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Galectin-1 sensitizes carcinoma cells to anoikis via the fibronectin receptor $\alpha_5\beta_1$ -integrin

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Running Title: Galectin-1 stimulates anoikis via $\alpha_5\beta_1$ integrin

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Abstract

Anoikis resistance is a hallmark of transformed epithelial cells. Here, we show that treatment of anoikis-resistant carcinoma cell lines with the endogenous lectin galectin-1 (Gal-1) promoted apoptosis via interaction with the unligated fibronectin receptor $\alpha_5\beta_1$ -integrin. Gal-1 efficiency correlated with expression of $\alpha_5\beta_1$ -integrin, and transfection of the α_5 -subunit into deficient cell lines conferred Gal-1 binding and anoikis stimulation. Furthermore, Gal-1 and the α_5 - and β_1 -integrin subunits coprecipitated in Gal-1-stimulated cells undergoing anoikis. Other members of the galectin family failed to be active. The functional interaction between Gal-1 and $\alpha_5\beta_1$ -integrin was glycan-dependent with $\alpha_2,6$ -sialylation representing a switch-off signal. Desialylation of cell surface glycans resulted in increased electrophoretic mobility of $\alpha_5\beta_1$ -integrin and facilitated Gal-1 binding and anoikis stimulation. On the level of signaling, Gal-1-stimulated anoikis was prevented by filipin, which impaired the internalization of $\alpha_5\beta_1$ -integrin via cholesterol-enriched microdomains, and by pretreatment with a caspase-8 inhibitor.

We propose that Gal-1/ $\alpha_5\beta_1$ -integrin interaction participates in the control of epithelial integrity and integrin sialylation may enable carcinoma cells to evade this Gal-1-dependent control mechanism.

Key words: anoikis, caspase-8, fibronectin receptor, galectin, integrin, sialylation

Abbreviations: ECM, extracellular matrix; Gal-1, galectin-1; ManNAc, N-acetylmannosamine; SNA, *Sambucus nigra* agglutinin; PNA, peanut agglutinin; Erk, extracellular signal-regulated protein kinase; PKB/AKT, protein kinase B; PCNA, proliferating cell nuclear antigen; poly-HEMA, poly(2-hydroxyethyl methacrylate)

Introduction

Epithelial cells require attachment to an appropriate extracellular matrix (ECM) in order to survive (1;2). This anchorage is provided by integrins, heterodimeric transmembrane receptors that recognize and bind ECM ligands and thereby induce reorganization of the actin-based adhesive structures of the cytoskeleton. In addition to this physical linkage, integrins act as sensors of the ECM environment (3). They initiate intracellular signal transduction and/or create signaling platforms that modify signals generated by other classes of cell surface receptors.

When bound to appropriate ECM components, integrins cooperate with growth factors to activate mitogenic and anti-apoptotic signaling pathways (1). However, failure to engage with proper ECM components will not only deprive epithelial cells of these mitogenic and protective signals, but generate pro-apoptotic signals reminiscent of death-receptor activation (4;5). In consequence, epithelial cells that detach from the ECM or lack appropriately composed ECM undergo apoptosis in a process named "anoikis" (Greek for homelessness) (2) or integrin-mediated cell death (6), respectively. Integrins thereby provide a central safeguard mechanism to preclude survival of epithelial cells in inappropriate locations. Conversely, escape from anchorage dependence is a hallmark of carcinoma cells.

Nonetheless, cancer cells frequently retain or increase integrin expression, and overexpression of individual integrins correlated to invasive and metastatic phenotypes in clinical cancer specimens (7). In experimental settings, ligated integrins promoted malignant cell behavior of carcinoma cells, but frequently fail to initiate cell death, when unligated. This failure may result from alterations either in downstream signaling cascades or integrin properties. In this context, changes in integrin glycosylation are receiving increasing attention (8). In fact, altered glycosylation has long been associated with the malignant phenotype and inflammation (9;10).

We previously identified galectin-1 (Gal-1) as a novel ligand that functionally interacts with the fibronectin receptor $\alpha_5\beta_1$ -integrin via binding to glycans (11). Gal-1 belongs to the galectin family of adhesion/growth-regulatory endogenous lectins, which share specificity for β -

galactosides and derivatives thereof, sequence similarity in the carbohydrate recognition domain and the jelly-roll folding (12). Structurally, Gal-1 is a proto-type homodimeric protein with one binding site per subunit (13). Cellular binding partners are suitable carbohydrate moieties of distinct glycoproteins and glycolipids (12;14). The ability for receptor-type-specific cross-linking renders it suited to modulate cell adhesion, migration and growth (9,12).

Gal-1 interaction with the fibronectin receptor on adherent carcinoma cells attenuated cell cycle progression via induction of p21 and p27 (11). Upon cell detachment biological responses to Gal-1 might also be modified, prompting us to analyze Gal-1/fibronectin receptor interaction in carcinoma cells subjected to anoikis conditions. Our results identify Gal-1 as a potent activator of pro-apoptotic $\alpha_5\beta_1$ -integrin signaling, which imposed anchorage dependence by activating integrin-associated caspase-8.

Results

Gal-1 sensitizes HepG2 cells to anoikis. In initial experiments, we examined the effects of Gal-1 on growth and survival, when cells were deliberately kept from attaching to the substratum. Extending previous work with adherent cells (11) hepatocellular HepG2 carcinoma cells were chosen as a representative Gal-1-responsive cell line with endogenous α_5 -integrin expression. These cells, when cultured on poly-HEMA, responded with a significant induction of apoptosis when treated with Gal-1, but not with vehicle or when kept under adherent conditions (Fig.1a). Importantly, anoikis was also induced, when adherent cells first received Gal-1 treatment and were subsequently transferred to suspension cultures without further addition of Gal-1 (Fig. 1b). Thus, Gal-1 treatment sensitized HepG2 cells to anoikis, prompting us to identify the functional binding partner.

The fibronectin receptor is required for anoikis induction by Gal-1. As Gal-1 effects on growth of adherent cells required $\alpha_5\beta_1$ -integrin (11), we assumed a similar interaction in Gal-1-stimulated anoikis of HepG2 cells and tested, whether anoikis could be prevented by coincubation with soluble fibronectin. Indeed, fibronectin prevented Gal-1-stimulated anoikis in a dose-dependent manner (Fig. 1c). Similarly, Gal-1-stimulated anoikis was prevented by a neutralizing $\alpha_5\beta_1$ -integrin-specific antibody (Fig. 1c). A functional interaction with $\alpha_5\beta_1$ -integrin was apparently required to elicit the pro-apoptotic effect, as was independently confirmed in a second cell line (supplementary Fig. 1).

Encouraged by this evidence for $\alpha_5\beta_1$ -integrin involvement in Gal-1-mediated anoikis, we characterized a panel of 44 tumor cell lines of different histogenetic origin with respect to its expression and anoikis stimulation by Gal-1 (Figure 2). The data collected provided the following insights: (i) Gal-1 significantly increased anoikis in 27 of the 44 cell lines (Fig. 2a); (ii) the six cell lines that were α_5 -subunit deficient were entirely insensitive to Gal-1 treatment; (iii) α_5 -integrin presence and susceptibility to Gal-1-mediated anoikis were significantly

correlated (Pearson $r=0.6028$, $p= 0.006$; Fig. 2b); and (iv) Gal-1 responsiveness differed between cell lines derived from tumors of different histogenetic origin.

To provide further evidence for a functional role of $\alpha_5\beta_1$ -integrin in Gal-1-mediated anoikis, we used the Caco-2 cell system described previously (11). Caco-2 wild-type (wt) cells are α_5 deficient and entirely resistant to both Gal-1-dependent cell cycle regulation and stimulation of anoikis. Following transfection with cDNA for the α_5 -integrin subunit, Caco-2 cells had become responsive to Gal-1-induced cell cycle inhibition (11). Using this cell model in suspension culture, enhanced Gal-1 surface reactivity (Fig. 3a) and Gal-1-stimulated anoikis were seen in α_5 -transfected Caco-2 cells, but not in their mock-transfected controls (Fig. 3b). Maximal anoikis rates were reached in clones that displayed the highest level of α_5 -integrin expression (Fig. 3b). Furthermore, anoikis induction in clones with minute α_5 -integrin subunit expression did not reach statistical significance, suggesting that a certain density of $\alpha_5\beta_1$ -integrin was required either for efficient Gal-1 binding or pro-apoptotic downstream signaling. Similar corroborating evidence was obtained from α_5 -integrin-transfected HT29 cell populations (Fig. 3c). Together, these experiments suggested that the fibronectin receptor conferred susceptibility to Gal-1-stimulated anoikis.

Anoikis stimulation by Gal-1 is galectin-type specific and inhibited by cognate sugar.

We next explored the galectin-type specificity of anoikis stimulation using galectins-3 and -7 and the N-terminal domain of galectin-4, which represent another proto-type galectin very similar to Gal-1 (Gal-7), the chimera type (Gal-3) and the N-terminal part of a tandem-repeat-type galectin (Gal-4), respectively. We found that these other galectin family members were unable to stimulate anoikis (supplementary Fig. 2). This observation was confirmed in two additional α_5 -integrin-positive, Gal-1-responsive cell lines, i.e. DU145 and 97TM1 (supplementary Fig. 3).

Gal-1 interacts with cellular effectors via lectin-carbohydrate/protein interactions (14). To document involvement of the carbohydrate recognition domain, the cognate sugar lactose, which inhibits binding of Gal-1 to glycans on the cell surface, was utilized. Addition of lactose

but not sucrose abrogated Gal-1-stimulated anoikis, whereas the haptenic inhibitor had no effect by itself (supplementary Fig. 2).

Sialylation modulates Gal-1 binding and Gal-1-stimulated anoikis. Overall, the correlation between α_5 -integrin expression and Gal-1-stimulated anoikis supported the concept that glycans of the $\alpha_5\beta_1$ -integrin represent the functional interaction partner. However, several cell lines did not follow the assumed reactivity pattern. They may have a saturating level of Gal-1, prompting us to explore Gal-1 expression in our panel of cell lines by immunoblotting (supplementary Fig. 4). However, Gal-1 (and also Gal-3) presence was variable, and high endogenous levels could occur in cell lines, which responded to addition of exogenous Gal-1, precluding a clear-cut correlation. These results turned attention to monitoring Gal-1 reactivity, testing PA1 and CRL1617 cells, which revealed less apoptosis induction than expected from their $\alpha_5\beta_1$ -integrin level, as well as U1752 cells, which were exceptionally responsive, although $\alpha_5\beta_1$ -integrin presentation was moderate (please see Fig. 2). HepG2 cells were also included, as they responded to Gal-1 exposure as predicted from their α_5 -integrin content. Using biotinylated Gal-1 (Gal-1-bio) as probe, we barely detected probe binding to PA1 or CRL1617 cells when compared to HepG2 or U1752 cells (Fig. 4a). Because terminal $\alpha_2,6$ -sialylation of glycans blocks Gal-1 binding (14), we hypothesized that low-level probe binding might result from sialylation of $\alpha_5\beta_1$ -integrin. Cells were thus cautiously treated with sialidase. The removal of $\alpha_2\rightarrow 6$ -linked sialyl moieties from PA1 and CRL1617 cells was confirmed by reduced binding of *Sambucus nigra* agglutinin (SNA; a probe for $\alpha_2,6$ -sialylated Gal/GalNAc residues). As a consequence of desialylation, the reactivities for Gal-1 and peanut agglutinin (PNA; a probe for sialic-acid-free type 1 O-glycans) were increased in sialidase-treated PA1 and CRL1617 cells, but not in HepG2 cells (Fig. 4a). The rather small changes for HepG2 cells indicate a small degree of sialidase sensitivity under the given conditions. Overall, removal of masking sialylation increased Gal-1-binding sites. Parallel immunoblot analyses of α_5 - and β_1 -integrin subunits revealed a sialidase-dependent increase in electrophoretic mobility primarily for the β_1 -integrin subunit

from PA1 and CRL1617 cells (Fig. 4b). In sum, Gal-1-stimulated anoikis in PA1 and CRL1617 cells was enhanced by sialidase treatment (Fig. 4c). Conversely, increased sialylation may reduce the responsiveness to Gal-1. As experimental test, we pretreated highly responsive U1752 cells for 48 hours with 5 mM N-acetylmannosamine (ManNAc), a biosynthetic precursor of sialic acid, to enhance cell surface sialylation. Addition of ManNAc to U1752 cells cultures reduced the ability of Gal-1 to stimulate anoikis (Fig. 5a), as it did with the extent of lectin binding to the cell surface (Fig. 5b). Gal-1 reactivity and Gal-1-stimulated anoikis of HepG2 cells remained nearly unaffected, although we could detect sufficient amounts of free sialic acid in cell supernatants (not shown). Detailed monitoring of α 2,3/6-sialylation on the cell surface with a panel of plant/human lectins after cell treatment with 5 mM and 20 mM ManNAc for 48 h fittingly revealed only slight increases (up to 12%), in full accord with the given data for Gal-1 (Fig. 5b). SNA binding, for example, increased from about 40% to 42-45% (percentage of positive cells) and 15.8 to about 16.7 (mean fluorescence intensity). Evidently, cell surface sialylation did not critically depend on synthesis of free sugar in these cells.

In sum, our data indicate that accessibility and/or functionality of a critical binding partner of Gal-1 were modulated by sialylation.

Gal-1 directly associates and functionally interacts with $\alpha_5\beta_1$ -integrin. Based on (i) the requirement for $\alpha_5\beta_1$ -integrin expression for Gal-1-stimulated anoikis, (ii) increased Gal-1 binding in α_5 -integrin-transfected cells, and (iii) anoikis protection by fibronectin and the $\alpha_5\beta_1$ -integrin-blocking antibody, Gal-1 likely associated with $\alpha_5\beta_1$ -integrin. The predicted complexes containing Gal-1 were found with a fibronectin-receptor-specific antibody in Gal-1-treated HepG2 cells (Fig. 6a, left). Addition of lactose, but not fibronectin abolished this co-immunoprecipitation. Using an antibody to Gal-1, the α_5 - and β_1 -integrin subunits were pulled down in the absence, but not in the presence of lactose (Fig. 6a, middle). Again, fibronectin did not prevent this complex formation, although it abrogated anoikis induction by Gal-1. Using Gal-1-coated magnetic beads $\alpha_5\beta_1$ -integrin was detected (Fig. 6a, right). Different from

immunoprecipitations, fibronectin reduced the recovery of $\alpha_5\beta_1$ -integrin from Gal-1-coated beads, likely due to the altered experimental conditions with disparate Gal-1 presentation and a 30-minute fibronectin pre-incubation prior to the application of Gal-1.

Taken together, Gal-1 appeared to physically interact with the fibronectin receptor outside the fibronectin-binding domain, fully in line with contact to integrin's N-glycans.

In the Caco-2 cell system, we coprecipitated the β_1 -subunit not only from Caco-2/ α_5 -expressing cells but also from parental α_5 -deficient cells with the Gal-1-specific antibody (Fig. 6b). Thus, Gal-1 can interact with the β_1 -integrin subunit independent of α_5 -integrin expression. Having revealed evidence for direct interaction, we addressed Gal-1-initiated signaling next.

Gal-1 engagement of $\alpha_5\beta_1$ -integrin results in caspase-8 activation. Because integrins may modulate survival via caspase-8 activation, we determined caspase-8 activity in Gal-1-treated HepG2 suspension cultures (Fig. 7). Treatment with Gal-1 resulted in rapid and progressive activation of caspase-8, when compared to untreated controls. To define the significance of this caspase-8 activation for Gal-1-stimulated anoikis, a specific caspase-8 inhibitor (Z-IETD-FMK) was added at various time points following Gal-1 stimulation. The inhibitor prevented Gal-1-dependent anoikis completely when added within five minutes of starting Gal-1 treatment (Fig. 7b). When added at later time points, Gal-1-mediated stimulation of anoikis could only be partially inhibited. Hence, Gal-1 stimulated anoikis required the activation of caspase-8. This observation prompted us to further explore the mechanism, whereby Gal-1/ $\alpha_5\beta_1$ -integrin complexes might activate caspase-8.

Gal-1-stimulated anoikis is associated with internalization of $\alpha_5\beta_1$ -integrin via cholesterol-enriched microdomains. Since cell detachment is followed by a rapid internalization of cholesterol-enriched microdomains that can result in cell death, we examined the internalization of Gal-1 and $\alpha_5\beta_1$ -integrin in our anoikis model. While detachment *per se* did not alter $\alpha_5\beta_1$ -integrin surface immunoreactivity, reactivity rapidly

declined following Gal-1 treatment in detached, but not adherent cells (Fig. 8a). Immunoblotting of membrane fractions corroborated the loss of $\alpha_5\beta_1$ -integrin (Fig. 8b), whereas the overall integrin content of whole cell lysates remained unchanged (supplementary Fig. 5). Immunofluorescence microscopy similarly illustrated a redistribution of $\alpha_5\beta_1$ -integrin membrane reactivity into the cytosol of Gal-1-treated detached HepG2 cells (Fig. 8c). Examination of other integrins (Fig. 8c and supplementary Fig. 6a) or stimulation with Gal-3 suggested that internalization of $\alpha_5\beta_1$ -integrin occurred specifically upon addition of Gal-1. We then compared Gal-1-stimulated internalization of $\alpha_5\beta_1$ -integrin in cell lines that differed with respect to extent of Gal-1-stimulated anoikis and observed internalization exclusively in cell lines, which underwent anoikis. To directly test the functional relevance of the internalization process for Gal-1-stimulated anoikis, we used the macrolide filipin to perturb microdomain integrity (Fig. 8d). Filipin treatment did not alter level of spontaneous anoikis, but potently reduced Gal-1-stimulated internalization of $\alpha_5\beta_1$ -integrin (Fig. 8d left) and anoikis (Fig. 8d right). Thus, microdomain integrity and Gal-1-induced internalization of its cognate integrin play a role for anoikis induction.

Discussion

The current study identifies human Gal-1 as a potent modulator of anchorage-independent survival in epithelial cancer cells. We provide evidence that (i) Gal-1 is capable of promoting anoikis in a series of cell lines of different histogenetic origin, (ii) Gal-1 binding depends on the presence of the fibronectin receptor and is modulated by sialylation of cell surface glycans, (iii) anoikis stimulation by Gal-1 requires a functional interaction with the fibronectin receptor, and (iv) complex formation with this integrin promotes internalization of the integrin via filipin-sensitive cholesterol-enriched microdomains and activation of caspase-8, a process cascade triggering anoikis.

Previously, we had found that Gal-1 interacted with the fibronectin receptor in adherent cells to induce cell cycle inhibition (11). Here, we focused on Gal-1 interactions with the unligated integrin in suspension culture, which intriguingly promoted anoikis. When screening a panel

of 44 carcinoma cells lines we found the majority anoikis-resistant despite expression of $\alpha_5\beta_1$ -integrin, suggesting the integrin had lost the capacity to impose anchorage dependence. Addition of Gal-1 restored anoikis susceptibility in most of these cells. Several lines of evidence indicated that the unligated fibronectin receptor represented the relevant functional target of Gal-1: (i) $\alpha_5\beta_1$ -integrin expression was mandatory for anoikis stimulation via Gal-1, (ii) overall, the extent of Gal-1-mediated anoikis correlated to the amount of α_5 -integrin surface expression, (iii) transfection with cDNA specific for the α_5 -integrin subunit conferred Gal-1 binding and responsiveness, and (iv) fibronectin engagement and a neutralizing antibody against $\alpha_5\beta_1$ -integrin, respectively, prevented Gal-1-stimulated anoikis. The latter findings are in full accord with the functional antagonism between Gal-1 and fibronectin noted under adherent conditions (11). In addition, Gal-1 also stimulated anoikis in cells, which failed to respond under anchorage-dependent conditions (Fig. 2a), suggesting differential downstream signaling. Glycans of integrins have previously been shown to interact with galectins, resulting in either impaired or enhanced integrin-mediated adhesion (15-17). Close association of the β_1 -integrin subunit and Gal-1 was evident from cross-linking and co-precipitation experiments, and Gal-1 has been implicated in β_1 -subunit surface presentation and trafficking (18;19). Also, Gal-1 was recovered from $\alpha_5\beta_1$ -integrin complexes in transfected K562 chronic myelogenous leukemia cells in a recent proteomic analysis (20). The requirement for $\alpha_5\beta_1$ -integrin in Gal-1-stimulated anoikis furthermore fits the profile of this integrin as a relevant determinant of anchorage dependence (21-23).

While expression of the fibronectin receptor was a prerequisite for Gal-1-stimulated anoikis, the capacity of the cell to produce Gal-1 turned out to be less predictive. Evidently, the signal intensity of Western blots was not correlated to cell reactivity, possibly due to insufficient routing to the integrin and/or presence of other binding partners. A coordinated upregulation of Gal-1 production and its surface presentation is orchestrated via reexpression of the tumor suppressor p16^{INK4a} in a pancreatic cancer cell line (24). Induction of Gal-1 was accompanied by reprogramming of glycosylation, leading to increased Gal-1 reactivity and potent induction of anoikis involving $\alpha_5\beta_1$ -integrin (24).

With respect to the cellular reactivity, accessibility of Gal-1-binding sites emerged as a crucial modifier. Specifically, enzymatic reduction of cell surface sialylation increased binding and anoikis response of cell lines that appeared resistant to Gal-1 despite abundant α_5 -integrin expression. Thus, consequences of integrin sialylation may extend beyond altered binding of protein ligands (8;25;26) due to an effective masking of lectin-reactive sites. Indeed, the presence of $\alpha_2,6$ -linked sialic acid precluded cell binding of Gal-1 (27), even in N-acetyllactosamine repeats (28). The current findings suggest $\alpha_2,6$ -sialylation of $\alpha_5\beta_1$ -integrin constitutes a relevant (patho-)physiologic response modifier for Gal-1-stimulated cancer cell anoikis. Interestingly, this type of N-glycan branch-end structure is increased in various malignant cells (10).

In line with this concept, our immunoprecipitation analyses confirm a physical, lactose-inhibitable interaction of Gal-1 with the fibronectin receptor. Presence of both integrin subunits appears mandatory, since Gal-1 binding and pro-apoptotic signaling are negligible in Caco-2 or HT29 cell lines with abundant β_1 -integrin expression, unless they are transfected to produce the α_5 -integrin subunit. Although fibronectin counteracts Gal-1-stimulated anoikis, it did not consistently displace Gal-1 from $\alpha_5\beta_1$ -integrin complexes, suggesting physically separate interaction sites. Information on the precise localization of the glycans acting as docking sites will be required to answer topological aspects of this question.

Which signals trigger anoikis following the binding of Gal-1 to $\alpha_5\beta_1$ -integrin? Initiation and execution of the anoikis process rely on the activation of caspases via integrins (1), though variability exists with respect to the sequence of caspase activation. Studies on $\alpha_5\beta_1$ - and $\alpha_v\beta_3$ -integrins describe an early and functionally relevant activation of procaspase-8 (5;29). In fact, procaspase-8 was shown to promote migration and invasion of cancer cells, unless turned into an apoptotic signal by activation (30). Here, we report a rapid procaspase-8 activation upon Gal-1 engagement of $\alpha_5\beta_1$ -integrin and impairment of Gal-1-stimulated anoikis with a selective caspase-8 inhibitor. Involvement of caspase-8 has also recently been

demonstrated for anoikis induction in p16^{INK4a}-reconstituted cells, which required autocrine Gal-1 availability (31).

Different mechanisms are proposed, whereby integrins activate or suppress caspase-8 activity in a ligand-dependent manner, including proximity-induced activation of integrin-associated procaspase-8 due to integrin clustering (6), prevention of autocatalytic cleavage by post-translational modifications (30;32), redistribution of endogenous caspase inhibitors (33), or increased availability of pro-apoptotic bcl-2 family members (34). Here, we observed rapid internalization of $\alpha_5\beta_1$ -integrin via cholesterol-enriched microdomains, a process required for full anoikis induction. Thus, Gal-1 may provoke clustering and/or repartition of the unligated $\alpha_5\beta_1$ -integrin into endocytotic microdomains and thereby enable or amplify its capacity to activate caspase-8. Enhanced caspase-8 activation by death receptors, such as CD95, following recruitment to lipid rafts and endocytosis provides an example for modulation of apoptotic signaling by endocytotic trafficking (35).

Association of ganglioside GM1, which by itself is a potent Gal-1 counterreceptor, with this integrin, as noted in T effector cells (36), may favor clustering in cholesterol-enriched microdomains, leading to enhanced Gal-1 reactivity (37) thereby supporting recruitment to microdomains and endocytosis.

In summary, we report Gal-1 as a counterreceptor of N-glycans of the $\alpha_5\beta_1$ -integrin. Binding to the unligated integrin initiates apoptosis. Thus, Gal-1 is capable of implementing anchorage dependence in epithelial cancer cells. Cancer cells may circumvent this control mechanism by reducing access of Gal-1 to its target(s) on the cell surface via α 2,6-sialylation. Thus, an acquired anoikis resistance of carcinoma cells may be encoded in glycans at the cell surface, besides being exerted by oncogenic alterations in downstream survival signaling pathways. Consequently, shifting the glycophenotype to a Gal-1-responsive profile offers an attractive alternate avenue for therapeutic intervention.

Materials and Methods

Cell culture

Human tumor cell lines were obtained from ATCC, the German Cancer Research Center (Heidelberg, Germany) (DanG cells), or the Japan Health Sciences Foundation (QGP-1), and were grown exactly as recommended. BON and LCC-18 neuroendocrine cancer cell lines were maintained in DMEM, supplemented with 10 % (v/v) FCS, 100 units/ml penicillin and 100 µg/ml streptomycin.

Induction and detection of anoikis

For determination of anoikis, 2×10^5 cells were cultured as suspension cultures on plates coated with poly(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma, Deisenhofen, Germany) for the indicated periods of time. Apoptotic cells were then quantitated from the pre-G₁ fraction in cell cycle analyses (23).

Production and purification of galectins

Human galectins -1, -3 and -7 and the N-terminal domain of rat tandem-repeat-type galectin-4 were purified with affinity chromatography as crucial step, rigorously checked for purity by one- and two-dimensional gel electrophoresis, mass spectrometry and gel filtration, and bioactivity was ascertained by solid-phase and cell assays as previously described (38, 39).

Antibodies

Antibodies for immunoblotting of integrins α_5 (clone 1), β_1 (clone 18), α_4 (clone 7), and α_v (clone 21) were from BD Transduction Laboratories (Heidelberg, Germany); the antibody utilized to immunoprecipitate $\alpha_5\beta_1$ -integrin and as blocking antibody (AB1950) was from Chemicon (Hofheim, Germany). The antibody to PCNA from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies used for flow cytometry were as follows: α_5 (CD49e R-PE CBL497P) and β_1 (CD29PE CBL481P) from Cymbus Biotechnology (Hampshire, UK).

Secondary antibodies were from Dianova (Hamburg, Germany). Polyclonal antibodies against Gal-1 free of cross-reactivity for other galectins have been described previously (24).

Protein extraction and Western blotting

Cells were lysed in 1 x RIPA buffer (50mM Tris-HCl pH 7.5, 0.15M NaCl, 0.25% SDS, 0.05% sodium deoxycholate, 1% NP-40, 1mM dithiothreitol, 1µg/ml aprotinin, 2mM leupeptin, 1mM Na₃VO₄, 1mM NaF, 1mM PMSF), and sonicated on ice. Aliquots (5-10 µg) were subjected to SDS-PAGE and were electroblotted onto PVDF membranes (NEN, Cologne, Germany). Blots were incubated overnight at 4°C with the respective antibodies (diluted 1:1000 in 5% non-fat dried milk in PBS with 0.5% Tween 20). Immunoreactive bands were visualized by enhanced chemoluminescence (NEN, Cologne, Germany).

Sialidase treatment

4x10⁵ cells were washed with PBS, resuspended in 50µl PBS and incubated with 10mUnits of neuraminidase from *Clostridium perfringens* (specific for α2,3/6-sialylation; Roche Diagnostic GmbH, Mannheim, Germany) 40 min at 37°C. For Western blot analysis 30-40µg protein extract were processed instead of whole cells.

Analysis of integrin content by flow cytometry

Approximately 10⁵ cells were resuspended in 300µl PBS with 10µl of subunit-specific phycoerythrin (PE)-labeled integrin antibodies (α₅, β₁). After incubation for 15 min at 4°C, cells were washed with PBS and fluorescence was recorded on a FACSCalibur (Becton Dickinson, Heidelberg, Germany) and analyzed with CellQUEST software.

Stable transfection of α_5 -integrin constructs

Full-length α_5 -integrin-subunit-specific cDNA was originally obtained from E. D. Kreuser (Department of Hematology and Oncology, University Medical Center Benjamin Franklin, Free University of Berlin, Germany) and was subcloned in pRC-CMV (pRC- α_5) (40). To generate stably transfected cells Effectene Transfection Reagent (Quiagen, Hilden, Germany) was used following the manufacturer's protocol. Subsequent selection of stably transfected cells was carried out with 0.8mg/ml G418 (11).

Determination of Gal-1 binding

Cells were incubated with 125 μ g/ml of biotinylated Gal-1, prepared and checked for activity and label incorporation as described (24), washed twice with PBS and bound Gal-1 (and also labeled plant lectins) was then detected by flow cytometry using a fluorescent streptavidin derivative (39). Carbohydrate-dependent binding was ascertained by controls with sugars.

Immunoprecipitation of integrin/galectin complexes

Immunoprecipitations were carried out as previously described (23). Briefly, cells were lysed in 50mM Hepes, pH 7.4, 150mM sodium chloride, 1mM EDTA, 2.5mM EGTA, 10% glycerol, 0.1% Tween 20, 1mM DTT, 1mM sodium fluoride, 10mM β -glycerolphosphate, 0.1mM sodium orthovanadate, 0.1mM PMSF, 3mg/ml aprotinin, and 2mM leupeptin. Cell lysates were immunoprecipitated with antibodies as indicated, and immune complexes were recovered with protein A-Sepharose or protein G-Sepharose ($\alpha_5\beta_1$ -integrin) beads (Sigma-Aldrich) overnight at 4°C. Precipitated proteins were separated on SDS-PAGE gels and electroblotted to PVDF membranes.

Determination of caspase-8 activity

Cells with activated caspase-8 were detected using the carboxyfluorescein-labelled derivative of the caspase-8 inhibitor Z-LETD-FMK (FAM-LETD-FMK) (Biocarta, Hamburg,

Germany), which irreversibly binds to activated caspase-8. Fluorescence intensity was evaluated by flow cytometry.

Magnetic separation of galectin-containing complexes

For separation of Gal-1-associated complexes, 2×10^8 tosyl-activated Dynabeads[®] M-280 (Dyna[®]) were coated overnight with either 250 μ g Gal-1 or BSA at 37°C. Dynabeads were recovered on a magnetic separation stand, washed twice with PBS, deactivated with 0.2M Tris-HCl pH 8.5 for 4 hr at 37°C and washed again with PBS. Beads were then added to 10^6 cells for 5 min at RT. Cells and beads were rinsed twice with PBS and homogenization buffer (20mM Tris-HCl pH 7.6, 10mM MgCl₂, 1 μ g/ml aprotinin, 2mM leupeptin, 1mM PMSF) was added. Proteins attached to the beads were washed twice with homogenization buffer and resuspended in SDS-DTT protein loading buffer.

Subcellular fractionation

All steps were carried out at 4°C. Cells were rinsed with PBS, resuspended in ice-cold homogenization buffer (20mM Tris-HCl pH 7.6, 10mM MgCl₂) containing protease inhibitors and lysed by pipeting. Following centrifugation (2 min at 40xg) the pellet (cell debris) was discarded and the supernatant centrifuged again (10 min at 750 x g). The pellet (nuclear fraction) was stored in homogenization buffer and the supernatant centrifuged 60 min at 100,000 x g. The supernatant (cytosolic fraction) was stored and the pellet (membrane enriched fraction) resuspended in homogenization buffer. Aliquots (20 μ g) of the membrane enriched fraction were separated by SDS-PAGE and further processed as described for Western blotting above.

Immunofluorescence microscopy

For immunofluorescence microscopy, adherent cells were grown on Lab-Tek chamber slides (Nalge Nunc Int., USA) and suspended cells were centrifuged onto microscope slides prior to analysis. Cells were fixed with cold methanol:acetone (2:1) for 10 min at -20°C.

The following monoclonal antibodies were used: α_5 (CD49e R-PE CBL497P), β_1 (CD29PE CBL481P; CD29F:P5.2 CBL481F) from Cymbus Biotechnology LTD (Hampshire, UK), α_2 , α_v , β_5 from Dianova (Hamburg, Germany), β_4 from Telios Pharmaceuticals (San Diego, CA, USA).

Statistical analysis

Unless indicated unpaired Student t-Test analyses were performed using Prism software (Prism, San Diego, CA). Data were considered significant at $p < 0.05$. Spearman correlation and Deming regression, respectively, were applied to describe the relation between α_5 integrin expression and extent of Gal-1-mediated anoikis.

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Conflict of interest

The authors declare no conflict of interest.

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Titles and legends to figures

Figure 1: Gal-1 stimulates anoikis in HepG2 cells. (a) HepG2 cells were cultured overnight either adherent or suspended on poly-HEMA-coated plates and received Gal-1 (125 µg/ml) as indicated. The pre-G₁ fractions were quantitated by flow cytometry to determine apoptosis (adherent) and anoikis (suspended). (b) Adherent HepG2 cells were pretreated with either vehicle or Gal-1 (125 µg/ml) and subsequently transferred to poly-HEMA-coated plates. Anoikis was then determined following overnight incubation in the presence or absence of Gal-1. Control cells received vehicle throughout the experiment, for the other samples conditions were as indicated (pretreatment/treatment). Pretreatment with Gal-1 was sufficient to sensitize HepG2 cells to anoikis. (c) Functional modulation of Gal-1-stimulated cell death by addition of the indicated concentrations of soluble fibronectin or 2 µg/ml of an $\alpha_5\beta_1$ -integrin-blocking antibody. Cells received the treatment immediately after detachment and were then kept in suspension for 24 hours before cell death rates were determined. Data represent mean \pm SEM of at least three independent experiments (**p<0.01, ***p<0.001 vs. Gal-1-treated cells).

Figure 2: Gal-1 stimulates anoikis in tumour cells that express the fibronectin receptor. (a) The α_5 expression level and anoikis stimulation by Gal-1 (125 µg/ml) were determined in 44 cell lines of different histogenetic origin. Gal-1-stimulated anoikis is given relative to control values of anoikis determined in untreated controls (fold control) and ratios >1.5 are highlighted in bold (*). For comparison, growth inhibition by Gal-1 in adherent cultures is indicated. Antiproliferative effects were determined based on cell numbers. Gal-1-treated cultures with a >30% reduction of cell numbers after 96 hours were considered as responsive (+). Cellular $\alpha_5\beta_1$ -integrin surface expression was determined in parallel by flow cytometry. Data are derived from three independent experimental series. (b) The linear regression analysis (Pearson r=0.6028, p= 0.006) between these two parameters is shown.

Figure 3: The fibronectin receptor confers capacity for Gal-1-stimulated anoikis to α_5 -deficient colon cancer cells. (a) Cell surface binding of Gal-1 in mock- and α_5 -transfected (clone $\alpha_5/1$) Caco-2 cells. Number inserts indicate the amount of biotinylated Gal-1 added ($\mu\text{g}/\text{ml}$). (b,c) Determination of Gal-1-stimulated anoikis after incubation with 125 $\mu\text{g}/\text{ml}$ Gal-1 for 24 hours (open bars) and α_5 expression levels (closed bars) in different clones of α_5 -transfected Caco-2 cells ($\alpha_5/1$ to $\alpha_5/4$), wild-type (wt) cells and mock control (panel b) and in two α_5 -transfected HT29 clones ($\alpha_5/1$ and $\alpha_5/2$), wt cells and mock control (panel c). Data represent at least three independent experiments (anoikis as fold stimulation without treatment, * $p<0.05$, ** $p<0.01$, *** $p<0.001$) and a representative determination of α_5 expression.

Figure 4: Sialidase treatment enhances Gal-1 binding and Gal-1-stimulated anoikis. (a) Flow cytometry analysis of binding of SNA, PNA or biotinylated Gal-1 to control (light) and (bold) sialidase-treated cells. Background fluorescence (shaded) was similar in the PBS and sialidase-treated cells. (b) Western blot for detection of α_5 - and β_1 -integrin subunits in cells with or without sialidase treatment. (c) Anoikis rates in HepG2, PA1 and CRL1617 cells with or without previous sialidase treatment measured after 24 hrs on suspension culture either in the presence or absence of Gal-1 are expressed as relative to the corresponding PBS-treated controls. Data represent mean \pm SEM of at least three independent experiments (* $p<0.05$).

Figure 5: ManNAc supplementation reduces Gal-1 stimulated cell death and Gal-1 binding. (a) Gal-1-mediated cell death in U1752 and HepG2 cells previously cultured for 48 hrs with 5 mM or without ManNAc supplementation. Cells were then subjected to suspension culture with continued ManNAc supplementation and Gal-1 was added as indicated. Anoikis rates were determined after 24 hours based on the pre-G1 fraction from DNA-histograms. Anoikis is expressed as fold of the respective controls in the absence of Gal-1. Data represent mean \pm SEM of at least three independent experiments (* $p<0.05$, one tailed t-

Test). (b) Binding of biotinylated Gal-1 to U1752 (left panel) or to HepG2 (right panel) cells that had been cultured for 48 hours in the presence of ManNAc (+ManNAc) or vehicle (control). Flow cytometry analysis shows control (light), ManNAc-treated (bold) cells and background fluorescence via binding of indocarbocyanine-streptavidin conjugate in the absence of biotinylated Gal-1 (shaded).

Figure 6: Gal-1 binds to the $\alpha_5\beta_1$ fibronectin receptor. Coimmunoprecipitation of Gal-1 with $\alpha_5\beta_1$ -integrin. (a) Whole cell lysates from HepG2 cells that had been cultured on PolyHEMA coated dishes and treated for one hour with (+) or without (-) 125 μ g/ml Gal-1. For co-incubation conditions, 300 μ g/ml fibronectin (FN), 20 μ M lactose (Lac), or 100 μ g/ml of α_5 -blocking antibody were added as indicated. Cells were lysed and immunoprecipitations were conducted with antibodies to $\alpha_5\beta_1$ -integrin (left panel) or Gal-1 (middle panel). In an alternative approach, HepG2 cells were incubated for five minutes with Gal-1-coated magnetic beads after pretreatment with 300 μ g/ml fibronectin (FN), 20 μ M lactose (Lac), or 100 μ g/ml of α_5 -blocking antibody, respectively (right panel). (b) Co-immunoprecipitation of Gal-1 and $\alpha_5\beta_1$ -integrin in α_5 -transfected Caco-2 cells and mock controls, using an antibody to Gal-1 after treatment with (+) or without (-) 125 μ g/ml Gal-1 for one hour. Immunoblots using antibodies to either α_5 - or β_1 -integrin subunits or to Gal-1 are shown.

Figure 7: Caspase-8 is activated in Gal-1-stimulated cells undergoing anoikis. (a) HepG2 cells were subjected to suspension culture in the presence or absence of Gal-1, and caspase-8 activity was determined at the indicated time points. (b) Cells were cultured and Gal-1 stimulation (125 μ g/ml) was conducted as above. At the time points indicated, the caspase-8 inhibitor Z-IEDT-FMK (20 μ M) was added to cells in suspension culture in the presence of Gal-1. After 24 hours, anoikis rates were determined by flow cytometry. Data represent mean \pm SEM of four independent experiments. (* p <0.05, ** p <0.01).

Figure 8: Gal-1-stimulated anoikis is associated with microdomain-mediated internalization of $\alpha_5\beta_1$ -integrin and prevented by filipin.

(a) HepG2 cells were cultured either under adherent conditions (adh.) or subjected to suspension culture and treated with 125 μ g/ml Gal-1 as indicated. After 30 min incubation time, an aliquot was withdrawn and native cells were stained with anti- α_5 or anti- β_1 integrin antibodies, respectively, to determine surface abundance of these integrins. The remaining cells were further incubated on poly-HEMA for confirmation of maintained anoikis induction (not shown). (b) Immunoblotting of membrane-enriched fractions (separated at 100,000 x g, 20 μ g protein/lane) obtained from HepG2 cells that were cultured on poly-HEMA and treated after with Gal-1 or vehicle for the indicated time periods. Both α_5 - and β_1 -integrin subunits were reduced in cells that had received Gal-1. (c) Immunofluorescence of α_5 - and β_1 -integrin subunits. Cells were cultured as described above and treated with Gal-1 for one hour, and fixed before α_5 - and β_1 - integrin subunits were examined by immunofluorescence microscopy. Left part shows integrin localization in adherent cells incubated without (a-d) or with Gal-1 (e-h) for one hour. Right part shows results from cells cultured on poly-HEMA treated without (i-l) or with Gal-1 (m-p) for one hour. Shown are immunoreactive fluorescence signals for α_5 - (a,e,i,m), β_1 - (b,f,j,n) subunits, overlay of α_5 - and β_1 - (c,g,k,o) and α_v - (d,h,l,p) integrin subunits. (d) Effect of filipin on Gal-1-stimulated $\alpha_5\beta_1$ -integrin internalization and anoikis. Cells were detached and incubated without or with Gal-1 in the presence or absence of filipin. Subsequently, $\alpha_5\beta_1$ -integrin immunoreactivity was determined in native cells (left). Labels are as follows: control cells (C), Gal-1 treated cells (G1) and cells pretreated with filipin (4 μ g/ml) for 60 min before Gal-1 incubation (F). To determine effects of filipin on anoikis, cells were cultured on PolyHEMA and treated with Gal-1 (125 μ g/ml) or filipin (4 μ g/ml) or a combination thereof. For filipin conditions, cells had also been pretreated for 60 min before they were transferred to suspension culture. Anoikis rates were determined after 24 hours. Results are expressed as fold of control and represent the mean \pm S.E.M. of at least three independent experiments. ANOVA was performed using Prism software (Prism, San Diego, CA). **p<0.01, ***p<0.001.

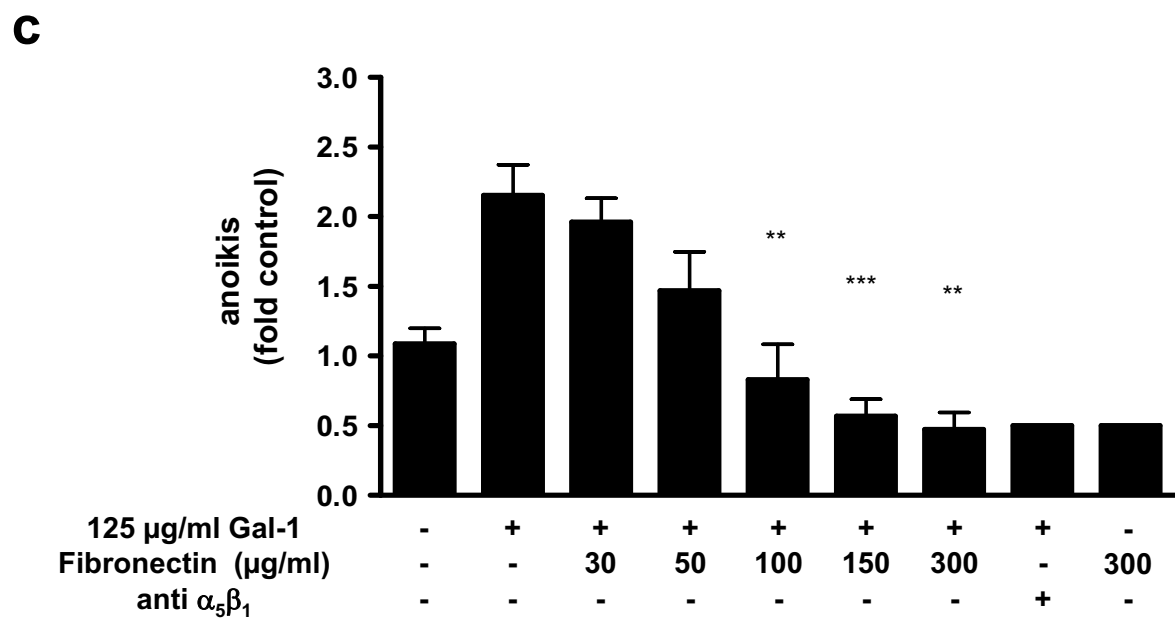
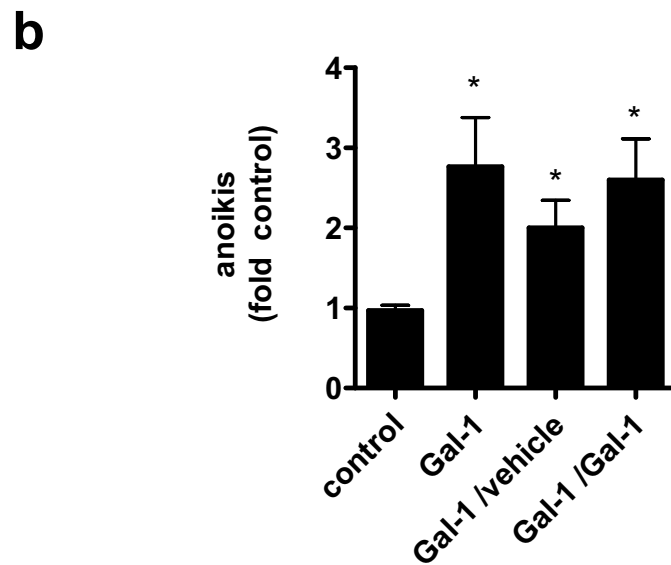
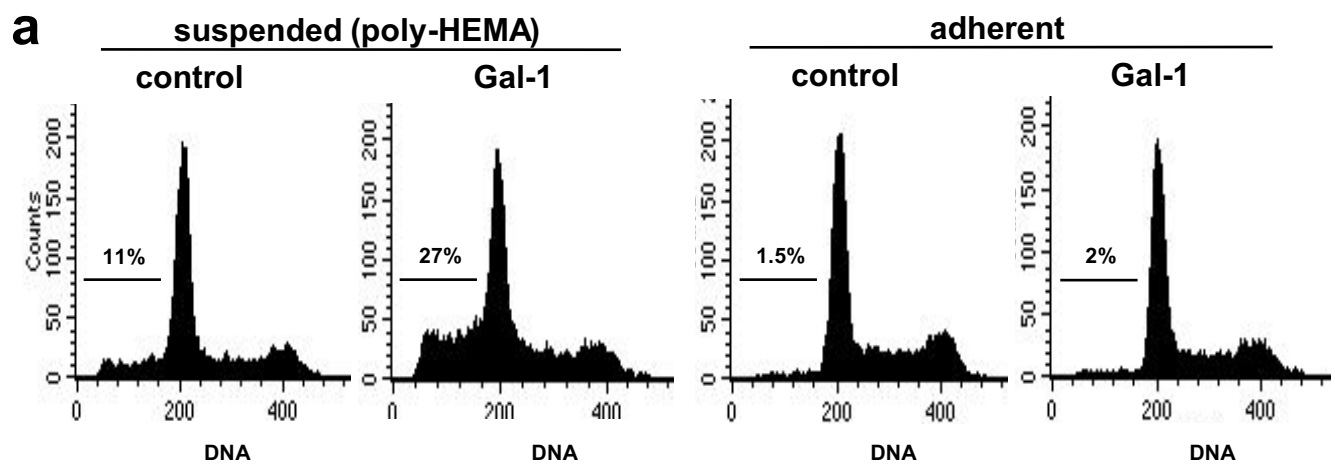


Figure 1 Sanchez-Ruderisch, H.

a

Tumor origin		Cell line	Gal-1 induced anoikis (fold control)	Gal-1 induced growth inhibition	α_5	β_1
Hepatocellular		HepG2	2.9 ± 0.16*	+	249	585
		Huh 7	2.2 ± 0.22*	+	80	497
		SK-Hep-1	2.5 ± 0.48*	+	173	1178
Prostata		LNCaP	1.7 ± 0.20*	–	177	519
		DU145	3.4 ± 0.49*	–	402	2564
		PC3	1.8 ± 0.17*	–	112	957
		22RV1	1.7 ± 0.50*	–	30	467
Lung	squamous	32M1	3.8 ± 1.20*	–	164	111
		U1752	6.7 ± 1.67*	–	138	890
	large cell	103H	2.4 ± 0.09*	–	445	949
		97TM1	2.4 ± 0.09*	+	208	772
	adeno	H23	3.4 ± 0.20*	–	297	1116
		A539	2.3 ± 0.30*	–	84	507
		H125	4.8 ± 1.10*	–	189	917
		H460	2.6 ± 0.50*	–	182	832
		H1573	1.3 ± 0.20	–	178	834
		H2009	1.7 ± 0.20*	–	80	513
		H2077	3.8 ± 1.10*	–	58	259
		H2126	2.0 ± 0.28*	–	33	798
Breast		T47D	2.7 ± 0.51*	+	55	408
		BT-474	1.1 ± 0.10	–	negative	266
		SK-BR3	1.2 ± 0.05	–	negative	41
		MB435	2.0 ± 0.04*	–	100	316
		MCF 7	3.6 ± 0.23*	–	70	902
Melanoma		MEL2a	1.4 ± 0.05	–	76	236
		Mewo	2.5 ± 0.70*	–	146	288
		CRL1617	1.5 ± 0.40*	–	139	227
		SKmel13	2.0 ± 0.12*	+	114	449
Ovary		OV90	1.5 ± 0.23*	+	47	899
		TOV21G	1.6 ± 0.31*	–	175	1524
		PA1	0.8 ± 0.09	–	112	530
Cholangiocellular		Egfr1	1.3 ± 0.13	–	25	negative
		TFK	1.2 ± 0.20	–	negative	969
Pancreas		Mia PaCa	1.3 ± 0.12	–	52	126
		Aspcl	1.3 ± 0.16	–	21	884
		Capan-1	1.2 ± 0.16	–	55	953
		Dan-G	1.2 ± 0.11	–	105	1426
Neuroendocrine		QGP-1	1.3 ± 0.09	–	112	1444
		Bon	1.3 ± 0.17	+	90	1423
		LCC-18	1.2 ± 0.10	–	negative	82
		Ags	1.1 ± 0.10	–	60	625
Colon		Lovo	1.0 ± 0.00	–	60	1009
		Caco-2	0.8 ± 0.17	–	negative	951
		HT29	1.0 ± 0.06	–	negative	959

b

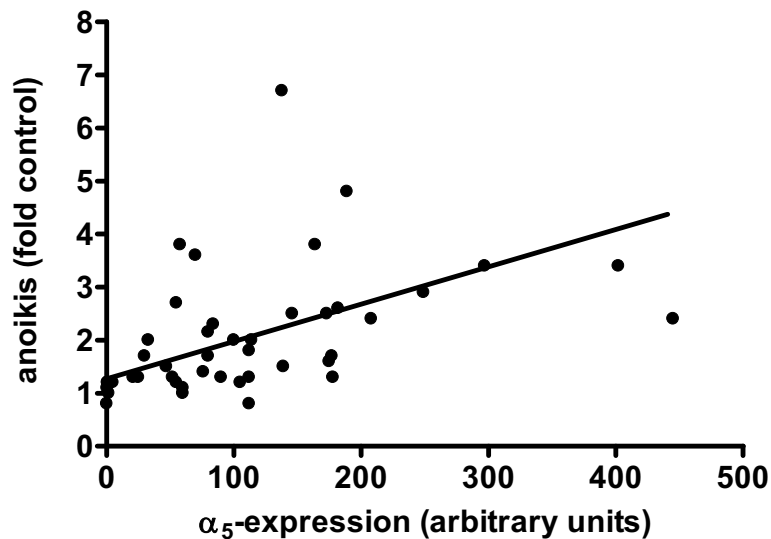
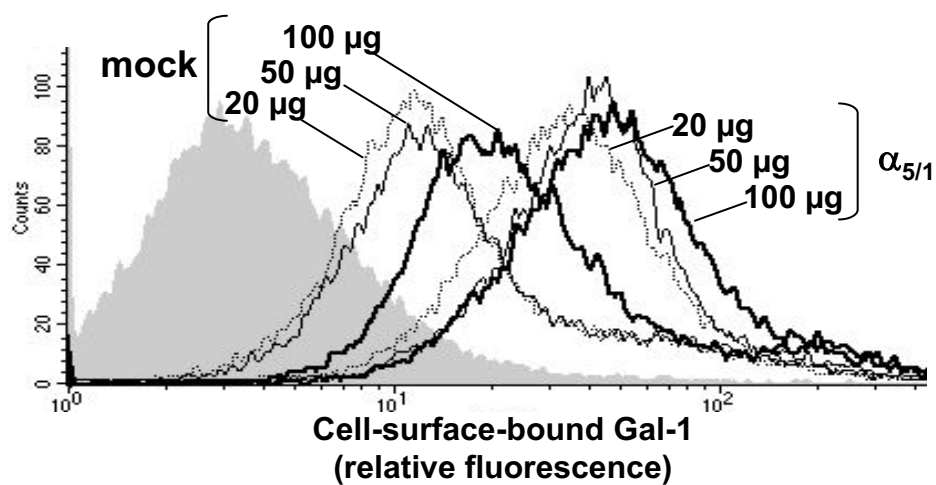
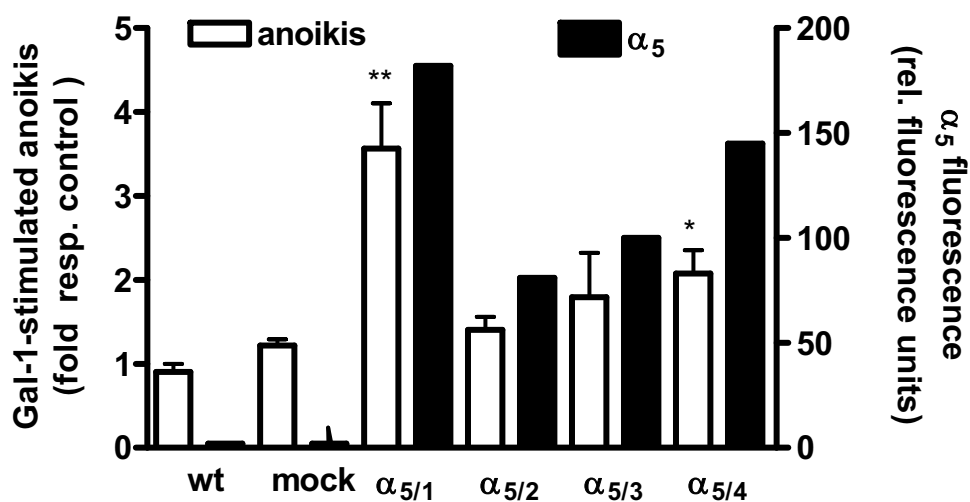


Figure 2 Sanchez-Ruderisch

a



b



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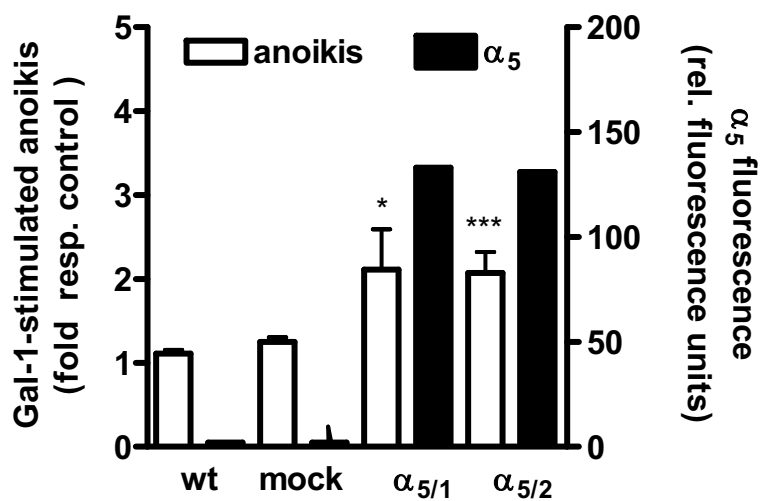
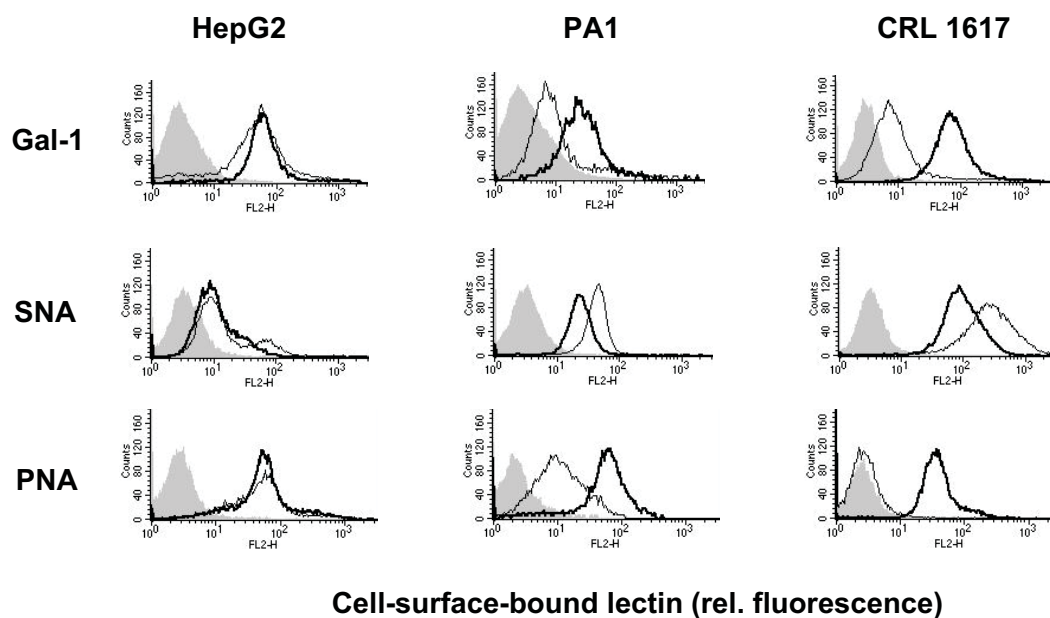
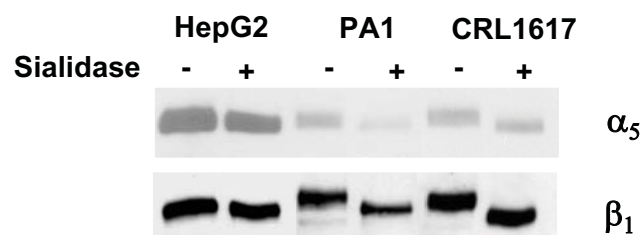


Figure 3 Sanchez-Ruderisch, H.

a



b



c

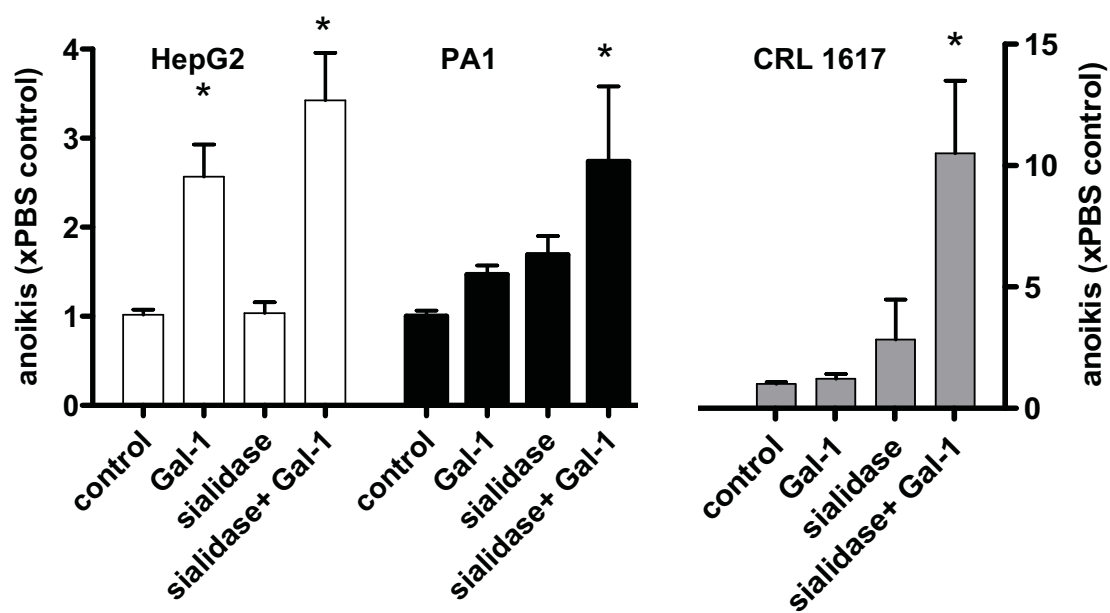
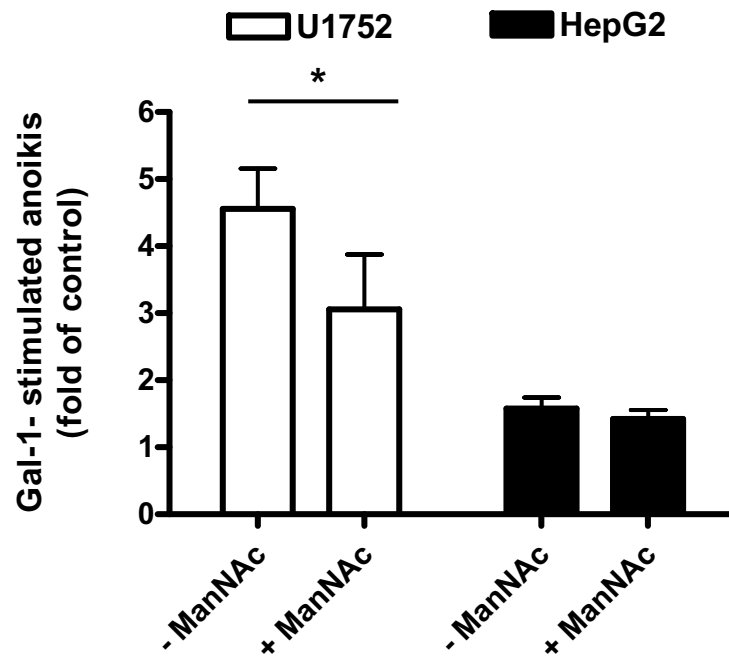


Figure 4 Sanchez-Ruderisch

a



b

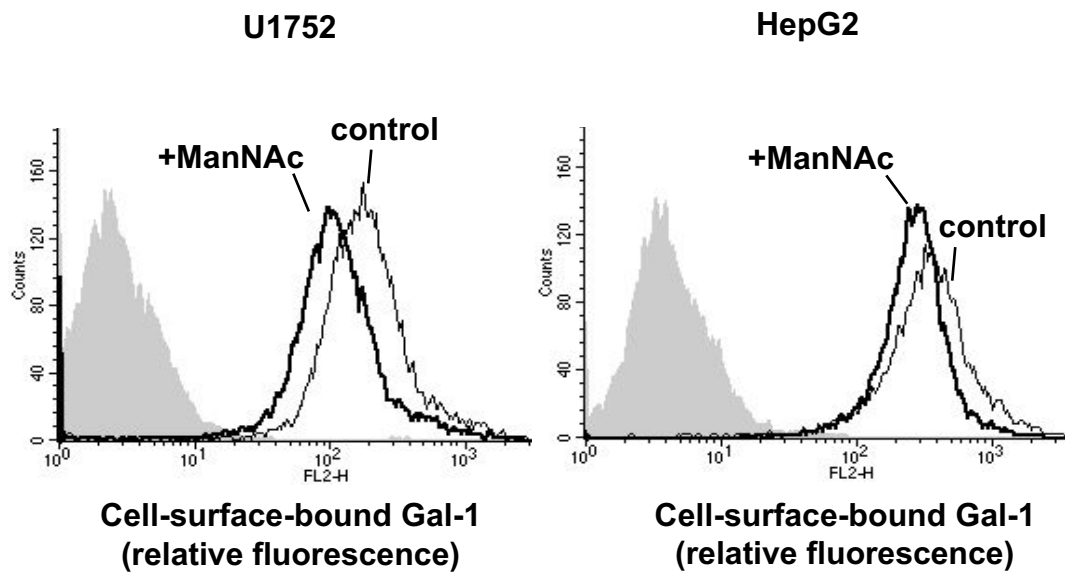
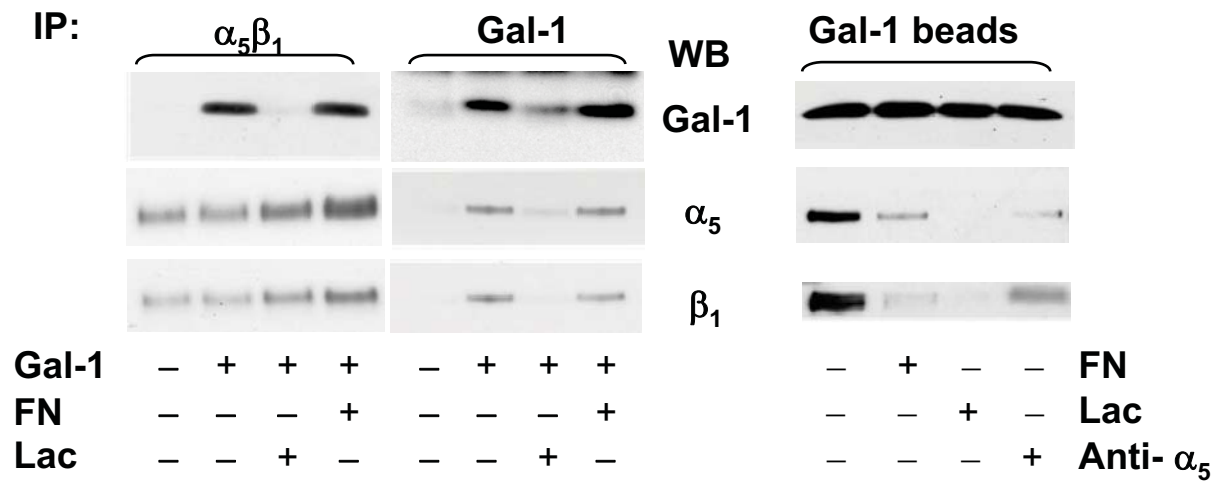


Figure 5 Sanchez-Ruderisch

a



b

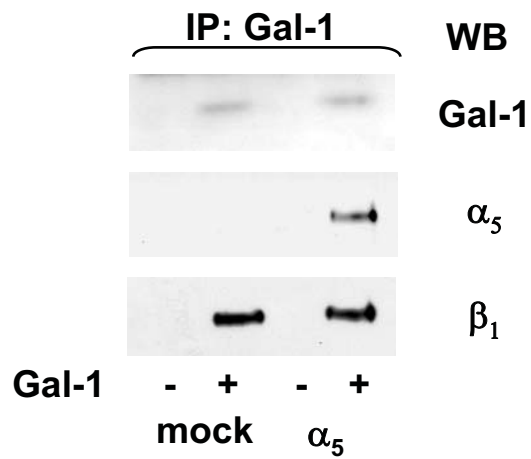
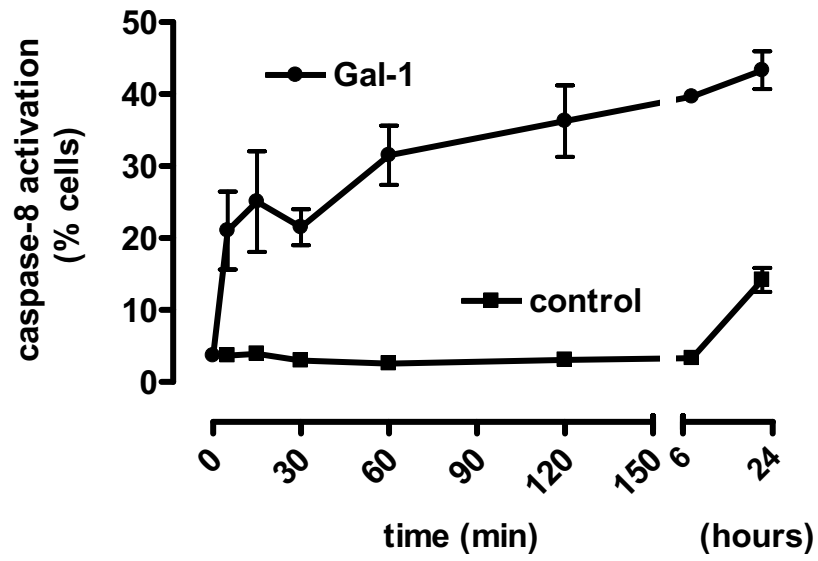


Figure 6 Sanchez-Ruderisch

a



b

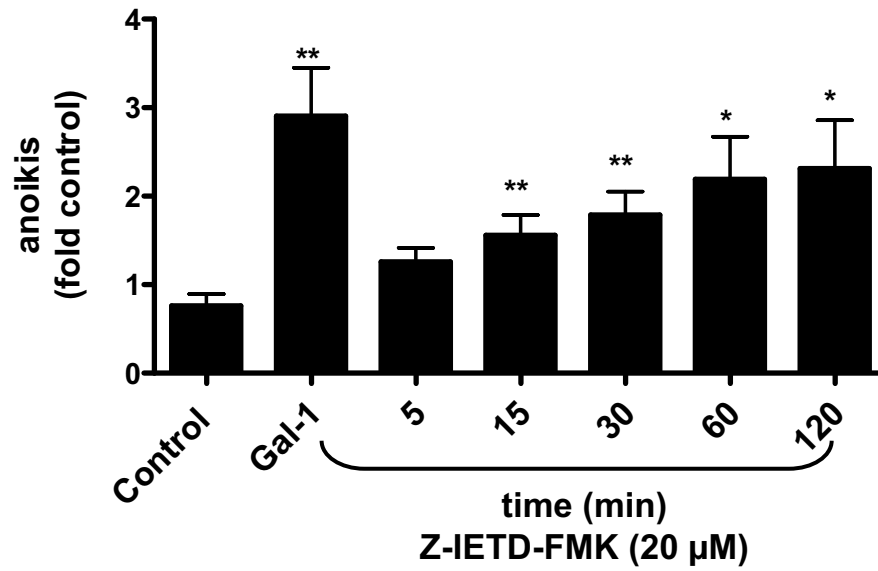


Figure 7 Sanchez-Ruderisch

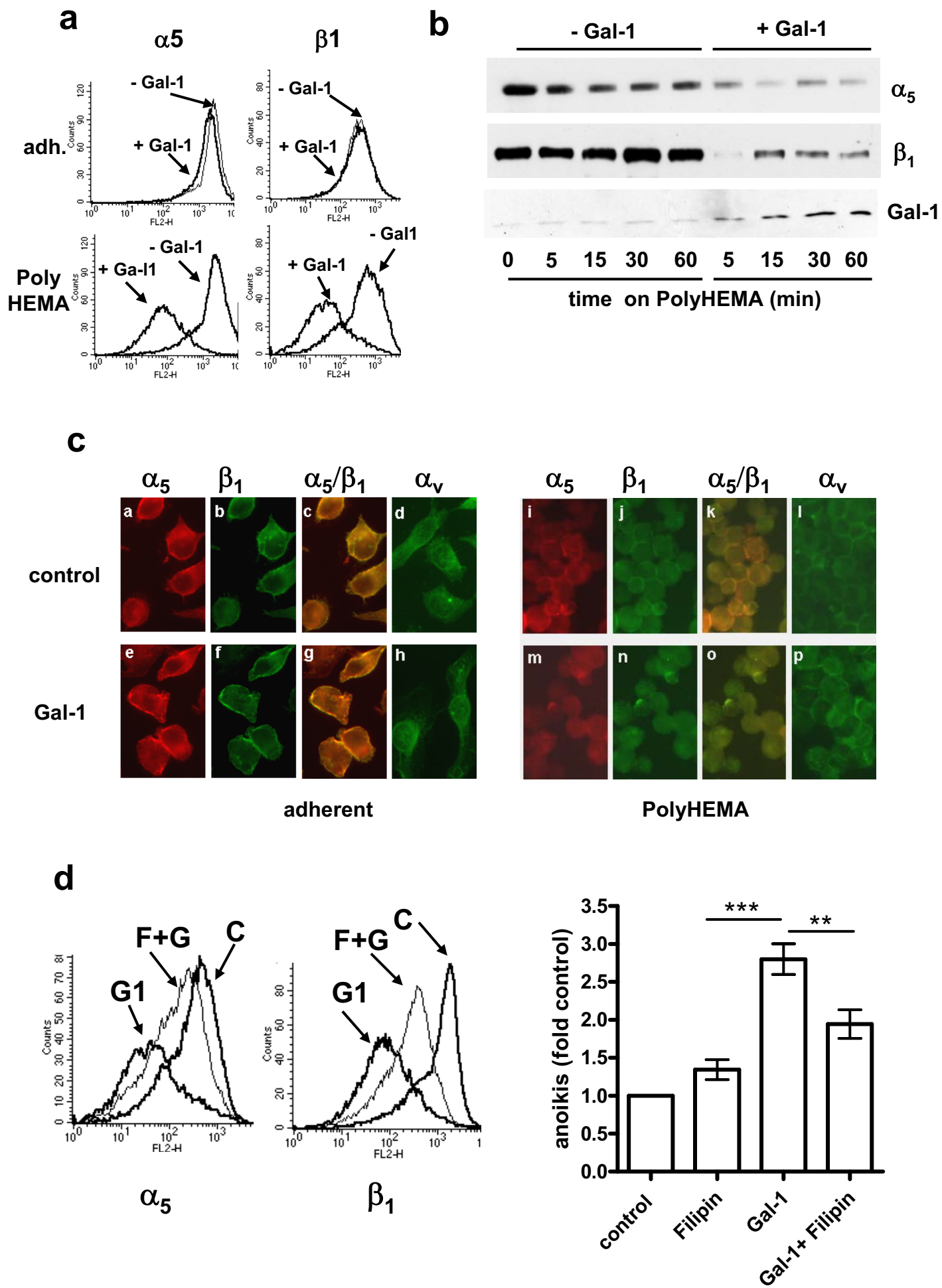


Figure 8 Sanchez-Ruderisch