (Bromberg et al. 1999).

Caveolae (“little caves”) are cholesterol-rich, 50-100 nm Ω mega-shaped indentations of the plasma membrane, with caveolins (Cav1-3) embedded in their lipid bilayer (Goetz et al. 2008). Caveolae are known to have a number of functions in the cell, including the regulation of signal transduction. Cav1 has been reported to sequester and *in*activate a large number of membrane signalling molecules through binding to the scaffolding domain (CSD) of Cav1 (Boscher and Nabi 2012; Chiu et al. 2011).

The effect of Cav1 upon Stat3 is still controversial. Early results, using the cholesterol chelator, methyl- β -cyclodextrin (MCD) to inhibit Cav1 function suggested that Cav1 might actually be *required* for Stat3 activation by IL6, thus denoting a *positive* role of Cav1 upon Stat3 activity (Sehgal et al. 2002). Similarly, in cell lines from metastatic lesions of lung carcinoma with high Cav1 levels, Cav1 downregulation inhibited Stat3 and arrested proliferation (Pancotti et al. 2012). Still, it was also shown that lung tissues from Cav1 knockout mice displayed high Stat3-tyr705 levels (Jasmin et al. 2004) which points to a *negative* role of Cav1 upon Stat3.

In all of the above publications the effect of confluence of cultured cells upon Stat3 activity was not taken into account. Since density can trigger a dramatic increase in Stat3-tyr705 levels, we recently revisited the question of the effect of Cav1 upon Stat3 in mouse fibroblasts and the human lung carcinoma SHP77 line, by testing cells at a range of densities. The results demonstrated that Cav1 downregulates Stat3-tyr705 through downregulation of cadherin-11 in these cells. This argues that Cav1 has a *negative* regulatory effect upon the cadherin-11/Stat3 axis (Geletu et al. 2018), and is in agreement with data from Cav1 knockout mice.

Since a variety of factors can affect both Cav1 and Stat3-tyr705 levels, we attempted to examine whether the cellular context might affect their intrinsic levels as well as the relationship between them. Our results reveal a further level of complexity in the relationship between Cav1 and Stat3-tyr705 than previously appreciated, which might account for some of the controversy in the literature. In addition, we demonstrate that, in a feed back loop, Stat3 inhibition upregulates Cav1 in HeLa cells but had no significant effect upon Cav1 levels in a number of other lung and breast cancer lines tested.

Materials and Methods

Cell lines, culture techniques and gene expression

The breast cancer lines (MDA-231, MDA-468 and MCF-7), lung cancer lines (A549, SHP77 and SK-Luci6), non-transformed human lung fibroblast line (MRC9), brain glioma line (U87), pancreatic cancer cell line (Panc-1) and the human cervical cancer line (HeLa) were purchased from ATCC. A query of the Cancer Cell Line Encyclopedia (CCLE) database revealed no Stat3 or Cav1 mutations in any of the lines examined.

All cell lines were grown as described (Geletu et al. 2018). Briefly, all lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Sigma), apart from SHP77 and MRC9 that were grown in RPMI-1640 supplemented with 10% FBS and antibiotics. Human keratinocytes (HaCat), were kindly provided by Dr. Diamandis (University of Toronto). Mouse fibroblast lines (Balb/c3T3, 10T1/2, and NIH3T3) have been described previously (Geletu et al. 2013a; Raptis et al. 1997; Raptis et al. 1985).

Cell confluence was estimated visually and quantitated by imaging analysis of live cells under phase contrast using a Leitz Diaplan microscope and the MCID-elite software (Imaging Research, St. Catharine's Ont.). Since cell to cell contact was shown to increase Stat3-tyr705 levels and activity, extra care was taken to ensure that the cells were evenly spread on the petri surface.

Fluorescence and phase-contrast digital images of EGFP- or mRFP-expressing cells were taken with a Nikon IX70 microscope equipped with epifluorescence or with a *BioTek Cytation 3* Cell Imaging Multi-Mode Reader.

MCF7 cells were transfected with Cav1-myc-mRFP or Cav1-F92A/Y94A myc-mRFP plasmids (Joshi et al. 2012; Meng et al. 2017), using the HilyMax transfection reagent (Dojindo Molecular Technologies, USA #H357-10), according to the manufacturer's protocol, and selected for G418-resistance. EGFP-Cav1 was expressed through transfection and puromycin resistance selection (Volonte et al. 1999).

For Cav1 downregulation, the MoMLV-based retroviral packaging line ψ 2 was used to generate two Cav1 shRNA retroviral vector particles (CAV1 MISSION shRNA TRCN0000112662 and TRCN0000112663, Sigma).

Sequence of Cav1-

shRNA1: CCGGCGACGTGGTCAAGATTGACTTCTCGAGAAGTCAATCTTGACCAC
GTCGTTTTTG

Sequence of Cav1-

shRNA2: CCGGTGAAGCTATTGGCAAGATATTCTCGAGAATATCTTGCCAATAGCTT
CATTTTTG

As control we used the MISSION shRNA plasmid control vector with the insert:
CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTGTTT

TT (Sigma #SHC002 MISSION pLKO.1-puro Non-Mammalian shRNA control vector with backbone: TRC1/1.5, insert: Non human or mouse shRNA). No effect upon Cav1 or Stat3-
ptyr705 levels was ever detected with this control, compared to untransfected cells.

For retroviral vector production, ψ 2 cells were grown in DMEM with 5% calf serum. Following Calcium-phosphate transfection, stable clones were obtained with selection for puromycin resistance and the culture supernatant was used as virus stock to infect mouse 10T1/2 fibroblasts (Vultur et al. 2005). Phoenix ecotropic cells were also used for transient production of retroviral vectors as described (Geletu et al. 2013a).

Adenoviral vectors: We used first generation, recombinant adenoviral vectors (serotype 5), Stat3 shRNA (Ad-Stat3i) and a scrambled Stat3 shRNA as a control (Ad-Stat3scr). The methods for adenoviral generation, purification, characterization and scale up have been previously described by our lab (Southgate 2000). HEK-293 cells were obtained from Microbix (Toronto, CA) and cultured in MEM supplemented with non-essential amino acids, 1% Pen-Strep, 1% L-Glutamine, (all from CellGro, Herndon, VA) and 10% FCS (Omega Scientific, Tarzana, CA). To generate Ad-Stat3i and Ad-Stat3scr, oligonucleotides encoding the shRNA were annealed (Stat3: Forward 5'-
GAGTCAGGTTGCTGGTCAAATTCAAGAGATTTGACCAGCAACCTGACTTTTTTCT
GCA-3' Reverse 5'-
GAAAAAAGTCAGGTTGCTGGTCAAATCTCTTGAATTTGACCAGCAACCTGACTC-3
' ; SOCS1: Forward 5'-
GATaAcGgTtCgtGggcAATTCAAGAGAAgcccCacGaAcCgTtAaTTTTTTCTGCA-3'
Reverse 5'-
GAAAAAAtTaAcGgTtCgtGggcTTTCTCTTGAATTgccCacGaAcCgTtAaTC-3') and ligated into pBSENU6-shRNA (Zhou et al. 2005). Assessment of effective inhibition of Stat3 expression and activity elicited by the Stat3 shRNA used to generate the Ad vector was performed as described in Supp Figure 1. To generate the Ad/U6-shR-Stat3/CMV-EGFP vector, the CMV promoter and enhanced green fluorescent fragment (CMV-EGFP) were cut from pcDNA-EGFP and inserted into XhoI and EcoRV sites of pDeltaE1sp1A (Microbix Biosystems, Inc., Toronto, Ontario, Canada). This generates the plasmid pDeltaE1sp1A/CMV-EGFP. The U6-shRNA-Stat3 expression cassette was then cut from pBSENU6-shRNA (Zhou et al. 2005) and inserted into the XhoI and PmeI sites of pDeltaE1sp1A/CMV-EGFP to generate Ad/CMV-EGFP/U6-Stat3shRNA. The Ad/CMV-EGFP/U6-STAT3scr was generated using a similar method except using the scrStat3 RNA.

Adenoviral vectors were scaled up by infecting HEK 293 cells with a multiplicity of infection of three plaque-forming units (pfu)/cell of vector seed stock. The cells were harvested 48 h later, lysed with 5% deoxycholate and deoxyribonuclease I, and the Ads were purified by ultracentrifugation over two cesium chloride step gradients. Vectors were titrated in triplicate by end-point dilution cytopathic effect assay and screened for the presence of replication-competent adenovirus (RCA) and for lipopolysaccharide (LPS) contamination (Cambrex, East Rutherford, NJ). Vector preparations were free from RCA and LPS contamination. HeLa cells were infected with both vectors at a multiplicity of 20 pfu/cell.

Stat3 inhibition was performed using the SH4-54 (Haftchenary et al. 2013) or CPA7 (Littlefield et al. 2008) inhibitors.

For apoptosis assays, PARP-1 cleavage assay was performed using the anti-cleaved PARP1 antibody (abcam #ab72805). As a control, apoptosis was induced in SHP77 cells through treatment with 1 μ M staurosporin (BioShop #STA001.1) for two hours.

Cell lysis and Western blotting were performed as before (Geletu et al. 2018). Briefly, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer: 20 mM Tris pH 7.4, 150 mM NaCl, 0.5% Sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS). Total protein was measured using the BCA Protein assay kit, (Pierce). 30 μ g of total protein of clarified extracts were resolved on a 4%-15% polyacrylamide-SDS gel and transferred to a PVDF membrane (BioRad). Membranes were blocked with 5% skim milk powder in TBST then cut to strips and incubated overnight at 4°C in primary antibody at a 1:1,000 dilution. Primary antibodies used were against: Cav1 (abcam, #ab2910), Stat3-tyr705 (Abcam, #ab76315), total Stat3 (Cell signaling #4904) and β -actin or Hsp90 as loading controls (Santa Cruz Biotechnology, # sc-835 and # sc-6970, respectively). Following washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibodies (Cell signaling, # 7076S) or fluorescently labeled secondary antibodies [Anti Rabbit IgG Alexa Fluor 488 (Cell signaling #4412s), anti mouse IgG Alexa Fluor (Cell signaling # 4410s), Anti mouse IgG Alexa Fluor 488 (Cell signaling # 4408s) or Anti rabbit IgG Alexa Fluor (Cell signaling # 4414s)], for 1hr at room temperature. Following washing, bands were visualized using clarity western ECL substrate luminal/enhancer solution and peroxide solution at 1:1 ratio for HRP secondary antibodies according to the manufacturer's instructions (BioRad, Cat# 102030882) then images captured and analyzed using Image lab software ChemiDoc™MP Imaging system (BioRad), or exposed to X-ray film. Representative results are presented for all experiments. When indicated, results are also given as means plus $\pm$ SEM of at least 3 independent experiments. The *P* value was calculated using the Student *t* test by comparing the means of 3 different independent experiments using graphpad prism software. A *P* value of 0.05 or less was considered significant.

Results

Cav1 vs Stat3-tyr705 levels in breast and lung cancer lines

We recently demonstrated that Cav1 downregulates cadherin-11, hence Rac and Stat3-tyr705 in mouse fibroblasts and human lung carcinoma SHP77 cells (Geletu et al. 2018). To examine whether the cellular context may affect the relationship between Cav1 and Stat3 activity, which might account for some of the controversy in the literature, we explored the intrinsic correlations between Cav1 and Stat3-tyr705 levels in a number of commonly studied breast and lung carcinoma lines. To avoid the complication of cell to cell adhesion-mediated Stat3 activation, cells were grown and extracted at 50% confluence. Cav1 and Stat3-tyr705 levels which correlate with Stat3 transcriptional activity (Geletu et al. 2013b), were subsequently examined by Western blotting (see Materials and Methods). As shown in Fig. 1, the breast cancer line MCF7 (Joshi et al. 2008; Kojic et al. 2007) (lane 3) and lung carcinoma SK-Luci6 (lane 5) expressed very low Cav1 and Stat3-tyr705 levels. On the

other hand, the breast cancer line MDA-MB-231 (Joshi et al. 2008; Kojic et al. 2007) (lane 1), A549 (lane 4), HaCat (lane 7), and mouse nontransformed Balb/c and NIH3T3 fibroblasts (lanes 10 - 11), expressed high Cav1 levels and very low Stat3-tyr705. The breast cancer line MDA-MB-468 (lane 2) and the pancreatic Panc-1 (lane 8) expressed low but significant amounts of Cav1, but very low Stat3-tyr705 levels. The U87 cell line (lane 6) had a significant amount of Cav1 and high Stat3-tyr705. The non-transformed Human lung fibroblast (MRC-9, lane 9) line had high Cav1 and high Stat3-tyr705. Taken together, these results demonstrate that the endogenous Cav1 levels, as well as the ratio Cav1: Stat3-tyr705 levels can vary substantially with the cell line (Table I). Together with the effect of density, this could, at least in part, account for the conflicting results in the literature regarding the interrelationship between Cav1 levels and Stat3 activity.

Negative regulation of Stat3-tyr705 by Cav1 in mouse fibroblasts and MCF7 breast carcinoma cells

The effect of Cav1 upon Stat3-tyr705 in different lines was examined next, by manipulating Cav1 protein levels. Since cell confluence was shown to have a dramatic effect upon Stat3 activity [(Arulanandam et al. 2009; Geletu et al. 2013a; Vultur et al. 2004) reviewed in (Geletu et al. 2013b; Raptis et al. 2009)], a fact that was not taken into account in most previous publications, we now examined Stat3-tyr705 levels at a range of cell densities from 100% of confluence to 100% plus two days, the range of densities where a dramatic increase in Stat3-tyr705 is observed in most lines.

Cav1 was knocked down with two different shRNAs, expressed with retroviral vectors in mouse 10T1/2 fibroblasts, followed by examination of their effect upon Stat3, tyr705 phosphorylation in independent clones (see Materials and Methods). As shown in Fig. 2, cell density *per se* triggered a dramatic increase in Cav1 levels (top panel, lanes 1 vs 2 and 3, 4 vs 5 and 6, 7 vs 8 and 9). Interestingly, expression of either of the two shCav1 constructs resulted in higher levels of Stat3-tyr705 at all densities examined (lanes 1-3 vs 4-9, middle panel), consistent with previous data from Balb/c3T3 cells (Geletu et al. 2018). Taken together, the above findings indicate that density triggers an increase in Cav1, while a reduction in Cav1 levels results in an increase in Stat3, tyr705 phosphorylation in mouse fibroblasts.

We next examined the effect of Cav1 overexpression upon Stat3-tyr705. However, a further increase in Cav1 levels in mouse fibroblast lines which express high Cav1 (Fig. 1), was found to be lethal to these cells. Therefore, to examine the effect of Cav1 overexpression we made use of MCF7, breast carcinoma cells, which naturally express low Cav1 levels (Fig. 1). Cav1 was introduced through transfection as a fusion protein with Green Fluorescence Protein (EGFP-Cav1) or red fluorescence protein (Cav1-mRFP). As shown in Fig. 3A (left panel), stable expression of either protein (lanes 1 and 2 vs 3, and 4 vs 6) triggered a significant reduction in Stat3-tyr705 levels. On the other hand, expression of the CSD mutant (Cav1-F92A/Y94A fused to mRFP) had no effect upon Stat3-705 (lane 5 vs 4), pointing to a requirement for the CSD domain. Taken together, the above findings argue for Cav1 as a **negative** regulator of Stat3 activity in MCF7 cells, by a mechanism requiring the CSD domain.

Cav1 overexpression triggers apoptosis in MCF7 and SHP77 cells

Studies from a number of labs have shown that Stat3 increases transcription of genes promoting survival such as *Bcl-xL*, *Mcl-1* and *survivin* (Catlett-Falcone et al. 1999; Epling-Burnette et al. 2001) through direct promotor binding, as well as by mitochondrial effects (Demaria and Poli 2011; Gao et al. 2012; Gough et al. 2009; Wegrzyn et al. 2009). Since Cav1 downregulates Stat3, we examined the effect of stable expression of Cav1-mRFP or the Cav1-CSD-mRFP mutant, upon apoptosis in MCF7 breast carcinoma and SHP77 lung carcinoma cells. As shown in Fig. 3B, expression of Cav1-mRFP triggered apoptosis in MCF7 cells which was clearly visible by phase-contrast and fluorescence microscopy (e, f), while expression of the CSD mutant had no effect (c, d). PARP cleavage analysis (Fig. 3C) showed apoptosis upon expression of Cav1-mRFP (lane 4), while expression of the CSD mutant-mRFP did not reveal any PARP cleavage fragment (lane 3).

The experiments were repeated with the lung carcinoma line SHP77. As shown in Fig. 3E, Cav1mRFP expression triggered apoptosis, by a mechanism requiring the CSD domain (a vs c). In addition, expression of the constitutively active mutant, Stat3C by transfection and puromycin-resistance selection reversed the apoptotic effect of Cav1 overexpression, pointing to a role for Stat3 (panel e, f). Taken together, the above data indicate that Cav1 overexpression reduces Stat3-705 levels and triggers apoptosis in both MCF7 and SHP77 cells, and that the scaffolding domain is required for this effect.

Downregulation of Stat3 upregulates Cav1 levels in HeLa cells

It was previously demonstrated that Stat3 binds to and downregulates the p53 promotor (Niu et al. 2005). Since p53 upregulates Cav1 (Razani et al. 2000), we examined the possibility that, in a feedback loop, Stat3 downregulation might, in fact, upregulate Cav1 levels. To this effect, Stat3 levels were downregulated through expression of shStat3 RNA with an Adenoviral vector in HeLa human cervical carcinoma cells (see Materials and Methods and Supplementary data, Fig. S1). HeLa cells were infected with the Adenovirus vector and three days thereafter detergent cell extracts were probed for Stat3-tyr705 and Cav1. As shown in Fig. 4, B, vector expression caused a dramatic increase in Cav1 levels in HeLa cells, while the same vector expressing a control, scrambled sequence had no effect (lane 2 vs 3). Stat3 inhibition with the SH4-54 inhibitor (Haftchenary et al. 2013) gave similar results (Fig. 4C, lanes 1 vs 3).

To examine the generality of this phenomenon, we reduced Stat3 activity with SH4-54 in A549, SK-LuCi6, H1299, SW1573 and MCF7 cells. As shown in Fig. 4D, treatment with the SH4-54 inhibitor did not detectably increase Cav1 levels. Similar results were obtained with the CPA7 Stat3 inhibitor [(Littlefield et al. 2008), not shown]. Taken together, these findings indicate that, in a feedback loop, Stat3 also downregulates Cav1 in HeLa cells but not in the breast or lung carcinoma lines tested.

Discussion

The effect of Cav1 upon Stat3 is a matter of controversy. To explore the reasons for the apparent discrepancies, we examined Cav1 and Stat3-tyr705 levels in a number of

extensively studied breast and lung cancer lines. The results revealed a variation in the naturally occurring levels of expression of both Cav1 and Stat3-tyr705, which might account for their different behavior upon manipulation of Cav1 or Stat3 levels as well.

Regarding the role of Cav1 in signal transduction, an early model proposed by Galbiati, Lisanti *et al* (Williams and Lisanti 2005), postulates that caveolae normally sequester and inactivate membrane signalling molecules such as a large number of receptor tyrosine kinases and components of signalling pathways, through binding to the CSD domain of Cav1. As a result, a reduction in Cav1 levels through shRNA expression increases the activity of Erk1/2, the downstream effector of a number of tyrosine kinases (Chen and Resh 2002; Galbiati et al. 1998), although other receptors such as the Insulin receptor, are positively regulated through Cav1 interaction (Vihanto et al. 2006; Yamamoto et al. 1998). In any event, the inhibitory effect of Cav1 can be subverted by phosphorylation by Src or other tyrosine kinases at Cav1-tyr14, which then forms a binding site for Grb7 and perhaps other signal transducers, with an increase in signal strength as a result (Galbiati et al. 1998; Patel et al. 2008). Thus, although Cav1 downregulation causes hyperactivation of Erk1/2 signalling in non-neoplastic NIH3T3 fibroblasts (Galbiati et al. 1998), and Cav1 ablation renders these cells more susceptible to transformation by oncogenes (Williams et al. 2004), Cav1 levels were also found to be increased at later stages of tumor progression in certain metastatic tumors (Yang et al. 1998). Therefore, different activation levels of various receptors and oncogenes such as Src, Grb7 and possibly other signal transducers in the different lines examined may explain at least in part their differences in Cav1 and Stat3-tyr705 levels observed.

Findings by Quest *et al* demonstrated that Cav1 expression reduces mRNA and protein levels of the IAP protein, survivin, through association of Cav1 with β -catenin, and that E-cadherin is required for this effect (Torres et al. 2007; Torres et al. 2006). We recently demonstrated that Cav1 may downregulate cadherins, potent Stat3 activators (Geletu et al. 2018), while Stat3, in turn, activates the survivin promoter (Gritsko et al. 2006). It follows that the reduction in survivin upon Cav1 upregulation may be mediated, at least in part, by E-cadherin/Stat3. Thus, through its effect upon the cadherin/Stat3/survivin axis, Cav1 may behave as a conditional tumor suppressor depending on the presence and potentially the levels of cadherins. In addition, a competition between the galectin lattice and oligomerized Cav1 microdomains for EGF receptor recruitment was shown to regulate EGFR signaling (and potentially Stat3 activity) in tumor cells (Goetz et al. 2008; Lajoie et al. 2007). In mammary tumor cells deficient for the *Mgat5* gene coding for β 1, 6N-acetylglucosaminyltransferase V, the reduction in EGFR binding to the galectin lattice permits the association of EGFR with Cav1, and suppression of EGFR signaling. Therefore, the Cav1/galectin balance could be key for receptor signaling, including Stat3-tyr705 levels. Whether such differences in oncogenes expressed, cadherins or *Mgat5* activity levels might be attributable to actual differences in stage along the carcinogenic progression of the cell lines described requires further investigation.

We also show that Stat3 downregulation in HeLa cells causes an increase in Cav1 levels, although there was no detectable increase in other lines (A549, SKLuCi6, H1299, SW1573 and MCF7). Two possible pathways could account for the Cav1 increase upon Stat3

inhibition: First, Stat3 was shown to be able to downregulate the Cav1 promotor through direct binding (Chiu et al. 2011). Second, Stat3 inhibits transcription of the p53 tumor suppressor through direct promotor binding (Niu et al. 2005), while p53 up-regulates caveolin-1 gene expression (Razani et al. 2000), so that Stat3 would downregulate Cav1 through p53 downregulation. The exact mechanism is currently under investigation.

Our results also demonstrate that Cav1 downregulation triggers an increase in Stat3-ptyr705 in mouse fibroblasts. Our findings are at variance to previous results showing that Cav1 inactivation through treatment with the cholesterol chelator, methyl-cyclo-dextrin (MCD) reduces Stat3-ptyr705 levels (Sehgal et al. 2002). The fact that Stat3-ptyr705 levels are dramatically affected by density of cultured cells (Fig. 2), which was not taken into account in previous publications, together with the greater specificity of the Cav1-shRNA retroviral vectors, may explain the apparent controversy.

In conclusion, our results reveal a further level of complexity in the relationship between Cav1 and Stat3-ptyr705 than previously thought, with a strong dependence upon the cell line under study. In addition, we demonstrate that, in a feed-back loop, Stat3 inhibition upregulates Cav1 in HeLa cells but not in a number of other breast or lung cancer lines. Since Stat3 is an oncogene and potent survival factor, the effect of Cav1 upon Stat3 in a given tumor could inform diagnosis and treatment decisions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

The authors thank Dr. Ferruccio Galbiati for precious advice and reagents and Dr. Eleftherios Diamandis for the HaCat cells.

The financial assistance of the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canadian Institutes of Health Research (CIHR), the Canadian Breast Cancer Foundation (Ontario Chapter), the Canadian Breast Cancer Research Alliance, the Ontario Centres of Excellence, the Breast Cancer Action Kingston (BCAK) and the Clare Nelson bequest fund through grants to LR and CIHR PJT-148698 to IRN is gratefully acknowledged. MG was supported by postdoctoral fellowships from the US Army Breast Cancer Program, the Ministry of Research and Innovation of the Province of Ontario and the Advisory Research Committee of Queen's University. PTG is supported by a Canada Research Chair, Canadian Foundation for Innovation, CIHR, NSERC and Canadian Cancer Society. RA was supported by a Canada Graduate Scholarships Doctoral award from CIHR, the Ontario Women's Health Scholars Award from the Ontario Council on Graduate Studies and a QGA.

References

- Arulanandam R, Vultur A, Cao J, Carefoot E, Truesdell P, Elliott B, Larue L, Feracci H, and Raptis L 2009 Cadherin-cadherin engagement promotes survival via Rac/Cdc42 and Stat3. *Molecular Cancer Research* 17: 1310–1327.
- Assi HH, Paran C, VanderVeen N, Savakus J, Doherty R, Petruzzella E, Hoeschele JD, Appelman H, Raptis L, Mikkelsen T, Lowenstein PR, and Castro MG 2014 Preclinical characterization of signal transducer and activator of transcription 3 small molecule inhibitors for primary and metastatic brain cancer therapy. *J. Pharmacol. Exp. Ther* 349(3): 458–469. doi: jpet.114.214619 [pii];10.1124/jpet.114.214619 [doi]. [PubMed: 24696041]
- Boscher C, and Nabi IR 2012 Caveolin-1: role in cell signaling. *Adv Exp Med Biol* 729: 29–50. doi: 10.1007/978-1-4614-1222-9_3. [PubMed: 22411312]

- Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, and Darnell JE Jr. 1999 Stat3 as an oncogene. *Cell* 98(3): 295–303. [PubMed: 10458605]
- Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernandez-Luna JL, Nunez G, Dalton WS, and Jove R 1999 Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* 10(1): 105–115. [PubMed: 10023775]
- Chen X, and Resh MD 2002 Cholesterol depletion from the plasma membrane triggers ligand-independent activation of the epidermal growth factor receptor. *J. Biol. Chem* 277(51): 49631–49637. [PubMed: 12397069]
- Chiu WT, Lee HT, Huang FJ, Aldape KD, Yao J, Steeg PS, Chou CY, Lu Z, Xie K, and Huang S 2011 Caveolin-1 upregulation mediates suppression of primary breast tumor growth and brain metastases by stat3 inhibition. *Cancer Res* 71(14): 4932–4943. doi: 0008-5472.CAN-10-4249 [pii];10.1158/0008-5472.CAN-10-4249 [doi]. [PubMed: 21622714]
- Demaria M, and Poli V 2011 From the nucleus to the mitochondria and back: the odyssey of a multitask STAT3. *Cell Cycle* 10(19): 3221–3222. doi: 17379 [pii];10.4161/cc.10.19.17379 [doi]. [PubMed: 21926478]
- Epling-Burnette PK, Liu JH, Catlett-Falcone R, Turkson J, Oshiro M, Kothapalli R, Li Y, Wang JM, Yang-Yen HF, Karras J, Jove R, and Loughran TP Jr. 2001 Inhibition of STAT3 signaling leads to apoptosis of leukemic large granular lymphocytes and decreased Mcl-1 expression. *J. Clin. Invest* 107(3): 351–362. [PubMed: 11160159]
- Galbiati F, Volonte D, Engelman JA, Watanabe G, Burk R, Pestell RG, and Lisanti MP 1998 Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. *EMBO J* 17(22): 6633–6648. [PubMed: 9822607]
- Gao X, Wang H, Yang JJ, Liu X, and Liu ZR 2012 Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. *Mol. Cell* 45(5): 598–609. doi: S1097-2765(12)00003-2 [pii];10.1016/j.molcel.2012.01.001 [doi]. [PubMed: 22306293]
- Geletu M, Arulanandam R, Chevalier S, Saez B, Larue L, Feracci H, and Raptis L 2013a Classical cadherins control survival through the gp130/Stat3 axis. *BBA-Molecular Cell Research* 1833: 1947–1959. [PubMed: 23541910]
- Geletu M, Guy S, Arulanandam R, Feracci H, and Raptis L 2013b Engaged for survival; from cadherin ligation to Stat3 activation. *Jaks-Stat* 2: e27363-27361–e27363-27368.
- Geletu M, Mohan R, Arulanandam R, Berger-Becvar A, Nabi IR, Gunning PT, and Raptis L 2018 Reciprocal regulation of the Cadherin-11/Stat3 axis by caveolin-1 in mouse fibroblasts and lung carcinoma cells. *Biochim Biophys Acta*. doi: 10.1016/j.bbamcr.2018.02.004.
- Goetz JG, Lajoie P, Wiseman SM, and Nabi IR 2008 Caveolin-1 in tumor progression: the good, the bad and the ugly. *Cancer Metastasis Rev* 27(4): 715–735. [PubMed: 18506396]
- Gough DJ, Corlett A, Schlessinger K, Wegrzyn J, Larner AC, and Levy DE 2009 Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. *Science* 324(5935): 1713–1716. [PubMed: 19556508]
- Gritsko T, Williams A, Turkson J, Kaneko S, Bowman T, Huang M, Nam S, Eweis I, Diaz N, Sullivan D, Yoder S, Enkemann S, Eschrich S, Lee JH, Beam CA, Cheng J, Minton S, Muro-Cacho CA, and Jove R 2006 Persistent activation of stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. *Clin. Cancer Res* 12(1): 11–19. [PubMed: 16397018]
- Haftchenary S, Luchman HA, Jouk AO, Veloso AJ, Page BD, Cheng XR, Dawson SS, Grinshtein N, Shahani VM, Kerman K, Kaplan DR, Griffin C, Aman AM, Al-Awar R, Weiss S, and Gunning PT 2013 Potent Targeting of the STAT3 Protein in Brain Cancer Stem Cells: A Promising Route for Treating Glioblastoma. *ACS Med. Chem. Lett* 4(11): 1102–1107. doi: 10.1021/ml4003138 [doi]. [PubMed: 24900612]
- Jasmin JF, Mercier I, Hnasko R, Cheung MW, Tanowitz HB, Dupuis J, and Lisanti MP 2004 Lung remodeling and pulmonary hypertension after myocardial infarction: pathogenic role of reduced caveolin expression. *Cardiovasc. Res* 63(4): 747–755. [PubMed: 15306231]

- Joshi B, Bastiani M, Strugnell SS, Boscher C, Parton RG, and Nabi IR 2012 Phosphocaveolin-1 is a mechanotransducer that induces caveola biogenesis via Egr1 transcriptional regulation. *J Cell Biol* 199(3): 425–435. doi: 10.1083/jcb.201207089. [PubMed: 23091071]
- Joshi B, Strugnell SS, Goetz JG, Kojic LD, Cox ME, Griffith OL, Chan SK, Jones SJ, Leung SP, Masoudi H, Leung S, Wiseman SM, and Nabi IR 2008 Phosphorylated caveolin-1 regulates Rho/ROCK-dependent focal adhesion dynamics and tumor cell migration and invasion. *Cancer Res* 68(20): 8210–8220. [PubMed: 18922892]
- Kojic LD, Joshi B, Lajoie P, Le PU, Cox ME, Turbin DA, Wiseman SM, and Nabi IR 2007 Raft-dependent endocytosis of autocrine motility factor is phosphatidylinositol 3-kinase-dependent in breast carcinoma cells. *J. Biol. Chem* 282(40): 29305–29313. [PubMed: 17690101]
- Lajoie P, Partridge EA, Guay G, Goetz JG, Pawling J, Lagana A, Joshi B, Dennis JW, and Nabi IR 2007 Plasma membrane domain organization regulates EGFR signaling in tumor cells. *J. Cell Biol* 179(2): 341–356. [PubMed: 17938246]
- Littlefield SL, Baird MC, Anagnostopoulou A, and Raptis L 2008 Synthesis, characterization and Stat3 inhibitory properties of the prototypical platinum(IV) anticancer drug, [PtCl₃(NO₂)(NH₃)₂] (CPA-7). *Inorg. Chem* 47(7): 2798–2804. [PubMed: 18269242]
- Meng F, Saxena S, Liu Y, Joshi B, Wong TH, Shankar J, Foster LJ, Bernatchez P, and Nabi IR 2017 The phospho-caveolin-1 scaffolding domain dampens force fluctuations in focal adhesions and promotes cancer cell migration. *Mol Biol Cell* 28(16): 2190–2201. doi: 10.1091/mbc.E17-05-0278. [PubMed: 28592633]
- Niu G, Wright KL, Ma Y, Wright GM, Huang M, Irby R, Briggs J, Karras J, Cress WD, Pardoll D, Jove R, Chen J, and Yu H 2005 Role of Stat3 in regulating p53 expression and function. *Mol. Cell Biol* 25(17): 7432–7440. [PubMed: 16107692]
- Pancotti F, Roncuzzi L, Maggolini M, and Gasperi-Campani A 2012 Caveolin-1 silencing arrests the proliferation of metastatic lung cancer cells through the inhibition of STAT3 signaling. *Cell Signal* 24(7): 1390–1397. doi: S0898-6568(12)00074-5 [pii];10.1016/j.cellsig.2012.02.015 [doi]. [PubMed: 22406084]
- Patel HH, Murray F, and Insel PA 2008 Caveolae as organizers of pharmacologically relevant signal transduction molecules. *Annu. Rev. Pharmacol. Toxicol* 48: 359–391. [PubMed: 17914930]
- Raptis L, Arulanandam R, Vultur A, Geletu M, Chevalier S, and Feracci H 2009 Beyond structure, to survival: Stat3 activation by cadherin engagement. *Biochemistry and Cell Biology* 87: 835–843. [PubMed: 19935869]
- Raptis L, Brownell HL, Wood K, Corbley M, Wang D, and Haliotis T 1997 Cellular ras gene activity is required for full neoplastic transformation by Simian Virus 40. *Cell Growth Differ* 8: 891–901. [PubMed: 9269898]
- Raptis L, Lamfrom H, and Benjamin TL 1985 Regulation of cellular phenotype and expression of polyomavirus middle T antigen in rat fibroblasts. *Mol. Cell. Biol* 5: 2476–2486. [PubMed: 2426583]
- Razani B, Altschuler Y, Zhu L, Pestell RG, Mostov KE, and Lisanti MP 2000 Caveolin-1 expression is down-regulated in cells transformed by the human papilloma virus in a p53-dependent manner. Replacement of caveolin-1 expression suppresses HPV-mediated cell transformation. *Biochemistry* 39(45): 13916–13924. [PubMed: 11076533]
- Sehgal PB, Guo GG, Shah M, Kumar V, and Patel K 2002 Cytokine signaling: STATS in plasma membrane rafts. *J. Biol. Chem* 277(14): 12067–12074. [PubMed: 11815625]
- Southgate T, Kingston P, and Castro MG 2000 Gene transfer into neural cells in vivo using adenoviral vectors *In Current Protocols in Neuroscience. Edited by* Gerfen CR, McKay R, Rogawski MA, Sibley DR, Skolnick P John Wiley and Sons, New York, New York, NY pp. 4.23.21–24.23.40.
- Torres VA, Tapia JC, Rodriguez DA, Lladser A, Arredondo C, Leyton L, and Quest AF 2007 E-cadherin is required for caveolin-1-mediated down-regulation of the inhibitor of apoptosis protein survivin via reduced beta-catenin-Tcf/Lef-dependent transcription. *Mol Cell Biol* 27(21): 7703–7717. doi: 10.1128/MCB.01991-06. [PubMed: 17785436]
- Torres VA, Tapia JC, Rodriguez DA, Parraga M, Lisboa P, Montoya M, Leyton L, and Quest AF 2006 Caveolin-1 controls cell proliferation and cell death by suppressing expression of the inhibitor of

- apoptosis protein survivin. *J Cell Sci* 119(Pt 9): 1812–1823. doi: 10.1242/jcs.02894. [PubMed: 16608879]
- Vihanto MM, Vindis C, Djonov V, Cerretti DP, and Huynh-Do U 2006 Caveolin-1 is required for signaling and membrane targeting of EphB1 receptor tyrosine kinase. *J Cell Sci* 119(Pt 11): 2299–2309. doi: 10.1242/jcs.02946. [PubMed: 16723736]
- Volonte D, Galbiati F, and Lisanti MP 1999 Visualization of caveolin-1, a caveolar marker protein, in living cells using green fluorescent protein (GFP) chimeras. The subcellular distribution of caveolin-1 is modulated by cell-cell contact. *FEBS Lett* 445(2-3): 431–439. [PubMed: 10094502]
- Vultur A, Arulanandam R, Turkson J, Niu G, Jove R, and Raptis L 2005 Stat3 is required for full neoplastic transformation by the Simian Virus 40 Large Tumor antigen. *Molecular Biology of the Cell* 16: 3832–3846. [PubMed: 15917293]
- Vultur A, Cao J, Arulanandam R, Turkson J, Jove R, Greer P, Craig A, Elliott BE, and Raptis L 2004 Cell to cell adhesion modulates Stat3 activity in normal and breast carcinoma cells. *Oncogene* 23: 2600–2616. [PubMed: 15007380]
- Wegrzyn J, Potla R, Chwae YJ, Sepuri NB, Zhang Q, Koeck T, Derecka M, Szczepanek K, Szelag M, Gornicka A, Moh A, Moghaddas S, Chen Q, Bobbili S, Cichy J, Dulak J, Baker DP, Wolfman A, Stuehr D, Hassan MO, Fu XY, Avadhani N, Drake JI, Fawcett P, Lesnefsky EJ, and Lerner AC 2009 Function of mitochondrial Stat3 in cellular respiration. *Science* 323(5915): 793–797. [PubMed: 19131594]
- Williams TM, Lee H, Cheung MW, Cohen AW, Razani B, Iyengar P, Scherer PE, Pestell RG, and Lisanti MP 2004 Combined loss of INK4a and caveolin-1 synergistically enhances cell proliferation and oncogene-induced tumorigenesis: role of INK4a/CAV-1 in mammary epithelial cell hyperplasia. *J. Biol. Chem* 279(23): 24745–24756. [PubMed: 15044451]
- Williams TM, and Lisanti MP 2005 Caveolin-1 in oncogenic transformation, cancer, and metastasis. *Am. J. Physiol Cell Physiol* 288(3): C494–C506. [PubMed: 15692148]
- Yamamoto M, Toya Y, Schwencke C, Lisanti MP, Myers MG Jr., and Ishikawa Y 1998 Caveolin is an activator of insulin receptor signaling. *J Biol Chem* 273(41): 26962–26968. [PubMed: 9756945]
- Yang G, Truong LD, Timme TL, Ren C, Wheeler TM, Park SH, Nasu Y, Bangma CH, Kattan MW, Scardino PT, and Thompson TC 1998 Elevated expression of caveolin is associated with prostate and breast cancer. *Clin. Cancer Res* 4(8): 1873–1880. [PubMed: 9717814]
- Yu H, Lee H, Herrmann A, Buettner R, and Jove R 2014 Revisiting STAT3 signalling in cancer: new and unexpected biological functions. *Nat. Rev. Cancer* 14(11): 736–746. doi: nrc3818 [pii];10.1038/nrc3818 [doi]. [PubMed: 25342631]
- Zhou H, Xia XG, and Xu Z 2005 An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. *Nucleic Acids Res* 33(6): e62. doi: 10.1093/nar/gni061. [PubMed: 15805121]

A **B**

Figure 1: Cav1 vs Stat3-tyr705 levels in cancer and nontransformed lines

A: The indicated cell lines were grown to 50% confluence. Cells were lysed and total protein extracted. The lysates were resolved by gel electrophoresis and western blots probed for total Cav1, Stat3-tyr705, total Stat3 and β-actin as a loading control. Note the variation in Cav1 and Stat3-tyr705 levels in the different lines.

B: Graphical presentation of the values obtained above.

A **B**

Figure 2: Cav1 downregulation increases Stat3-tyr705 irrespective of cell density in 10T1/2 mouse fibroblasts

A: Lysates from mouse 10T1/2 fibroblasts before (lanes 1-3) or after (lanes 4-6 and 7-9) stable expression of two different shCav1 constructs (clone 1 and 2), grown to the indicated densities were resolved by SDS-PAGE and probed for Cav1, Stat3-tyr705 or Hsp90 as a loading control.

B: Stat3-tyr705 levels were quantitated by scanning of the blots in three separate experiments. Results represented as means $\pm$ SEM and analysis was performed using GraphPad prism software. The independent two-tailed test (*t*-test) was used to calculate statistical significance.

**** denotes $P = 0.0001$,

*** denotes $P = 0.0003$

Figure 3:

A: Cav 1 overexpression downregulates Stat3-ptyr705 through the CSD domain and triggers apoptosis in breast carcinoma MCF7 and lung carcinoma SHP77 cells

Left panel: Cav1mRFP (lane 1), EGFP-Cav1 (lane 2) or the Cav1-CSDmutant-mRFP (CSDmut, lane 5) were expressed in MCF7 cells through transfection. Cell lysates were probed for Stat3-ptyr705, Cav1 or β -actin as a loading control. Note the reduction in Stat3-ptyr705 upon wt-Cav1mRFP or EGFP-Cav1 expression (lanes 1,2 vs 3) and the absence of reduction upon expression of the CSD-Cav1 mutant (lane 5 vs 6). Control (lanes 3, 4): untransfected cells. Numbers at the left refer to Mwt markers.

Right panel: Control cells transfected with EGFP- or mRFP-expressing plasmids alone, or untransfected (lane 1), as indicated.

B: Cav1 overexpression triggers apoptosis in MCF7 cells.

Cav1 mRFP, or the CSD mutant were expressed through transfection in MCF7 cells. Digital images of cells were taken under phase contrast (a, c, e) or fluorescence (b, d, f) illumination using a Nikon IX70 microscope. Note the dramatic change in morphology upon expression of wt-Cav1 (e, f), but not the CSD mutant (c, d).

C-D: PARP cleavage analysis. Same cells as in (B) above were tested for apoptosis by Western blotting analysis probing with a cleaved PARP antibody (C) or Cav1 (D). Lane 2: MCF7 cells treated with 1 μ M staurosporin for 2 h, as a positive control.

E: Cav1 overexpression triggers apoptosis in SHP77 cells.

Cav1-mRFP, or the CSD mutant were expressed through transfection in SHP77 cells. Digital images of cells were taken under phase contrast (a, c) or fluorescence (b, d) illumination as above. Note the dramatic change in morphology upon expression of wt-Cav1 (a, b), but not the CSD mutant (c, d). e, f: Constitutively active Stat3 mutant, Stat3C was expressed through transfection in SHP77 cells expressing EGFP-Cav1-wt. Arrows point to cells with normal morphology. g, h: EGFP-expressing, control cells. i, j: mRFP-expressing, control cells.

Figure 4: A-C: Downregulation of Stat3 upregulates Cav1 in HeLa cells

A: A Stat3-specific, siRNA was expressed in HeLa cervical carcinoma cells with an Adenoviral vector with a GFP marker (see Materials and Methods). Digital images were taken 3 days later under phase-contrast (a), or fluorescence (b) illumination.

B: Stat3 downregulation through expression of siRNA with the Adenoviral vector in HeLa cells increases Cav1 levels. Three days following infection with the vector expressing shStat3 or a scrambled control, detergent cell lysates were probed for Cav1 or Stat3-ptyr705 or Hsp90 as a loading control. Note the increase in Cav1 levels upon Stat3 downregulation (lanes 3 vs 2).

C: Stat3 inhibition in HeLa cells with SH4-54 increases Cav1 levels. Cells were treated with 30µM of the SH4-54 Stat3 inhibitor and levels of Cav1 and Stat3-ptyr705 examined. Note the increase in Cav1 levels upon Stat3 downregulation (lanes 3 vs 2).

D: Stat3 inhibition does not affect Cav1 levels significantly in the indicated breast or lung cancer lines. Cells were treated with 30 μ M of the SH4-54 Stat3 inhibitor and levels of Cav1 and Stat3-ptyr705 examined.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table I

Relative levels of Cav1 and Stat3-tyr705 in cancer and non-transformed lines.

Cell line	Cav1 ^a	Stat3-tyr705 ^b	Cav1/Stat3-tyr705
Breast cancer lines			
MDA-MB-231	19.0±4.8	4.0±2.9	4.7
MDA-MB-468	2.9±0.9	0.2± 0.1	14.5
MCF-7	0.4±0.1	0.12±0.05	3.3
Lung cancer lines			
SHP77	0.3±0.1	17.0±2.7	0.0176
A549	14.8±2.3	3.0±1.4	4.9
SK-Luci6	0.25±0.1	0.4±0.1	0.625
Brain glioma			
U87	9.4±0.2	13.0±2.1	0.723
Human keratinocytes			
HaCat	16.9±2.7	0.4±0.2	42.3
Pancreatic cancer			
Panc-1	5.3 ± 1.3	0.3± 0.1	17.7
Non-transformed lines			
Human lung			
MRC-9	13.1± 1.7	12.9±0.8	1.0
Mouse fibroblasts			
Balb/c3T3	12.0 ± 0.8	1.3±0.1	9.2
NIH3T3	16.0 ± 2.9	3.7±2.9	4.3

^{a,b}: Levels were quantitated by Western blotting analysis as in Fig. 1. Values refer to arbitrary units obtained by Imaging analysis (see Materials and Methods). Averages of three experiments±SEM are presented.