UNIVERSITA' DEGLI STUDI DI TORINO

DIPARTIMENTO DI SCIENZE BIOMEDICHE

DOTTORATO DI RICERCA IN: "Complex systems applied to post-genomic biology"

CICLO: XX

Functional screening for genes conferring anchorage-independence to human immortalized breast cells

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ANNI ACCADEMICI: 2004-2007

SETTORE SCIENTIFICO-DISCIPLINARE DI AFFERENZA*: BIO/17

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Abstract

Cells sense their location through specific interactions with the extracellular matrix (ECM) and neighbouring cells. As well as providing positional and mechanical cues for the organization of cells in tissues, the ECM is able to control fundamental cellular fates including growth, differentiation, survival and movement. Integrin-mediated regulation of cell survival is thought to contribute to maintenance of tissue homeostasis by ensuring that cells remain in their proper tissue environment. Disruption of this connection leads to a specific type of apoptosis known as anoikis in most non transformed cell types. Conversely, tumor cells display significant resistance to anoikis, as most tumor-derived epithelial cells survive in the absence of adhesion. Anchorage independent growth is a critical step in the metastatic transformation of a tumor since malignant cells, once they begin to metastasize, acquire properties rendering them able to detach from the primary tumor and to escape apoptotic mechanisms. Many oncogenes mimic integrin-dependent signals, thus allowing cancer cells to survive under conditions in which normal cells undergo apoptosis. The identification and characterization of novel genes or proteins promoting anchorage-independent growth, and thereby potentially driving a malignant phenotype, will likely provide candidate targets for innovative anticancer therapies.

Functional genomics aims to assign functional information to each of the genes in an organism in a high-throughput, systematic manner. Several technologies have recently become available to identify gene function in mammalian cells through “gain” or “loss” of function screening; however actual genetic screening are hampered by difficulties in the cloning and identification of integrated ORFs giving a particular phenotype, rendering them still high-demanding and labour-intensive. Here we conceived a high-throughput gene functional screening named “Xenoarray analysis” in which standard gene expression arrays are used for tracing the abundance of exogenous cDNAs derived from the library, before and after a selection. The screening is carried out by applying a selective pressure on the transduced cell population, so that cells transduced with genes conferring a selective advantage are enriched. Comparing microarray signal intensities for the exogenous genes before and after selection allows simultaneous detection of all advantageous ones. This

technology has the advantage to overcome previous problems related to genetic screens by detecting selected enriched ORFs in one single experiment. In this study, we transduced MCF10A cells (spontaneously immortalized human breast epithelial cells), with a retroviral mouse testis library and applied a selection by growing cells in the absence of anchorage. By DNA microarray analysis, we were able to trace the abundance of the enriched ORFs conferring a growth advantage in the absence of anchorage. In two independent infections and selections, the most reproducible enriched gene resulted to be GAB2. Gab2 cDNA transduction in MCF10A cells enabled the validation of its role in anchorage-independent growth.

Introduction

Tissue Morphogenesis and the control of cell-matrix interaction

Tissue plasticity is a fundamental feature of multicellular organisms, which is necessary for the progressive acquisition of a functional identity from undifferentiated cells in embryo development, and for maintenance of the functional integrity of the organism in adult life. Cell adhesion is crucial for the assembly of individual cells into the three-dimensional tissues of animals (Cukierman E et al 2002). A variety of cell adhesion mechanisms underlie the way that cells are organized in tissues. Stable cell interactions are needed to maintain the structural integrity of tissues and dynamic changes in cell adhesion participate in the morphogenesis of developing tissues (Adams JC et al 1993). Stable interactions actually require active adhesion mechanisms that are very similar to those involved in tissue dynamics. Adhesion mechanisms are highly regulated during tissue morphogenesis and are intimately related to the processes of cell migration and cell motility.

Regulation of cell adhesion can occur at several levels, including affinity modulation, clustering, and coordinated interactions with the actin cytoskeleton. Indeed, regulation of tissue morphogenesis requires complex interactions between the adhesion receptors, the cytoskeleton and network of signaling pathways (Aplin AE et al 1998).

Cells sense their location through specific interactions with the extracellular matrix (ECM) as well as neighbouring cells. The ECM is a complex mixture of matrix molecules, including the glycoproteins fibronectin, collagens, laminins, proteoglycans, and non-matrix proteins such as growth factors (Zamir E et al 2001). It provides pliable, but resistant scaffolds for cellular support that are present in all tissues and organs and exerts an extraordinary control on the behaviour of cells (Meredith JE et al 1993). Cell adhesion to the ECM induces discrete cell surface structures tightly associated with the matrix termed cell-matrix adhesions, which mediate direct interactions of the cell with its extracellular environment.

Cell-matrix adhesions are essential for cell migration, tissue organization, differentiation and they play central roles in embryonic development, remodeling, and homeostasis of tissue and organ systems. The biological programs that concur in the specification of tissue dynamic changes are orchestrated by a variety of stimuli leading to cell proliferation, survival, and active migration across the extracellular matrix. As a result, matrix adhesion signals cooperate with other pathways to regulate biological processes such as cell survival, cell proliferation, wound healing, and tumorigenesis.

Cellular contacts with the ECM are mediated via cell adhesion molecules (CAM), among which are integrins, members of the immunoglobulin superfamily, cadherins, and membrane-bound proteoglycans, such as syndecans. However, the effects of the ECM on cells are mainly mediated by the integrins (Miranti CK et al 2002, Danen EH 2003, Hynes RO. 1987) a large family of cell-surface receptors that bind — and therefore mediate adhesion to — ECM components, organize the cytoskeleton, and activate intracellular signalling pathways. The integrins are heterodimers of α and β subunits. Both subunits are type I transmembrane proteins with short cytoplasmic extensions. The mammalian system has 8 α and 18 β subunits, which are known to combine to form 24 distinct integrins. Although each integrin has its own binding specificity, many bind to the same ligand or to partially overlapping sets of ligands. Thus the major adhesive components of the matrix, such as fibronectin and laminin, are recognized by multiple integrins. In addition to matrix ligands, certain integrins also bind to counter-receptors, generally members of the Ig superfamily, expressed on other cells, and hence they mediate cell-to-cell adhesion.

Integrins cluster in specific cell-matrix adhesions to provide dynamic links between extracellular and intracellular environments by bi-directional signalling and by organizing the ECM and intracellular cytoskeletal and signalling molecules (Humphries MJ 2004, Giancotti FG 1999). They transmit both mechanical and chemical signals, imparting polarity to the cell and organizing and remodelling its cytoskeleton during adhesion and migration.

Signals generated locally by the adhesion receptors themselves are involved in the regulation of cell adhesion. These regulatory pathways are also influenced by extrinsic signals arising from the classic growth factor receptors. Furthermore, signals

generated locally by adhesion junctions can interact with classic signal transduction pathways to help control cell growth and differentiation. This coupling between physical adhesion and developmental signaling provides a mechanism to tightly integrate physical aspects of tissue morphogenesis with cell growth and differentiation, a coordination that is essential to achieve the intricate patterns of cells in tissues (Schwartz MA 2002, Giancotti FG 2003).

ECM remodeling and integrin signaling are clearly important for branching organs (Sakai T 2003), such as lung (Moore KA 2005), kidney and salivary gland. For instance, branching morphogenesis involves a number of repetitive steps, starting with formation of a cleft or indentation in the basement membrane, which is a specialized ECM located at the basal surface of differentiated epithelial cells (Bernfield M.R 1984).

Branching morphogenesis is a multi-step process that controls the formation of polarized tubules starting from hollow cysts. Its execution entails a series of rate-limiting events which include reversible disruption of cell polarity, dismantling of intercellular contacts, acquisition of a motile phenotype, stimulation of cell proliferation, and final re-establishment of cell polarity for creation of the definitive structures. Branching morphogenesis takes place physiologically during development, accounting for the establishment of organs endowed with a ramified architecture such as glands, the respiratory tract and the vascular tree. In cancer, aberrant implementation of branching morphogenesis leads to deregulated proliferation, protection from apoptosis and enhanced migratory/invasive properties, which together exacerbate the aggressive features of neoplastic cells.

Another example in which ECM remodeling plays important roles is in the execution of invasive growth (Brakebusch C 2002). Invasive growth is a complex morphogenetic program in which proliferative responses are integrated by apparently independent events such as migration, survival, matrix degradation, and induction of cell polarity (Gentile A et al, 2004, Comoglio P.M 2001). Invasive growth plays a central role in a wide variety of physiological and pathological events. During embryogenesis, it determines events such as gastrulation and nervous system development, and in adult organism regulates inflammatory processes and tissue remodeling during wound healing (Schmidt C et al 1995). The pathological facets are

cellular mechanisms that cause local invasion and metastasis (Comoglio PM et al 2002). In this scenario, cells proliferate without control, loose contact-inhibition, move from their primitive sites and invade distant tissues, where they survive and grow in an aberrant way and form secondary colonies. Indeed, from both a biological and clinical point of view, a tumor is defined as malignant when neoplastic cells acquire the ability to move from their natural context into the adjacent surroundings and colonize tissues and organs that are distant from the original site of growth.

In the first step of this sequence a cell within a colony or solid tissue is instructed to disrupt cadherin-based intercellular junctions and acquire a fibroblastoid, motile phenotype, initiating detachment from the primary site of accretion. This dramatic reshaping is accompanied by cytoskeletal rearrangements and enhanced production of matrix proteases, which digest basal lamina components and facilitate cell movement through the surrounding environment. During this phase, invading cells must induce a constant and dynamic remodeling of integrin-mediated adhesive contacts with the ECM, which provides a mechanical support for cell migration and prevents the induction of apoptosis (Trusolino L et al 2000).

Cell depolarization and invasion are followed by stimulation of cell growth, which allows new regions of the extracellular environment to become populated with cells, setting the stage for the restoration of normal tissue complexity. Ultimately, these cells stop dividing, repolarize, and start terminal differentiation, arranging themselves into three-dimensional structures that are usually organized as branching tubules.

It has long been known that many cytokines and growth factors can produce many of the signal features of invasive growth: proliferation, differentiation, chemotaxis, and protection from apoptosis. The program of invasive growth is orchestrated in its specific aspects by a variety of cytokines and growth factors, like epidermal growth factor (EGF), the insulin-like growth factor-1 (IGF-1) and the fibroblast growth factor (FGF). The prototype of the scatter factors family is Hepatocyte Growth Factor (HGF) (Trusolino L et al 2002).

Cell adhesion and Anchorage-dependent growth

Cell adhesion plays an important role in the regulation of cellular growth by giving consensus signals leading to cell proliferation (Aplin AE et al 1999). Proliferation of

mammalian cells is tightly regulated by multiple environmental influences, primarily adhesion to the extracellular matrix, cell-cell adhesion and soluble factors (i.e. polypeptide growth factors or inhibitors, mitogenic lipids, inflammatory cytokines and hormones). Of these environmental cues, soluble growth factors and integrin mediated adhesion are most crucial (Howe A et al 1998), as loss of adhesion generally results in complete G1 phase cell cycle arrest (Assoian RK et al 1997). For susceptible cell types, loss of adhesion leads to apoptosis (Ruoslahti E et al 1994), which might be regarded as the extreme case in which cell numbers decrease rather than remain stable. Both integrins and growth factor receptors use multiple cytoplasmic signaling pathways to regulate G1 phase cyclins and associated kinases that determine cell cycle progression (Assoian RK et al 2001, Mettouchi A et al 2001), strongly emphasizing the point that growth factors and the ECM are partners in cell cycle control, with each providing essential signals that allow for proper induction of the G1 cdks (cyclin-dependent kinases) and phosphorylation of their substrates. Cyclin D1 is the primary D type cyclin for several anchorage- dependent cell types, and recent studies indicate that the ECM and mitogens are jointly required to induce cyclin D1 expression (DeGregori et al 1995): both stimuli are required for its mRNA expression and for its translation. In addition, the induction of cyclin A is strongly dependent on signals from the ECM, and several lines of evidence indicate that a large part of this effect is a consequence of adhesion-dependent pRb/p107 phosphorylation (Schulze A et al 1996). On the contrary, in suspended cells, mitogens are unable to induce the expression of cyclin D1 and subsequent formation of cyclins-cdk complexes which correlates with increased expression of cell cycle inhibitors (p21 and p27) contributing to cell cycle arrest. Indeed, it seems that the coordinated control of the G1 cyclin-cdks by growth factors and the ECM underlies the well-established anchorage requirement for the proliferation of untransformed cells, since formation and spread of tumors, is closely associated with decreased dependence on ECM for growth and survival (Schwartz M 1997, Kang JS et al 1996).

Anoikis

Tissue homeostasis reflects a dynamic balance of cell proliferation, differentiation and apoptosis. In 1994, Frisch et al chose the term “anoikis” (Greek: state of

homelessness) to describe the form of apoptosis triggered in response to inappropriate cell/ECM interactions (Frisch SM et al 1994, Frisch, S. M. and Ruoslahti E. 1997). Anoikis seems to play an important role in the physiological induction of apoptosis during development and maintenance of tissue homeostasis in the organism. During vertebrate embryogenesis the inner endodermal cells loose anchorage to the underlying matrix and subsequently undergo anoikis permitting cavitation to occur (Coucouvanis E et al 1995). Non-transformed mammary epithelial cells are anchorage dependent and normal mammary gland development depends on cell-matrix anchorage. Degradation of the underlying laminin-rich matrix by metalloproteinases causes anoikis of these cells and contributes to the involution of the mammary gland (Talhouk RS et al 1992, Lund LR 1996). Similarly, the involution of the prostate gland is likely to reflect anoikis as growth factor withdrawal induces dissolution of cell-cell contacts as well as degradation of extracellular matrix with subsequent apoptosis, i.e. anoikis.

Likewise, in the gut, intestinal epithelial cells (IEC) nicely reflect how a microenvironment can change the fate of a cell within a few days (Shanmugathasan M et al 2000). IEC are generated by stem cells in the crypt and die of apoptosis within 3–5 days as they reach the luminal surface where they loose cell anchorage and shed into the intestinal lumen. This process is mediated by alterations of cell-cell anchorage, cell-matrix-anchorage and the expression of pro/antiapoptotic proteins within IEC, culminating in shedding of the dying enterocyte (Grossmann J et al 1998). In addition, there is evidence that keratinocytes undergo apoptosis/anoikis as they loose anchorage to the substratum and eventually shed from the skin (Haake AR et al 1993). Clearly, anoikis plays essential role in tissue homeostasis by maintaining correct cell number of high turnover epithelial tissues. The clearest evidence for this is that the breakdown of anoikis contributes to neoplasia.

Anchorage-independent growth and cancer progression

Dominant mutations in oncogenes (RAS, RAF, SRC, CRK) and recessive mutations in tumor-suppressor genes (PTEN, p53) disrupt the regulatory circuits that control cell fate, conferring on neoplastic cells the ability to survive and proliferate even if appropriate extracellular cues are not available (Kang JS et al 1996, McFall A et al

2001, Eckert LB et al 2004, Brabek J et al 2004). As a consequence, cells that have undergone neoplastic transformation are much less dependent on ECM adhesion for their survival and proliferation. The acquisition of anchorage-independent growth is therefore regarded as a critical step during the metastatic transformation of a tumor (Schwartz M et al 1997, Reddig PJ et al 2005). In fact, malignant cells, once they begin to metastasize, have obviously acquired properties rendering them resistant to loss of cell anchorage and anoikis—i.e. are able to detach from the primary tumor without undergoing apoptosis. Metastatic dissemination of cancer cells is characterized by multiple processes which involve their migration through the interstitial connective tissue to the capillaries or lymphatics, their diapedesis through the basement membrane and the endothelial cell lining of the capillary wall (intravasation), their transport with the lymph or blood stream and their adhesion to capillary walls followed by extravasation into the tumor host organ, where the tumor cells proliferate and form a secondary tumor (Fidler IJ et al 2003). Despite their relative anchorage independence, cancer cells still benefit from integrin signals, both during tumor initiation and tumor progression. It is increasingly clear that neoplastic cells relocalize or enhance the expression of integrins that favor their proliferation, survival and migration; whereas they tend to lose expression of the integrins that exert the opposite effect (Guo W et al 2004, Eble JA et al 2006). Certain integrins seem to mediate survival in non-transformed cells ensuring tissue homeostasis while other integrins seem to be upregulated particularly during neoplastic transformation providing survival signals during invasion and metastatic growth (Janes SM et al 2004). This concept has been further substantiated by the finding that α6β4 can serve an adhesion unrelated adaptor function for the tyrosine kinase Met, the receptor for HGF. β4 integrin conspires with the tyrosine kinase Met for efficient execution of anchorage independent growth by channeling Met signals towards activation of the Ras- MAPK–ERK oncogenic pathway (Bertotti et al 2006). Alternatively, the integrin repertoire of a tumor cell can predict the site of metastatic implantation and growth (Janes SM et al 2004, Hood JD et al 2002).

Signalling pathways relevant for anchorage-independent growth and anoikis

ECM-ligand binding to an integrin initiates signals regulating various cell functions, such as morphological changes, survival, adhesion, migration and gene activation. Integrins can function in an analogous way to growth factor receptors (GFR), or in combination with, and indeed activate many of the same downstream pathways, giving rise to a reciprocal crosstalk between the two systems (Clark EA et al 1995, Miyamoto S et al 1996, Colognato H et al 2002). Physical interaction between integrins and growth factor receptors results in different modes of signalling cooperation. (a) Signals triggered by growth factor receptors and those induced by integrin engagement might follow parallel but superimposable pathways, with additive activation of signaling cascades that converge on common downstream effectors. (b) Integrin ligation by matrix components can lead to adhesion-dependent, ligand independent activation of integrin-associated growth factor receptors. (c) Integrins can participate in growth-factor dependent signals irrespective of their adhesive function as in the case the Met receptor for HGF and α6β4 integrin (Bertotti et al 2005). Cells react to the simultaneous presence of an adhesive consensus and a growth factor molecule by mounting a double signaling response that ultimately converges onto common downstream effectors for their optimal activation (Yamada KM et al 2002, Brunton V.G et al 2004). Cell adhesion is therefore necessary to implement activation of growth factor receptors, and growth factors are necessary to stimulate cell adhesion, migration and the ensuing integrin-dependent signals. From a biological standpoint, this results in the enhancement of growth factor-dependent responses — for example, cell proliferation, motility or protection from apoptosis. Similarly, cell detachment results in cellular desensitisation to growth factors, for instance by targeting the receptor for degradation via the ubiquitin/proteasome system (Baron V et al 2000). Different factors such as cell type, composition of the surrounding matrix, tissue origin, state of differentiation or state of neoplastic transformation dictates which set of integrins are critical for transducing the survival signals to the interior of the cell. Anchorage-independent growth of tumor cells may

be caused by loss of normal integrin and growth factor coupling, allowing inappropriate survival signals to be maintained in the absence of adhesion.

Role of protein kinase signaling pathways in anchorage-independent growth

Numerous kinase/phosphatase signaling molecules have been identified as central regulators of anchorage-independent growth. Among the major players is the Ras pathway since it was shown that Ras activation could induce anchorage-independent growth (Frisch SM et al 1994, Eckert LB et al 2004) and that one of its effectors, the kinases PI3K (phosphoinositide-3 kinase) could induce similar effects (Khwaja A et al 1997). The serine-threonin kinase PKB/AKT represents a central element of cell survival signaling as integrins, growth factors and cell-cell anchorage-mediated signal transduction converge to the activation of this kinase. Activation of PKB/AKT leads to promitotic transcriptional activation, inhibition of the apoptotic machinery through inactivation of caspase-9 and the proapoptotic protein Bad, the Forkhead family transcription factors and activation of NF-kB (Persad S et al 2000, Datta SR et al 1997). In partcular Akt kinase has been associated to anchorage-independent growth in several cell types (Khwaja A et al 1997, Wei L et al 2001). A major integrin signaling molecules that has been linked to PI3K/Akt survival pathway activation is Fak (focal adhesion kinase). Fak plays a central role in transmitting adhesion dependent survival signals (Frisch SM et al 1996, Sieg DJ 2000) as constitutive active FAK mutants can promote anchorage-independent growth (Hungerford JE et al 1996). Detachment of epithelial cells from the extracellular matrix leads to rapid caspase 3-mediated degradation of FAK (Gervais et al 1998), cessation of the PI3 kinase-mediated survival signal, and anoikis (Frisch et al 1996). Interestingly, FAK is overexpressed in most human colon tumors, with additional increases in expression correlating with tumor progression (Weiner et al 1993).

Activation of the epidermal growth factor receptor (EGFR) and subsequent stimulation of the Erk/MAPK cascade paly important roles in anchorage-dependent and independent growth (Le Gall M et al 2000, Chen Q et al 1994). Erk (extracellular signal-regulated kinase) activation is required for both cell cycle progression and survival. An example of coordinate crosstalk between integrin and EGFR signalling results from the regulation of a specific apoptotic target involved in detachment-

induced apoptosis. Studies have examined the connection between the EGFR-Erk/MAPK survival pathway and the BH3-only protein Bim in ancorage-independent growth and showed that the Erk/MAPK cascade directly leads to the phosphorylation of Bim leading to its degradation in anoikis resistant cells (Ley R et al 2003). BH3-only proteins appear to act as sensors of cellular stress and have no intrinsic ability to induce cell death on their own. Two members of this family Bim and Bmf appear to be important sensors of changes in the actin cytoskeleton (Puthalakath H et al 2001). In adherent cells, activated EGFR represses Bim expression through the Erk/MAPK pathway. Conversely, the loss of β1 integrin engagement in detached cells represses expression of the EGFR and increased Bim expression. Thus, the negative regulation of Bim expression by adhesion and EGFR signalling were implicated in the suppression of anoikis in breast epithelial cell lines (Reginato MJ et al 2003).

C-Src is the prototype of a closely related family of nine genes encoding non-receptor membrane-associated protein tyrosine kinases (PTK). In a variety of cell types, Src family members participate in regulation of diverse functions, including proliferation, cell cycle, migration, adhesion, and differentiation (Thomas and Brugge 1997). Src activation has also been implicated in anchorage-independent growth and anoikis resistance (Coll et al 2002). Detachment of normal epithelial cells from the extracellular matrix leads to dephosphorylation of FAK and dissociation of FAK/Src complexes, followed by apoptosis (Frisch and Francis 1994, Khwaja et al 1997). Conversely, v-Src transformed cells possess FAK/Src complexes resistant to dissolution upon cellular detachment, decreased degradation of FAK and resistance to anoikis (Frisch et al 1996). Thus, increases in Src activity through FAK association contributes to anoikis resistance. Moreover, Src overexpression occurs in several human tumors and coorelates with poor prognosis (Karni et al 1999, Yeatman et al 2000).

Clearly, the biochemical and/or structural nature of the interface between cell adhesion and growth factor signaling is very complex and regulated by intricate networks. In this view, the existence of an anchorage-dependent 'signalosomes' has been postulated. The elements responsible for collaboration between cell adhesion and

growth factors may represent a prime target for specific, therapeutic manipulation of anchorage-independent cell growth characteristic of cancerous cells.

Functional Genomics and Targeted therapies

Various technologies can be used to produce genome-scale, or 'omics', data sets that provide systems-level measurements for virtually all types of cellular components in a model organism. The study of genes and their functions within a biological mechanism, both physiological and pathological, is an absolute necessity to better understand pathways and to setup novel and more effective therapies, especially if we consider that at present, functional information is available for only ~15% of human genes (Venter JC et al 2001). One successful approach to doing so requires the integration of omics data derived from genomics, transcriptomics, proteomics, metabolomics, glycomics, lipidomics and the protein-protein interactome (Joyce AR et al 2006).

The objective of functional genomics is to assign functional information to each of the genes in an organism in a high-throughput, systematic manner (Steinmetz LM et al 2004). Within this context, functional oncogenomics can be defined as the attempt to systematically define gene functional alterations in cancer. The final goal of oncogenomics is the identification of molecules with altered functional role against which cancer therapeutics might be targeted.

The study of a gene function requires the observation of its given phenotype when it is absent (removed) or expressed using forward or reverse genetics. Thanks to the availability of large collections of gene-perturbing reagents it is now possible to perform high-throughput functional assays to tackle this problem (Brummelkamp TR et al 2003). The use of genome-wide collections has the advantage that each component of the collection is sequenced and it is easily identifiable, therefore representing standardized and reliable tools. Several new technologies have recently become available to identify gene function in mammalian cells using high-throughput genetic screens (Bernards R et al 2006, Grimm S et al 2004).

An alteration in cellular phenotype can be accomplished through the introduction of exogenous genetic material into cells. It is most straightforward to select for a phenotype that confers a new trait to cells, such as the ability to form tumors in

experimental animals, growth in semi-solid media or acquisition of a transformed morphology. In each case, it is important that the frequency with which the recipient cells spontaneously acquire the selected phenotype is very low. In fact, engineering the proper cell system to carry out a genetic screen is often far more time-consuming and demanding than the performance of the genetic screen itself. As the novel phenotype is introduced in a dominant fashion by the foreign DNA, such genetic screens are referred to as 'gain-of function'screens (Kitamura, T et al 1995, Toshihiko Okia et al 2003).

Most genetic targets will result from a "gain of expression", as in the case of genes that are activated by either a point mutation or a chromosomal rearrangement that creates a new protein sequence. Targets can also be proteins with a normal sequence that are pathologically overexpressed by genomic amplifications, promoter mutations or upstream pathway activation. The best example of a targeted, small-molecule oncogene inhibitor that interrupts a crucial signalling pathway is Imatinib (better known by its commercial name, Gleevec), which is already in clinical use. Although Gleevec was initially developed as an inhibitor of the Bcr-Abl kinase, it is now known to inhibit a selected family of tyrosine kinases, including KIT and PDGFR, in a variety of tumor types. The application of Gleevec to a specific, oncogene dependent form of sarcoma, known as gastrointestinal stromal tumor (GIST), had spectacular anti-tumor effects and provided proof that both haematopoietic and solid tumors are amenable to targeted therapy.

On the other hand, genetic screens that aim to identify gene function through inactivation of a gene (or its corresponding mRNA) are referred to as "loss of-function screens" (Berns K et al 2004). Various technologies have been developed to study the effects of gene suppression in mammalian cells, including genetic suppressor elements (GSEs), antisense vectors, ribozymes, aptamer libraries and, more recently, RNA interference (RNAi). Designing compounds that are toxic to cells on the basis of "loss of expression" might be an alternative way to target cancer cells; this approach could be adopted in the case of missing tumor-suppressor proteins, a genetic event that is common in tumor development and could be addressed by chemical synthetic lethal screenings (Kaelin WG et al 2005, Aza-Blanc, P et al 2003).

For instance, inhibition of a gene that shows a synthetic lethal interaction with loss of TP53 will only be toxic in cells that contain a mutation in TP53, making it a tumor cell-specific drug target. Inhibition of such genes in normal cells (that contain wild-type TP53) will be much less toxic, providing a therapeutic window for cancer treatment. The problem in identifying siRNA vectors that show a synthetic lethal interaction with cancer-specific mutations is that these vectors are specifically lost from cells, making it hard to identify interactions when such screens are performed in a polyclonal format.

To perform "gain-of function screens", different methods have been developed based on two molecular biology techniques. One is the generation of expression vectors containing the full length cDNA for genes of interest (Whitehead I et al 1995, Simonsen H et al 1994) the second is the transduction of such vectors into target cells. A retroviral vector for instance can stably integrate its genome containing the coding sequence of interest in the genome of a target cell, which will express that gene.

Two approaches for selective screenings take advantage from the described techniques. In the first, a single ORF is transduced into the target cell line, then a stress capable to strongly reduce the viability and the proliferative potential of cells is applied. If the overexpression of the transduced gene confers resistance to the selective stress, then the fraction of cells expressing the exogenous gene has to increase. This technique requires a selection experiment for each ORF to be analyzed, which is a problem for a high-throughput screening.

In the second approach, an expression library is transduced into the target cell line. An expression library is the population of full length cDNAs generated from the total RNA extracted from cells or tissues of interest by reverse transcription, cloned into a retroviral expression vector. After transfection into the packaging cells, a population of viruses containing the transcripts expressed by the original sample is generated. A selection (for instance a cytotoxic drug) is then applied to the target cell line transduced with the retroviral library and resistant clones are analyzed to identify exogenous genes (library derived) capable to confer resistance to the cells. However, a huge amount of work is typically required to identify the integrated cDNAs in the resistant colonies, and to verify that they mediate the selective advantage. Moreover, the selection has to be drastic to avoid the emergence of spontaneously resistant

colonies, which would dramatically increase the number of false hits. As a consequence, this approach does not allow identification of genes conferring a limited advantage *per se* and potentially synergizing with others.

Xenoarray Technology

To overtake the problems of these two approaches, the setup of a new technology of selective screening was strongly needed to allow the use of expression libraries but also to trace the relative abundance of the transduced enriched ORFs in one single measurement, with no necessity of cloning and identification of integrated sequences.
Here we conceived a high-throughput gene functional screening combining transduction of mammalian cells with a retroviral cDNA expression library and DNA microarray technology, to trace the abundance of exogenous cDNAs derived from the library in the entire cell population. The screening is carried out by applying a selective pressure on the transduced cell population, so that cells transduced with genes conferring a selective advantage are enriched. Then microarray on RNAs from transduced cells prior and after selection with a gene chip, allows to measure exogenous transcripts signals and to identify ORFs enriched in selected cells. Comparing microarray signal intensities for the exogenous genes before and after selection therefore allows simultaneous detection of all advantageous ones. It is possible that such ORFs codify for proteins capable, under selection conditions, to confer resistance to cells.
The innovation of the Xenoarray approach brings several advantages to the traditional selective screening. Firstly, since the abundance of exogenous genes is easy to be detected within the host cells genome, the screening is much more sensitive, the selection doesn't need to be so drastic and even genes having a partial role in a pathway or potentially synergizing with others can be identified. Secondly, the microarray analysis allows a much faster and systematic identification of genes conferring a selective advantage, by establishing a direct cause-effect relationship. Finally, they overcome the labor-intensive task of cloning resistant cells to identify the integrated genes and verify that they effectively mediated the selective advantage.

Aim of the work

The aim of the work was to identify candidate genes involved in anchorage-independent growth by setting up an innovative functional screening based on cDNA expression library transduction and DNA microarray technology to recover, trace and quantify the abundance of enriched ORFs conferring the selective advantage. For this purpose, we chose to transduce MCF10A cells (human breast epithelial cells) since they rely on anchorage for cell growth and maintenance and represent an ideal model to study anchorage-(in)dependent growth (Debnath J et al 2005). In fact, when MCF10A cells are cultured in the absence of anchorage, for instance on polyhema coated plates, which inhibits cellular attachment and spreading; they undergo growth arrest and programmed cell death, namely anoikis (Reginato MJ et al 2003). Furthermore, MCF10A cells are spontaneously immortalized human breast cells (Soule HD et al 1990), thus not transformed, and therefore well suited to study the effects of genes conferring oncogenic properties such as the capacity to grow independently from anchorage. We exploited these features and transduced MCF10A cells with an expression library of full length cDNA derived from mouse testis or with a retroviral vector encoding for GFP as a control. The screening was then carried out by applying a selective pressure (anchorage-independent growth) on the transduced cell population, so that cells transduced with genes conferring a selective advantage were enriched. Comparing microarray signal intensities for the exogenous genes before and after selection allowed us to detect all the advantageous ones. These genes, for instance, could mediate the survival, growth or inhibition of apoptosis of MCF10A selected cells. The aim of this project therefore was to:

- Select control and library-transduced MCF10A cells able to grow in the absence of anchorage;
- Screening for enriched genes conferring growth advantage in the absence of anchorage by Xenoarray technology;
- Identify candidate hits;
- Validate and functionally characterize the most enriched gene by over expression in MCF10A cells;
- Give insights on the mechanisms of anchorage-independent growth conferred by the identified gene.

Results

Transduction and Selection of MCF10A cells

Library transduction and Xenoarray analysis

By Xenoarray technology it is possible to transduce cells with an expression library and to trace exogenous transcripts abundance after a selection using DNA microarray technology (Figure 1).

Here we focused on human MCF10A cells as a possible model for expression cloning of genes conferring anchorage-independent growth. For the functional screening, MCF10A cells were transduced with a retroviral mouse testis expression library (from Stratagene) or with a vector encoding for GFP only as control. The control vector was the same used to construct the expression library. Infections were performed in duplicate (A and B), using in all cases a multiplicity of infection around 1, to avoid multiple integrations in the same cells.

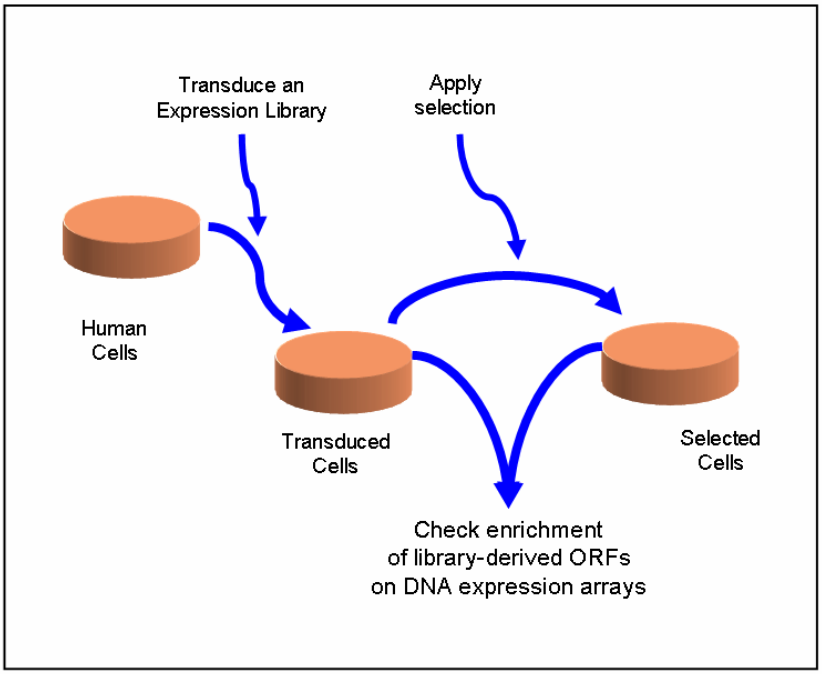

Figure 1.Cartoon of the Xenoarray design and selection strategy. Cells transduced with an expression library are subjected to a specific selection and DNA microarrays are used to identify ORFs conferring a selective advantage.

This led to the generation of four populations of cells: GFP-transduced unselected A (GFP-UNS-A), GFP-transduced unselected B (GFP-UNS-B), library-transduced unselected A (LIB-UNS-A) and library-transduced unselected B (LIB-UNS-B).

The advantage of having double, independent infections enabled us to verify both the efficiency of infection as well as the reproducibility of the selective process so to increase the robustness of the screening. In particular, the repeated infection-selection procedure allows to rule out two main possible artifacts: (i) enrichment deriving from deregulation of an endogenous gene by insertional mutagenesis; (ii) a very small subpopulation of resistant cells may exist from the beginning, and a cDNA transduced in these cells may get reproducibly enriched by carryover.

To measure the library-derived transcripts by Xenoarray analysis, total RNA was extracted from confluent cells and processed to produce a cDNA using a library-specific primer in order to increase the abundance of transcripts derived specifically from the library as described in Methods. Hybridization of the resulting cRNAs was then carried out using an Illumina murine gene chip, to allow specific detection of library transcripts (Figure 2).

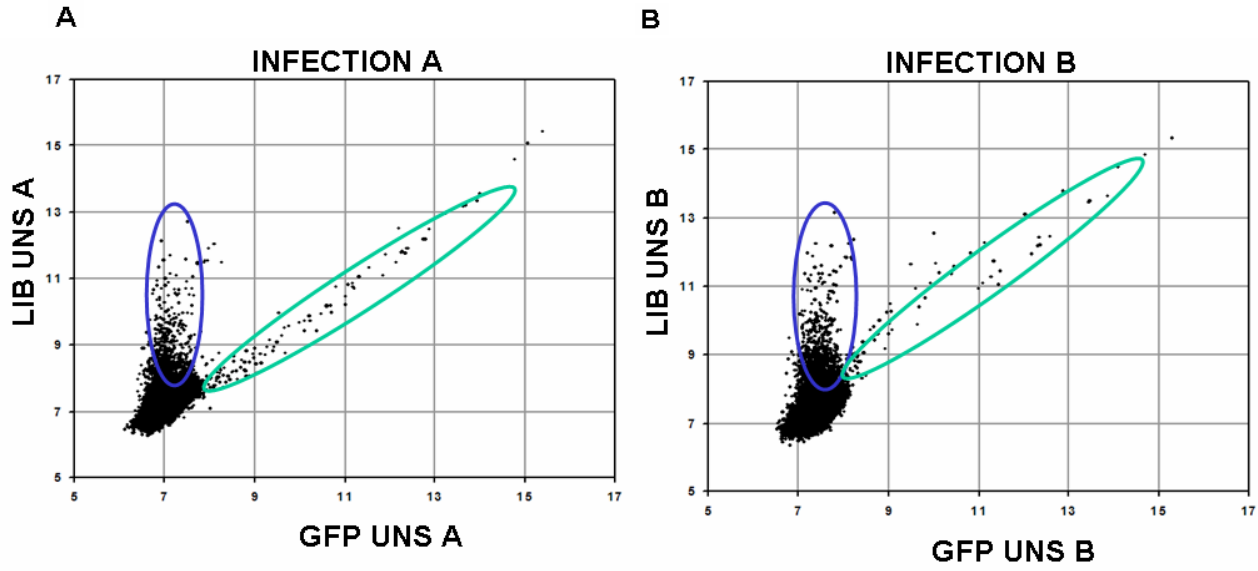

Figure 2. Xenoarray analysis on transduced MCF10A cells. Dot plot comparing the signal intensity for GFP-transduced and library-transduced cells for infection A (A), and infection (B).

The Xenoarray analysis pipeline was performed with Illumina BeadStudio software and subdivided in 3 main tasks: (i) Removal of probes cross-hybridizing to endogenous transcripts. All probes with a Detection value higher than 0.99 in at least one sample of GFP-transduced cells were excluded from the subsequent steps. (ii) Data preprocessing to improve reproducibility, and reduce technical noise. Briefly, to improve the sensibility of the system and reduce technical noise, we subtracted the signal corresponding to the 2/3 of the minimum values from the raw signal across all the chips. Then data was scaled on the 10^{th} percentile. For the filtering of probes giving unspecific signals, probes detected in cells transduced with GFP but not the library (before and after selection) were filtered out. (iii) Identification of probes giving higher signal after selection. The software dot-plot package was used to compare probe signals from transduced cells before and after the selection, and to calculate for each probe the $\log_2$ of the ratio between the signals before and after selection. The scatter plot in Figure 2 shows the detection of library-derived ORFs corresponding to infection A and B (blue circles) over a background of endogenous genes (green circles). Respectively, we estimated that the number of probes giving significant signal only in transduced cells was around 600 in infection A and 620 in infection B.

Anchorage-independent growth selection

When MCF10A cells are cultured in the absence of anchorage, for instance on polyhema coated plates; they undergo growth arrest and programmed cell death. We exploited this feature for a selective screening of anchorage-independency using the Xenoarray approach, on mouse testis library-transduced and GFP-transduced MCF10A cells (Figure 2). The selection was carried out by culturing GFP or mouse testis library-transduced MCF10A cells on polyhema coated plates for a period of 48h, followed by 24h of recovery on regular plates. Both replicates, A and B, of control (GFP) and transduced (LIB) cells were subjected to the selection, which was repeated for six cycles in three weeks. Interestingly, we were able to recover cells from all four selections, namely: GFP-transduced selected (GFP-SEL-A), GFP-transduced selected B (GFP-SEL-B), library-transduced selected A (LIB-SEL-A) and library-transduced selected B (LIB-SEL-B). This means that resistant cells may also emerge from control populations, transduced with GFP only.

Biological Assays on selected and unselected cells

Selected and unselected cells were assayed for different biological parameters. Firstly, a growth analysis was performed on selected and unselected populations (Figure 3) and increased growth was observed for only library-transduced selected populations.
In fact, the LIB-SEL populations displayed significantly higher proliferation rate compared to control cells transduced with GFP only. Interestingly, GFP-SEL cells could not overcome the LIB-SEL growth rate, highlighting a "library effect" not explainable with insertional mutagenesis but likely independent from the selection itself and deriving from the expression of exogenous transcripts. The increase in proliferation could be seen already after 48h and was further augmented after one week of cell culture.

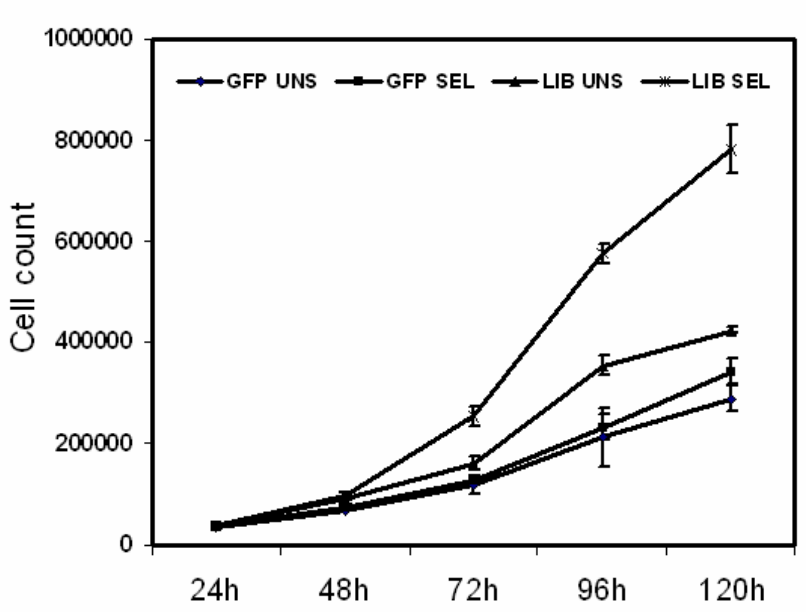

Figure 3. Growth curve of unselected and selected cells. Cells were counted after 24h, 48h, 72h, 96h and 120h and the mean and standard deviation of triplicate values was represented as function of time in culture.

Secondly, we evaluated cell growth in the absence of anchorage. Cells were plated for different times either on plastic or on polyhema coated plates in complete medium or in "starved" medium lacking EGF and serum (Figure 4). Cell vitality was measured by a MTT assay which allows the detection of metabolically active cells and

expressed as a ratio between the growth of LIB-SEL cells and GFP-SEL cells (CTRL), in respect of the percentage of plated cells the day of the assay, as described in Methods.

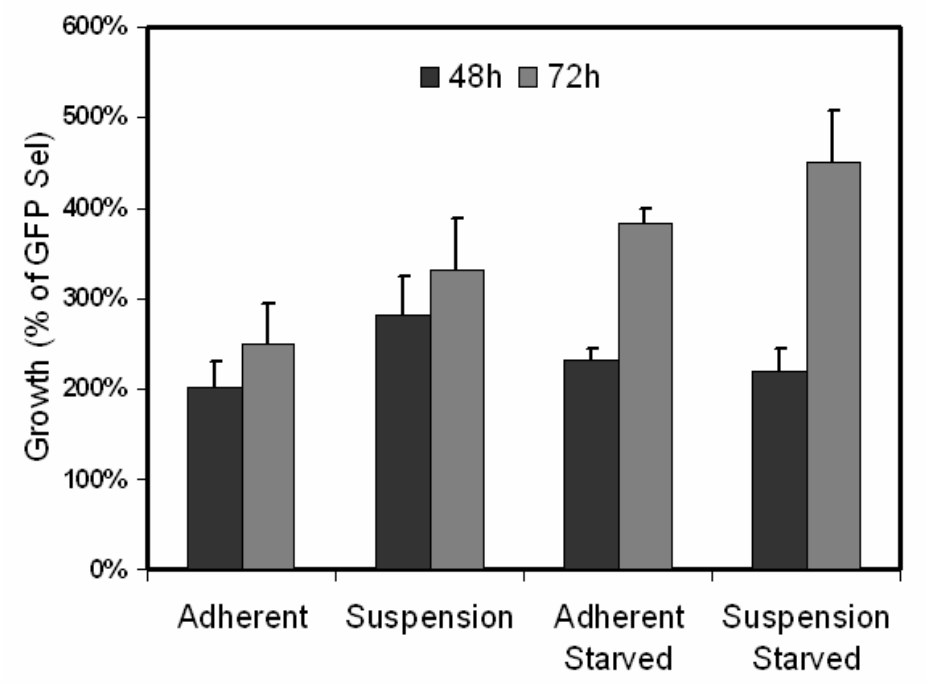

Figure 4. Growth curve on selected populations in adhesion or suspension, in complete or starving medium for 48h ad 72h respectively. Cell vitality, expressed as a ratio between control (GFP SEL) and library-transduced selected cells, was normalized to the percent of viable plated cells at time 0. The data represent the mean and standard deviation of triplicate values.

Similarly to adherent conditions, the LIB-SEL cells displayed a higher vitality after 48h or 72h of suspension cultures compared to control cells. In fact, LIB-SEL cells displayed a 2.6 time fold and 3.2 time fold increase in proliferation at 48h and 72h respectively of suspension culture. The addition of a starving medium had little effect on the growth of adherent LIB-SEL cells, while dramatically inhibiting the proliferation of control cells, indicating that LIB-SEL cells acquired the capacity to grow independently from growth factors. More importantly, this advantage was more evident when cells were kept in suspension for the same time points.

Subsequently, we evaluated the ability of selected cells to grow in soft agar, an in vitro hallmark of cell transformation.

As can be seen in Figure 5, LIB-SEL cells formed very large colonies while GFP-SEL cells formed few and small colonies, with the majority of cells remaining as single units and not aggregates, similarly to GFP-UNS cells. This enforces the "library effect" hypothesis for which it is the expression of exogenous transcripts to drive a biological phenotype possibly involved in anchorage-independent growth and neoplastic transformation.

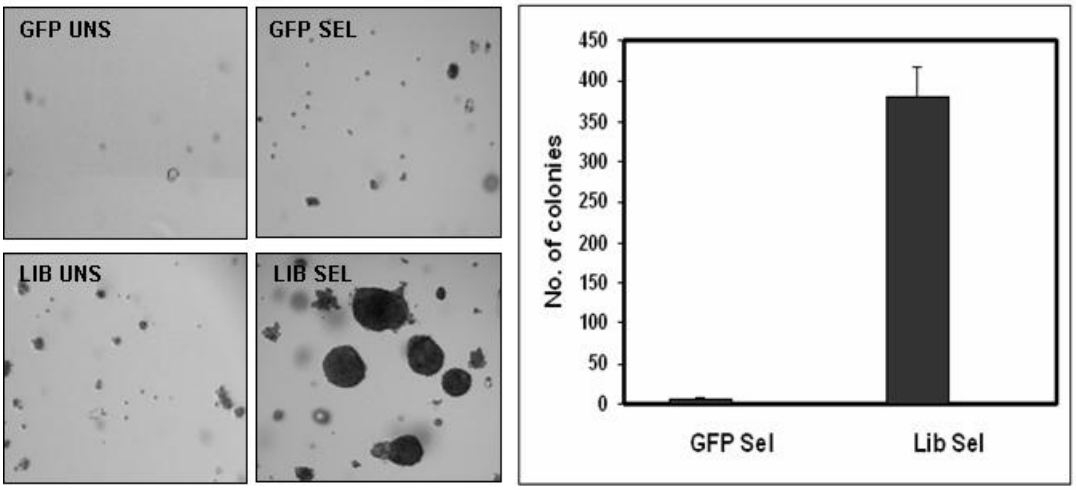

Figure 5. Soft agar assay on unselected and selected cells. Phase-contrast images were captured by a BD Pathway microscopic station (BD biosciences) after 3 weeks in agar, and quantification of cell colonies was performed by Attovision 1.5 software (BD biosciences).

Xenoarray analysis on selected cells

To evaluate if transcripts derived from the library were enriched in both selections A and B, RNA was extracted from LIB SEL (A and B) cells. RNA was then processed to produce a cDNA using the library-specific primer as previously described and the corresponding probes were used to hybridize murine gene chips. After removing from the analysis probes giving detectable signal in untransduced cells, comparisons were made on library-transduced cells, before and after the selection, for both replicates A and B (Figure 6). We observed a significant number of transcripts detected at higher levels in selected cells (blue circles). However, selection B resulted to be more stringent than selection A since a lower fraction of transcripts were detected. Surprisingly, the majority of the transcripts corresponding to the library were lost. This could be due to a high rate of dying cells observed after selection, so that only a

small fraction of the cells composing the initial population was represented at the end of the selection. To see if the same transcripts were enriched in both selections, we calculated an "enrichment score" (ES) for each transcript, i.e. the log(2) ratio between the signals in selected and unselected cells. The scatter plot presented in Figure 7, shows that several genes were reproducibly enriched in both selections.

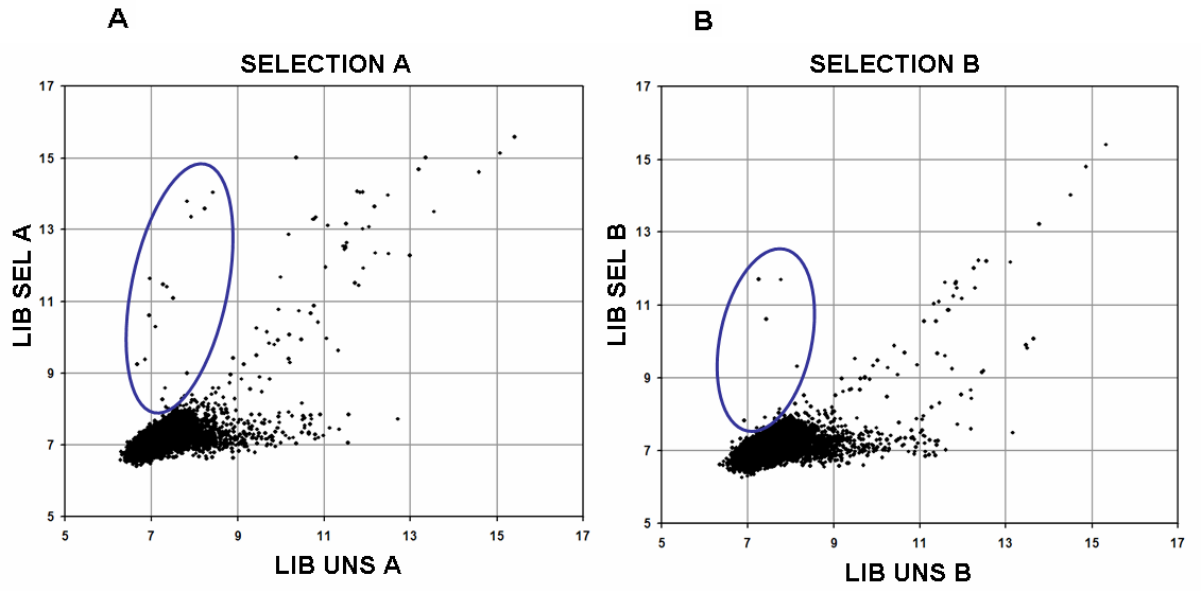

Figure 6. Xenoarray analysis on library-transduced MCF10A cells before (x-axis) and after (y-axis) selection, performed for infection A (A) and infection B (B).

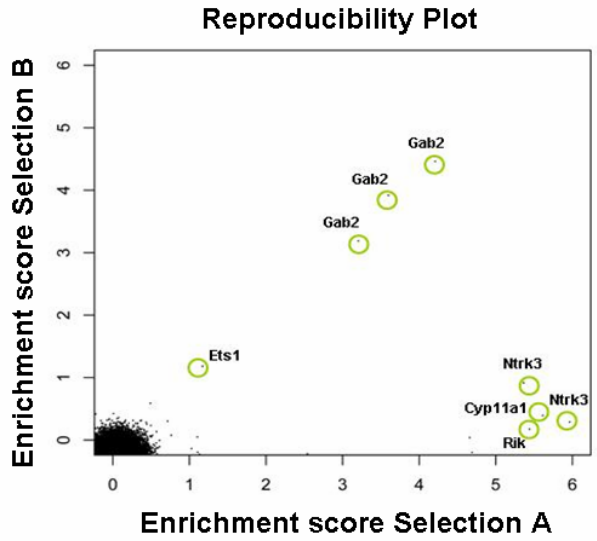

Figure 7. Scatter plot comparing library transcripts enrichment scores (log2 ratio) between the two selections. Green circles represent the transcripts displaying significant enrichment scores in one or both selections.

The names and the ES of the enriched transcripts which emerged from selection A and selection B are listed respectively in Table 1 and Table 2.

Table 1. Enriched transcripts from Selection A.

Gene Symbol	Description	ES
LOC218993	hypothetical LOC218993 (LOC218993)	2.54
4932701A20Rik	RIKEN cDNA 4932701A20 gene (4932701A20Rik)	2.56
4932701A20Rik	hypothetical Glycoside hydrolase family 56/EGF-like domain containing protein	4.69
4933414E15Rik	hypothetical Glycoside hydrolase family 56/EGF-like domain containing protein	3.65
Gab2	growth factor receptor bound protein 2-associated protein 2 (Gab2)	3.20
Gab2	growth factor receptor bound protein 2-associated protein 2 (Gab2)	3.59
Gab2	growth factor receptor bound protein 2-associated protein 2 (Gab2)	4.20
Ets1	E26 avian leukemia oncogene 1, 5 domain (Ets1)	1.17
Ntrk3	neurotrophic tyrosine kinase, receptor, type 3 (Ntrk3), transcript variant 1	5.36
Cyp11a1	cytochrome P450, family 11, subfamily a, polypeptide 1 (Cyp11a1)	5.61
Dnm2	dynamin 2 (Dnm2)	1.26
Ntrk3	neurotrophic tyrosine kinase, receptor, type 3 (Ntrk3), transcript variant 1	5.96
Thbs1	thrombospondin 1 (Thbs1)	0.76
Ntrk2	neurotrophic tyrosine kinase, receptor, type 2 (Ntrk2)	1

Table 2. Enriched transcripts from Selection B.

Gene Symbol	Description	ES
Ntrk3	neurotrophic tyrosine kinase, receptor, type 3 (Ntrk3), transcript variant 1	0.91
Gab2	growth factor receptor bound protein 2-associated protein 2 (Gab2)	4.46
Ets1	E26 avian leukemia oncogene 1, 5 domain (Ets1)	1.18
Cyp11a1	cytochrome P450, family 11, subfamily a, polypeptide 1 (Cyp11a1)	0.40
Gab2	growth factor receptor bound protein 2-associated protein 2 (Gab2)	3.18
Gab2	growth factor receptor bound protein 2-associated protein 2 (Gab2)	3.91

Notably, some of the enriched genes from selection A corresponded to uncharacterized genes (RIKEN) and some transcripts were detected by more than one probe. For instance, Ntrk3 gene was detected twice and another member of the same gene family (Ntrk2) resulted to be enriched too. Interestingly, Gab2 transcript was detected in both selections by 3 probes each designed in different regions of the transcript. In particular Gab2 transcript (detected by the three different probes) and Ets1 showed the higher correlation among the selections. However, Gab2 displayed the highest enrichment score. Other genes including Ntrk3 and Cyp11a1 emerged from both selection but with a differential enrichment score which was markedly higher in selection A. Genes identified only in one transduction-selection experiment are not necessarily false hits, given the variability in the complexity of the integrated cDNA repertoire in independent populations of transduced cells. This is particularly true for rare transcripts, which may not be represented in one of the two populations, or may integrate in unfavorable regions of the host genome.

On the basis of these results, we decided to focus our attention on Gab2, Ntrk3, Cyp11a1 and Ets1 transcripts for further validation. Notably, the identification of genes already known to be involved in anchorage-independent growth could represent

an internal control of the screening and confirm its effectiveness. Such genes were readily identified as two members of the neurotrophic tyrosine receptor kinase (NTRK) family, Ntrk3 (TrkC) and Ntrk2 (TrkB), which were previously shown to play a key role in anoikis resistance and were enriched in our selections. Interestingly, TrkB was identified in a functional screen for suppressors of anoikis in rat intestinal epithelial cells and was shown to possess highly tumorigenic and metastatic capacities (Geiger TR et al 2007).

Validation of Ntrk3, Cyp11a1 and Gab2 enrichment by Real-time PCR

To validate the actual enrichment of the selected genes (Gab2, Ntrk3, Cyp11a1, Ets1) in the LIB-SEL A and LIB-SEL B populations, we set up a Real-Time PCR exploiting specific primers to evaluate the expression of only the exogenous transcripts derived from the library and not the endogenous ones. The template for the reaction was obtained from the same RNA used in the Xenoarray and as control we used cDNA corresponding to LIB-UNS A and LIB UNS B cells. As shown in Figure 8, Ntrk3, Cyp11a1 and Gab2 enrichment in the selected cells was confirmed by Real Time PCR.

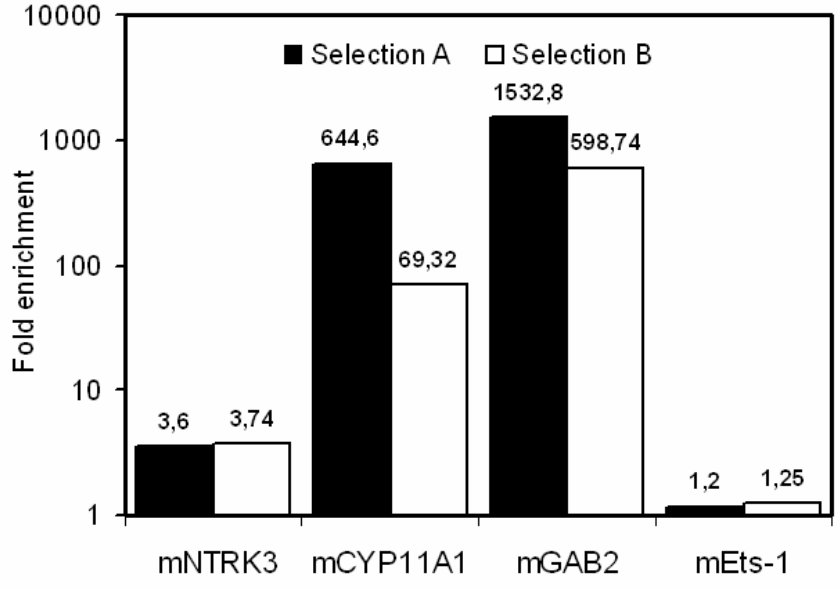

Figure 8. Real time PCR validation of enriched transcripts in both selections. Primers were designed with Primer Express software (Applied Biosystems) to detect only library-derived transcripts. The y-axis represents the relative increase in abundance of the transcripts in selected cells compared to unselected cells.

Unfortunately, we were not able to validate the enrichment for Ets1, probably due to difficulties in primer design resulting from little dimensions of the transcript. Ntrk3 enrichment was around four fold compared to unselected cells, and consistent in both selections. For Cyp11a1, the enrichment was particularly evident in selection A, with enrichment around 650-fold, and lower but still notable in selection B (70-fold). The gene which showed the highest enrichment score by Real-Time PCR resulted to be Gab2, confirming the Xenoarray data in both selections. In fact, Gab2 expression was found to be dramatically increased in selection A with an enrichment of around 1530-fold and also in selection B with a fold enrichment of around 600. This result showed that the candidate genes identified in the functional screening were effectively true positives with an exception of Ets1 and that we were able to validate, by tracing their abundance in Real-Time PCR, the effective enrichment of three of them. Such genes may play a crucial role in the capability acquired by these cells to grow in the absence of anchorage, thus they may be involved in transformation and/or invasion processes taking place during cancer onset and progression. In this view, Gab2 displayed the highest and most reproducible enrichment in the two independent selections. Therefore, we focused our attention on this gene and validated its enrichment also at the protein level by Western blot (Figure 9).

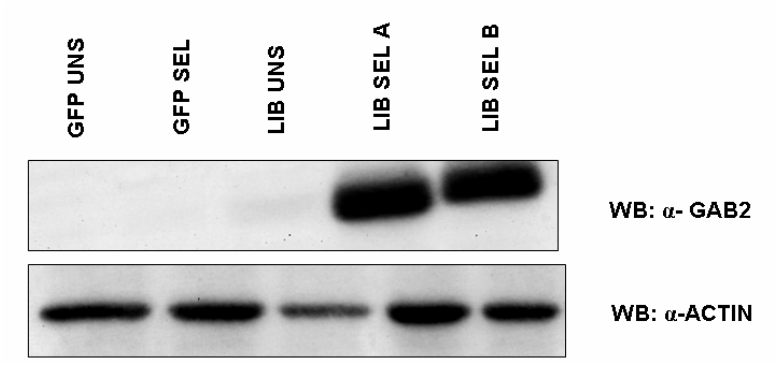

Figure 9. Western blot analysis on control (GFP) and library-transduced cells before and after selection to detect Gab2 protein levels.

As shown in Figure 9, the Gab2 protein was undetectable in GFP-transduced cells, irrespectively of the selection. Gab2 became barely detectable in library-transduced

cells and was extremely elevated in these cells after selection, further supporting a specific effect of the exogenous transduced Gab2 cDNA in the anchorage-independent growth selection.

Validation of GAB2 function

Gab proteins, including mammalian Gab1, Gab2, Gab3, comprise a growing family of scaffolding/docking molecules involved in multiple signaling pathways mediated by receptor tyrosine kinases (RTKs) and non-RTK receptors (Nishida K et al 2003). Upon receptor activation, these proteins localize to the plasma membrane and, following tyrosine phosphorylation at multiple sites, act to recruit a variety of SH2 domain-containing signaling proteins. As such, scaffolding proteins function as plasma membrane proximal assembly platforms that act locally to enrich the quantity and diversity of signaling proteins necessary to elicit a defined response to receptor activation. Gab2 for instance, is a key signaling adaptor which activates PI3K/AKT and ERK pathways downstream the EGFR (Meng S et al 2005) and recent evidence highlighted its role in mammary tumorigenesis (Ke Y et al 2007).
The result of our screening suggested that Gab2 may promote anchorage-independent growth following overexpression. To directly assess this hypothesis, we transduced MCF10A cells with the Gab2 coding sequence cloned in a retroviral vector (gift of R. Daly).

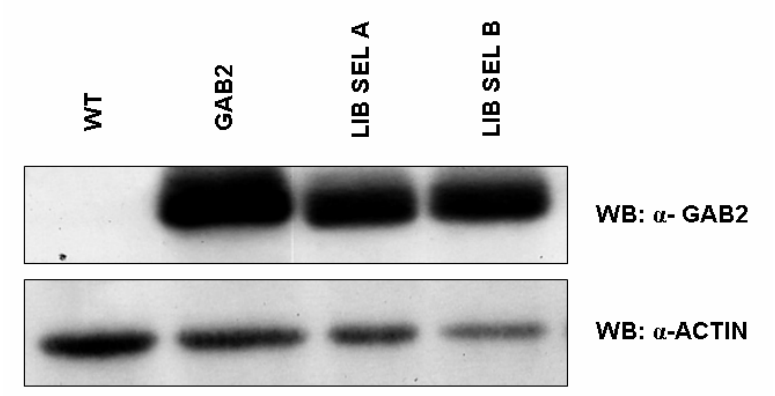

Figure 10. Western blot analysis on wild-type, Gab2 transduced MCF10A cells and library selected cells comparing Gab2 protein levels.

As shown in Figure10, retrovirally transduced Gab2 reached levels comparable to those of library-transduced selected cells, if not higher. We then analyzed the effect of

Gab2 overexpression in a series of cell-based assays aimed at measuring cell proliferation during adhesion or detachment, cell growth in soft agar, apoptosis and cell motility. We first analyzed the effects on growth, under both adherent and suspension conditions, at 48h and 72h. The effect of removing EGF and lowering the serum to 1% (starving medium) was also taken into account. Cell vitality was measured at the indicated times and expressed as the ratio between Gab2-MCF10A cells and wild-type cells (see details in Methods). As shown in Figure 11, adherent GAB2-overexpressing cells showed a significant increase in proliferation both at 48h and 72h (260% and 290% respectively). Interestingly, Gab2-sustained growth advantage was further increased in the absence of anchorage, both at 48h and 72h of suspension culture (310% and 320% respectively).

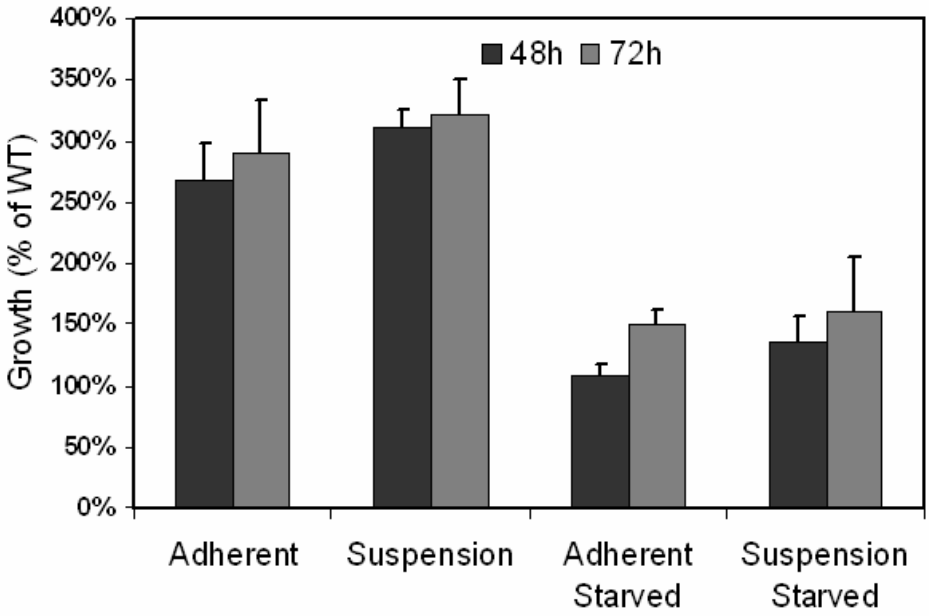

Figure 11. MTT growth assay on wild-type and Gab2-transduced MCF10A cells in adhesion or suspension, in complete medium or starving medium for 48 and 72h respectively. Cell vitality was normalized to the percent of viable plated cells at time 0, and then expressed as a ratio between wild-type and Gab2-transduced selected cells. The data represent the mean and standard deviation of triplicate values.

Notably, Gab2-sustained growth advantage was almost totally lost when cells were kept in starving medium. This demonstrates the importance of Gab2 in promoting proliferation independently from cell-anchorage but still dependent from the presence

of growth factors and/or serum. We next monitored the ability of Gab2 overexpressing cells to grow in soft agar (Figure 12). Compared to wild-type cells, which are not able to form aggregates in soft agar, Gab2 expressing cells formed large and detectable colonies. This result is of particular significance because these cells were not selected in any way for growing in suspension. Probably, the even larger colonies formed by the LIB-SEL cells are due to a combination of exogenous gene expression plus selective enrichment.

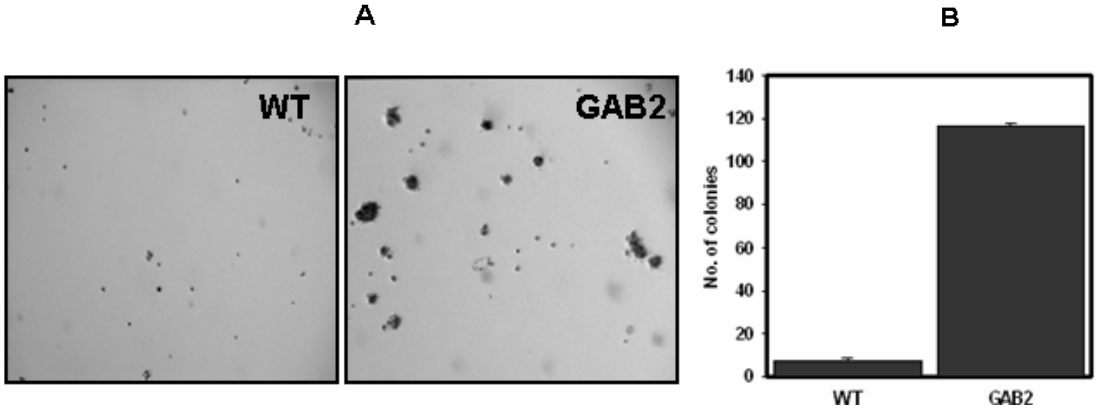

Figure 12. Soft agar growth of wild type and Gab2-transduced cells. Phase-contrast images were captured by a BD Pathway microscopic station (BD biosciences) after 3 weeks in agar (A), and quantification (B) of cell colonies was performed by Attovision 1.5 software (BD biosciences).

Loss of function of exogenous Gab2 by RNA interference attenuates anchorage independent growth of library-transduced selected population

To prove that Gab2 could have an instrumental role in the anchorage-independent growth of LIB SEL cells and rule out possible false interpretations we decided to study the effects of a loss of function of the exogenous Gab2 on cell proliferation under adherent and suspended conditions. Therefore we infected LIB SEL cells with a lentiviral vector carrying a specific murine shRNA targeting the library-transduced Gab2 transcript and assessed cell vitality using the MTT assay as described before. To exclude potential off-targets, we used an empty lentiviral vector provided by the manufacturer to infect LIB SEL cells as control. The vitality of stable LIB SEL cells expressing the shRNA for Gab2 and control cells was evaluated after 48h from plating either on regular plates or on polyhema coated plates and expressed as % vitality of plated cells (Figure 13).

Gab2 knock down by RNA interference had a drastic effect on the growth of LIB SEL cells, inducing a pronounced and similar reduction of the vitality of both adherent and detached cells. We could assert that this inhibition was effectively Gab2-specific since the shRNA completely abrogated Gab2 protein levels.

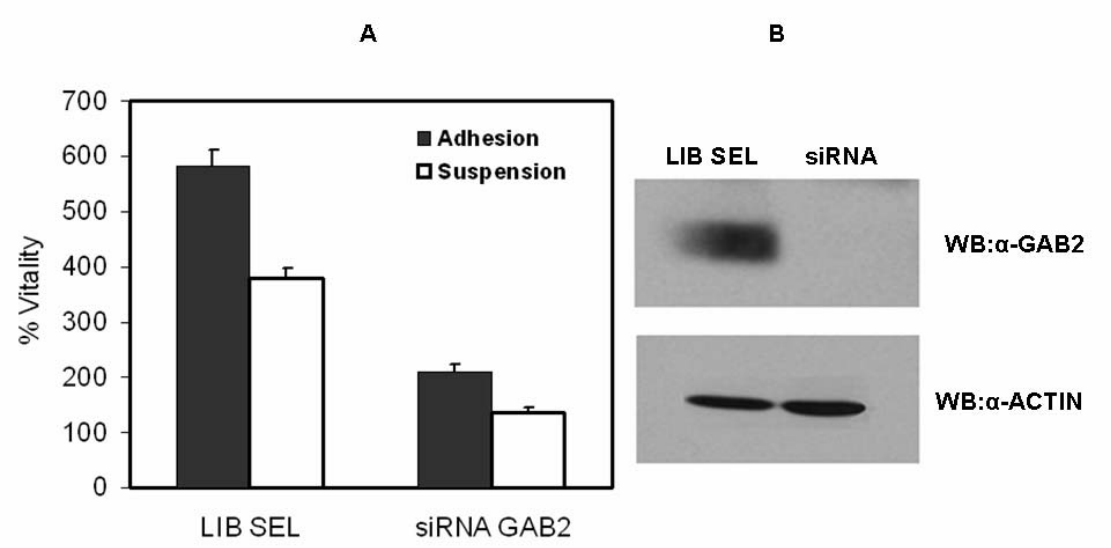

Figure 13. (A) Growth curve of library-transduced cells stably expressing a Gab2 siRNA compared to library-transduced cells transduced with control vector, after 48h in adhesion or suspension. Cell vitality was normalized to the percent of viable plated cells at time 0 and visualized independently for both cells. The data represent the mean and standard deviation of triplicate values. (B).Western blot analysis to evaluate Gab2 protein knock down.

To see if Gab2 loss of function could affect the transforming capacity of LIB SEL cells, we compared the growth of control LIB SEL cells and shRNA expressing cells in soft agar. As shown in Figure 14 (A), shRNA LIB SEL expressing cells formed less colonies compared to control cells. Moreover the size of the colonies was smaller as depicted by a higher frequency of occurrence of colonies with the minimum diameter and a reduced fraction of colonies with the maximum diameter observed (B). Taken together these data indicate that high levels of Gab2 are essential to fulfill the anchorage-independent growth of LIB SEL cells.

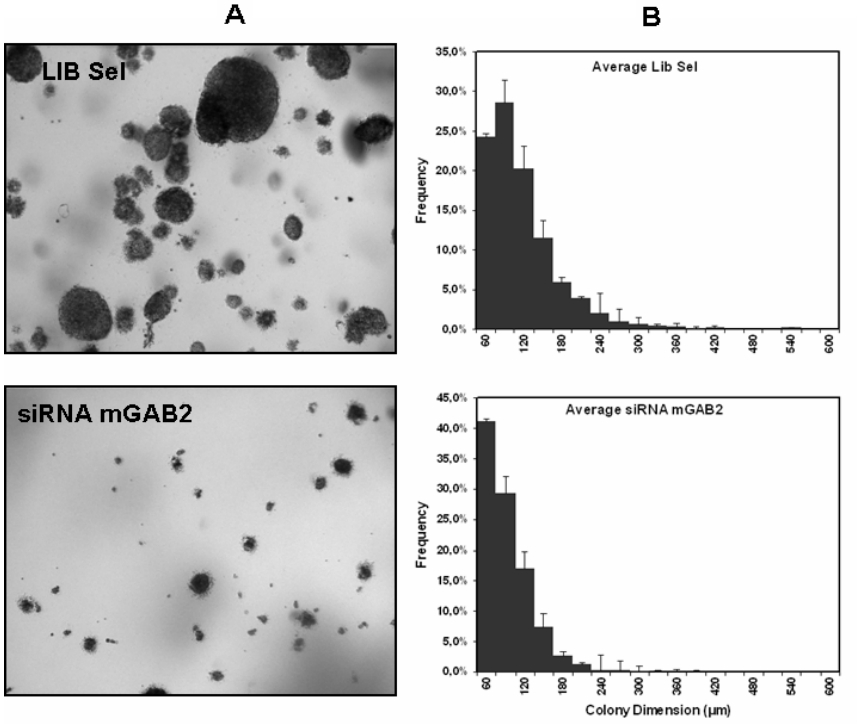

Figure 14. (A). Soft agar growth of library transduced cells expressing Gab2 siRNA or control vector. Phase-contrast images were captured by a BD Pathway microscopic station (BD biosciences) after 3 weeks in agar. (B) Distribution of colony size frequencies above 60µm measured by Attovision 1.5 software (BD biosciences).

Loss of function of endogenous Gab2 by RNA interference attenuates anchorage independent growth of wild-type and Gab2 overexpressing MCF10A cells

To confirm Gab2 function in anchorage-independent growth by a loss of function approach also of the human Gab2 transcript, we infected wild-type cells and Gab2-overexpressing cells with a lentiviral vector carrying an shRNA targeting the human gene and assessed cell vitality using the MTT assay as described before. As a control, we used an empty lentiviral vector provided by the manufacturer to infect both cells. The vitality of stable MCF10A cells expressing the shRNA for Gab2 and control cells was evaluated after 48h from plating either on regular plates or on polyhema coated plates and expressed as % vitality of plated cells (Figure 15).

Noticeably, the human shRNA was less efficient than the shRNA targeting the library-derived Gab2, as revealed by the residual protein which was detected in the Gab2 overexpressing cells. This defect could not be highlighted in WT cells since

they express very low levels of endogenous Gab2. Reasonably, the high levels of Gab2 protein derived from the overexpression, combined with a less specific property of the shRNA could account for the incomplete protein suppression.

Interestingly, Gab2 knock-down was sufficient to reduce the growth of WT and Gab2 overexpressing cells both on adhesion as well as in suspension. In WT cells, this inhibition was slightly more effective in adherent cells (2.2-fold inhibition) than in suspended cells (1.9-fold inhibition), while Gab2 overexpressing cells displayed a similar growth inhibition around 2.2-fold in both adherent and suspended cells. Therefore, in Gab2 overexpressing cells, the loss-of function abrogated growth advantage in suspension and rendered cells sensible to apoptosis to the same extent as WT cells.

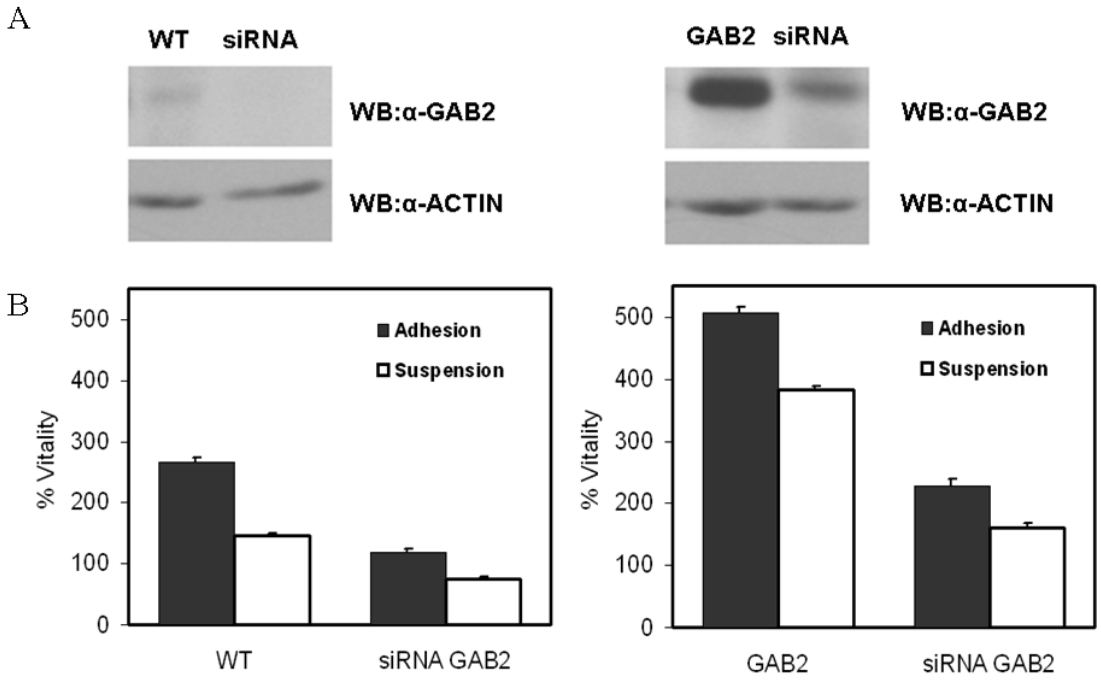

Figure 15. (A) Western blot analysis to validate Gab2 protein knock-down. (B). Growth analysis of library-transduced cells stably expressing a Gab2 siRNA compared to library-transduced cells transduced with control vector, after 48h in adhesion or suspension. Cell vitality was normalized to the percent of viable plated cells at time 0 and represented independently for both cell types. The data represent the mean and standard deviation of triplicate values.

Loss of function of exogenous Gab2 by RNA interference completely abrogates anchorage-independent growth of human breast cancer cells

To evaluate the effect of Gab2 downregulation in cancer cells, we selected a panel of breast cancer cell lines which endogenously expressed significant levels of Gab2 protein, namely the MDA-MB 435, MDA-MB-231 and MCF7 cells. Indeed, Daly and coworkers (Daly RJ et al 2002) reported a marked overexpression of Gab2 in a subset of breast cancer cell lines relative to normal breast epithelial strains and a trend for increased Gab2 expression in estrogen receptor (ER)-positive lines, such as the MCF7 cells. Interestingly, Gab2 was overexpressed also in a small subset of the ER-negative cell lines including the MDA-MB 231 and MDA-MB 435. We therefore decided to investigate the effects of Gab2 knock-down on the anchorage-independent growth of these cells. MCF7, MDA-MB 435 and MDA-MB-231 cells were infected with the empty lentiviral vector (control) or with the lentiviral vector carrying the shRNA for human Gab2, as described before.

We were able to obtain infected cells from all cell lines, although Gab2 shRNA-transduced cells displayed a modest reduction in cell growth, highlighting a minor negative effect of Gab2 shRNA (data not shown). The Gab2 protein was downregulated in all three cell lines, with the strongest differential observed in MDA-MB-231 (Figure 16 B). To assess the role of Gab2 specifically in anchorage-independent growth of these cells, we performed a soft agar assay on control and GAB2-shRNA-transduced cells (Figure 16A). Clearly, Gab2 knock-down determined a marked reduction, if not complete abrogation, of soft agar growth for all three cell lines, which displayed different transforming capacities, as highlighted by the differences in colony number and dimension of control cells. Although MDA-MB 435 cells formed the fastest-growing and largest colonies, Gab2 knock down still inhibited soft agar growth to a similar extent as the other cells considered. In MDA-MB-231 cells, which express the highest levels of endogenous Gab2 protein, the Gab2 siRNA caused a very pronounced reduction in cell colony formation, as very few, smaller colonies were observable. This result reflects Gab2 prominent role in the anchorage-independent growth and eventually in the transforming ability of different breast cancer cells, thus supporting its function in breast tumorigenesis.

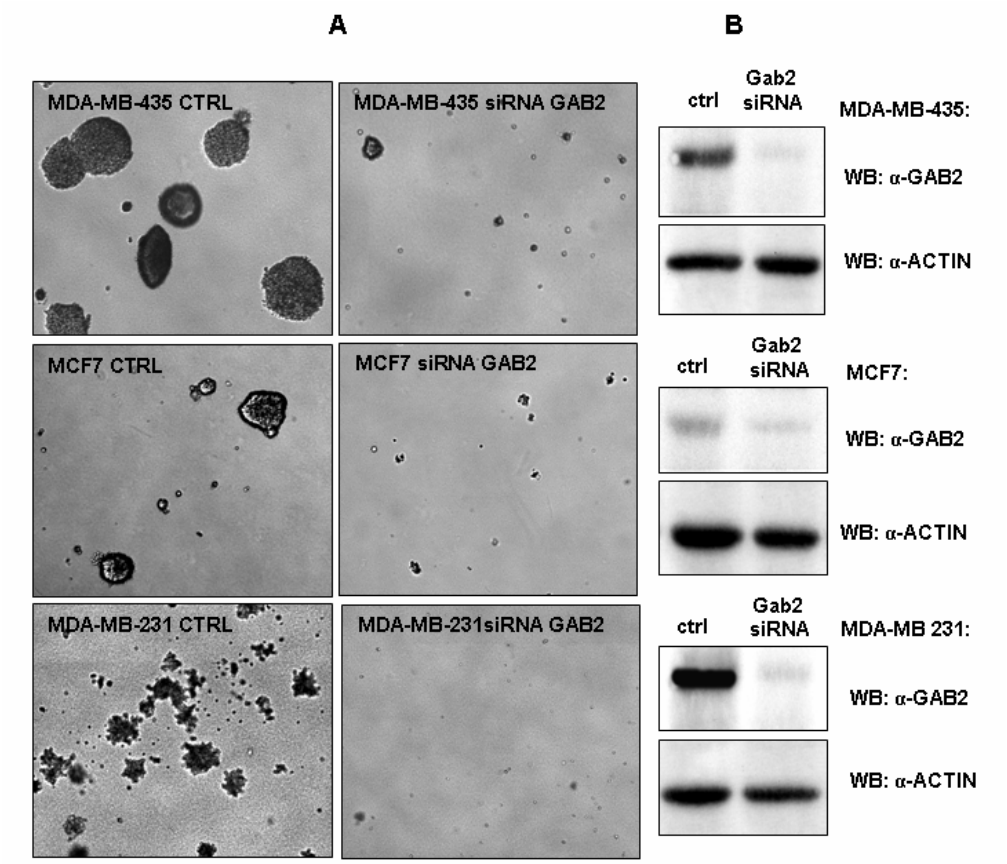

Figure 16. (A). Soft agar growth of different breast cancer cells expressing Gab2 siRNA or control vector. Phase-contrast images were captured by a BD Pathway microscopic station (BD biosciences) after 3 weeks in agar. (B). Western blot analysis to validate Gab2 protein knock-down.

Gab2 overexpression does not induce significant protection from detachment-induced apoptosis but sustains and promotes cell cycle progression during long-term suspension cultures

To evaluate whether Gab2 expression has a role in anoikis resistance, we estimated the % of apoptotic cells after 48h of suspension cultures. The % of apoptotic cells was assessed as cytofluorimetric measurement of the propidium iodide (PI) signal, which detects plasma membrane integrity.

Data acquisition was performed using the CellQuest software and data analysis was done using WinMDI software. Propidium iodide (PI) fluorescent signals (FL2) were displayed as histograms on a logarithmic scale versus the number of recorded events. The peak on the right represents cells with intact DNA, while that on the left refers to

apoptotic cells, which exhibit sub-G1 PI incorporation (hypodiploid/fragmented nuclei). Two bars (M1 and M2) were set on each histogram experiment by experiment, to delineate the percentage of PI positive cells (dead cells) and the % of cells in G1-S transition of the cell cycle (proliferating cells) respectively. These were kept constant in all of the conditions of each experiment for the two cell types tested (wild-type and Gab2 overexpressing cells). For each bar, the corresponding % of cells is depicted. The % of apoptotic cells in suspension (SUSP) was analyzed in respect to wild-type and Gab2 overexpressing cells cultured on regular plates (ADH) for the same period of time.

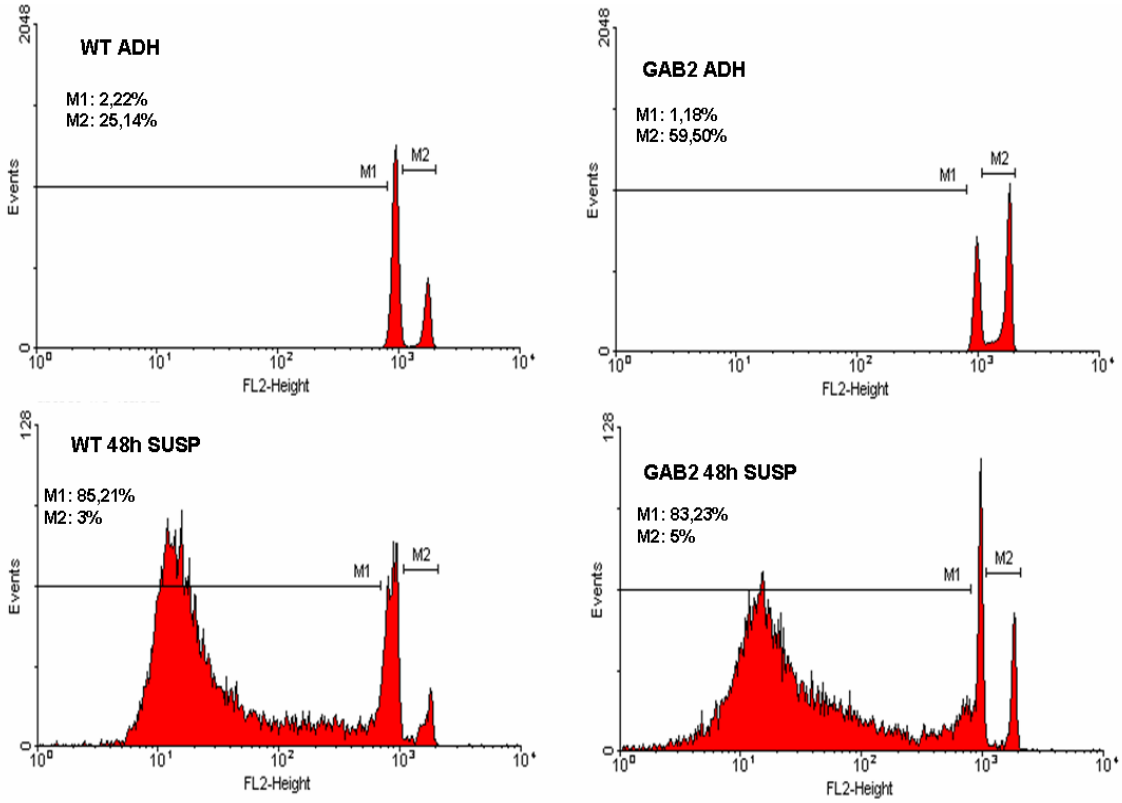

Figure 17. Detachment-induced apoptosis assay and flow cytometry analysis of apoptosis induction. Cell death was measured after 48h either in adhesion or supsension, by assessing the number of hypodiploid nuclei with the DNAcon3 kit (ConsulTS, Rivalta, Italy). DNA index was measured by cytofluorimetric analysis using a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA). Data acquisition was performed using CellQuest software and data analysis with WinMDI software. PI (FL2) fluorescent signals were displayed as histograms and shown as units of fluorescence on a logarithmic scale. The PI signal was calculated on the histogram window of WinMDI after discriminating apoptotic and non apoptotic subpopulations with a logical gate tool (M1).

Surprisingly, we could detect a comparable fraction of apoptotic cells between wild-type (85,21%) and Gab2 overexpressing cells (83,23%) after 48h of polyhema plating, with a slight majority of apoptotic WT cells, indicating that Gab2 does not seem to be directly involved in the protection from apoptosis of MCF10A cells during anchorage suppression (Figure 17).

Interestingly, cell cycle analysis revealed a marked difference in the percentage of actively proliferating vital cells, which was readily evident in adhesion (Figure 18).

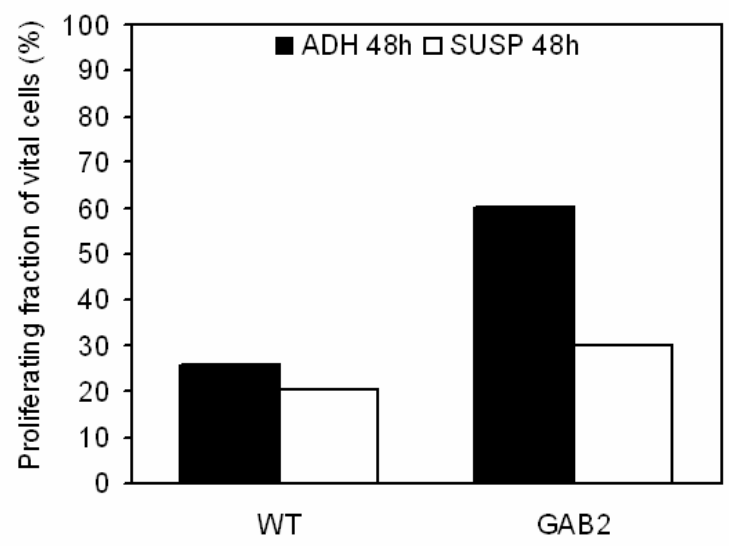

Figure 18. Cell cycle analysis of wild-type and Gab2 overexpressing cells. For each sample analyzed at the FACS, and indicated in the panel, the fraction of cells with a DNA content higher than 2n was calculated. This is the fraction of cells undergoing DNA synthesis and mitosis.

For instance, in these conditions, the proliferating fraction (60,21%) of Gab2 overexpressing cells was 2.3 times greater than WT cells (25,71%). Strikingly, although, detachment caused a pronounced reduction in the fraction of proliferating cells regardless of Gab2 status, still a significant advantage (1,5-fold) was maintained for the Gab2 overexpressing cells after 48h of suspension culture. Together, these data suggest that Gab2 function is not primarily centered in the inhibition from detachment-induced apoptosis, rather than in the sustenance and execution of the cell cycle, independently from an adhesive consensus. This is supported by reports

addressing Gab2 role in 3D morphogenesis of MCF10A cells indicating that this docking protein does not prevent apoptosis of luminal cells despite the positive proliferative effect conferred (Bentires-Alj et al 2006).

Inhibition of MAPK and SRC pathways specifically reduce Gab2 capacity to promote growth in the absence of anchorage

Scaffolding proteins have been shown to play a pivotal role in transducing signals from activated RTKs. In addition to being constitutively bound to signaling molecules, these specialized types of polypeptides also become tyrosine phosphorylated upon recruitment to RTKs. These tyrosine phosphorylation events establish high affinity phoshotyrosine-based binding sites for the recruitment of additional signaling molecules. Biochemical analyses and yeast two-hybrid screens have identified several signaling molecules that can bind to Gab2 upon receptor activation, including the tyrosine phosphatase Shp2 (Yu M et al 2006) (leading to Erk activation), the p85 subunit of the phosphoinositide 3-kinase (Bouscary D et al 2001), PI3K, (leading to Akt activation), Src family kinases (Kong M et al 2003, Yu M ET AL 2006, Zhu QS et al 2004) PLCγ, Crk, CrkL (leading to JNK activation) and also GC-GAP (Zhao C et al 2003), the GTPase-activating protein (GAP) for Rho family GTPases, involved in regulation of Rac and Cdc42 activities. To characterize the signaling pathways downstream Gab2 that could mediate the anchorage-independent growth driven by its overexpression, we examined the effects of a panel of specific small molecule inhibitors targeting key signaling kinases. WT and Gab2-MCF10A cells were incubated in adhesion or suspension in the presence or absence (CTRL) of a MEK inhibitor (PD98059), PI3K inhibitor (LY294002), SRC inhibitor (PP2), or JNK inhibitor (SP600125). Cell vitality was assessed after 24h of treatment and the drug effect was expressed as a percent inhibition of cell vitality in respect to untreated cells (Figure 19).

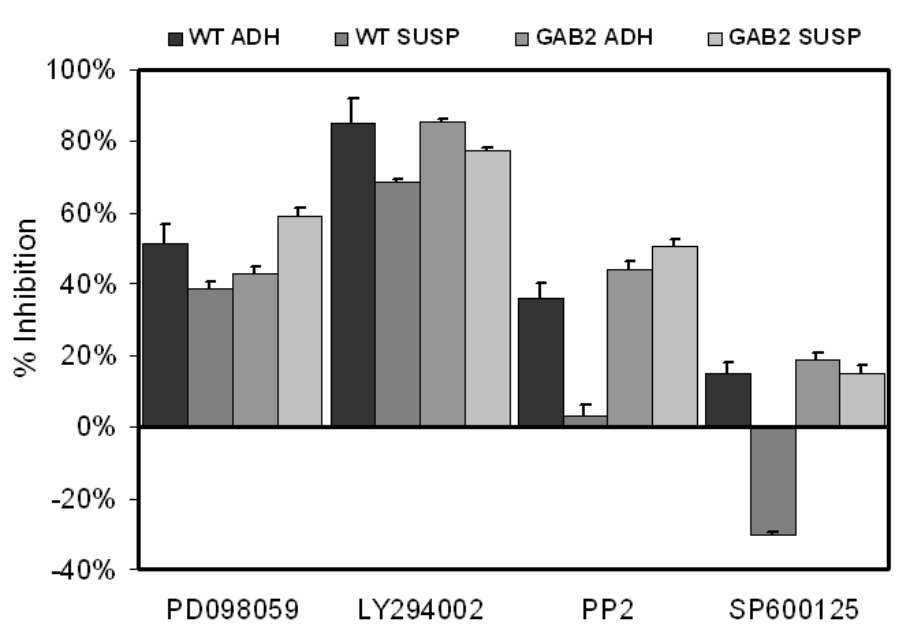

Figure 19. Evaluation of the contribution of different signaling pathways to Gab2-mediated enhancement of cell growth. Cells were incubated in adhesion or suspension in the presence or absence (CTRL) of a MEK inhibitor (PD98059, 40μM), PI3K inhibitor (LY294002, 50μM), SRC inhibitor (PP2, 10μM), or JNK inhibitor (SP600125, 10μM). Cell vitality was assessed 24h from the treatment and the drug effect was expressed as a percentage of cell vitality inhibition in respect of untreated cells. The data represent the mean and standard deviation of triplicate values from two independent experiments.

As shown in Figure 19, the various inhibitors displayed different sensitivity and specificity on the vitality of Gab2-MCF10A and WT cells. PI3K inhibition was the most effective in inhibiting anchorage-dependent and independent growth, but with no differential effect between Gab2-MCF10A or WT cells, showing a general involvement of this pathway in the survival of MCF10A cells. On the contrary, MEK inhibitor most effectively inhibited the growth of Gab2-MCF10A cells in suspension, inhibiting cells at lower levels in all the other conditions, suggesting a specific role of MAPK/ERK pathway in mediating anchorage-independent growth by Gab2. Most interestingly, PP2 displayed the highest specificity on Gab2 overexpressing cells in suspension: the reduction in vitality was of 55%, compared to a mere 10% of reduction in WT cells grown in suspension. This indicates that SRC pathway could play an important role in Gab2-dependent, anchorage-independent, growth.

The inhibition of the JNK pathway did not significantly influence the vitality of MCF10A cells, even not in the presence of high levels on Gab2, suggesting any specific involvement of this pathway downstream of Gab2 and implicated in anchorage-independent growth. Taken together, these data point to possible cross-talks between Gab2, MAPK\ERK and SRC pathway in mediating anchorage-independent growth, However, on the basis of the higher specificity of PP2 towards Gab2 overexpressing cells we could claim a major role of SRC pathway in controlling Gab2-enhanced growth. This is consistent with previous studies showing that SRC inhibition could inhibit Gab2-associated downstream events including EGF-induced PI3-kinase activation and DNA synthesis (Kong M et al 2003).

Gab2 overexpression abrogates Bim transcriptional upregulation by detachment, thereby potentially promoting anoikis resistance

To investigate whether Gab2 could be involved in the regulation of proapototic genes related to anoikis of MCF10A cells, we examined Bim mRNA levels during detachment-induced apoptosis of WT, GFP unselected and Gab2 overexpressing cells, specifically after 24h of suspension. Recent data from Brugge and co-workers, in fact showed that among the different BH3-only proteins, Bim was specifically induced during anoikis of MCF10A cells and that hyper-activation of the EGFR/ERK/MAPK signaling pathway was responsible for the negative regulation of Bim leading to anoikis suppression (Reginato MJ et al 2003). Using real-time RT–PCR to examine transcription of Bim mRNA during anoikis, we found that Bim transcripts were upregulated five-fold after cell detachment of WT cells and slightly less in GFP-transduced cells. This upregulation was inhibited in cells expressing Gab2 (Figure 20). This result indicates that Gab2 presence negatively regulates upregulation of a proapoptotic gene relevant for MCF10A cells death during detachment. However, this effect is likely not sufficient to protect Gab2 overexpressing cells from anoikis, as demonstrated in the above assays of detachment-induced apoptosis (Figure 17).

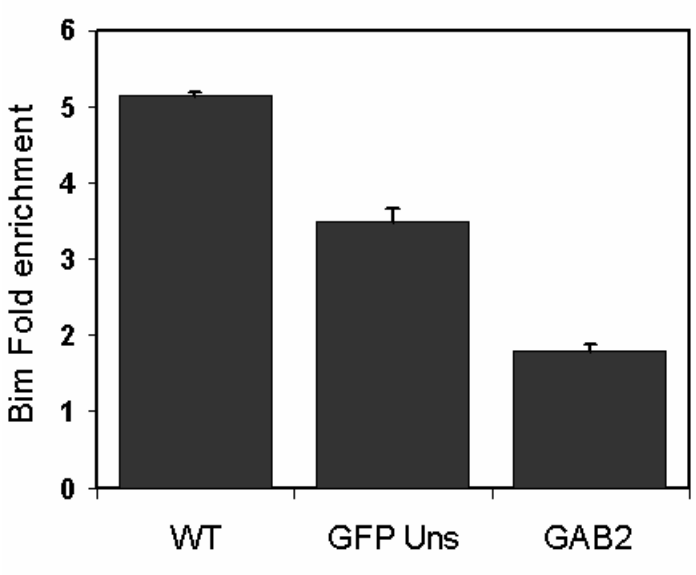

Figure 20. Gab2 overexpressing cells fail to upregulate Bim in response to detachment. Total RNA was collected from WT, GFP unselected and Gab2-overexpressing cells after 0 or 24 h in suspension and used to assay Bim transcripts using realtime RT–PCR. Data represent Bim fold enrichment after 24h of suspension and are expressed as relative Bim expression normalized to time zero from an average of three independent experiments.

Overexpression of Gab2 disrupts AKT and ERK dynamics during anoikis of MCF10A cells, but rescues ERK activation during long-term suspension

To judge the implication of these signaling pathways in the anchorage-independent growth of Gab2 overexpressing cells also from a biochemical point of view, we examined the activation of downstream targets in regard of the adhesion status of the cells. Therefore, we evaluated the activation of ERK and AKT by Western blot analysis on cell lysates from WT, LIB SEL and Gab2 overexpressing cells which were kept in adhesion or suspension for 24 or 48h in growth medium. To see whether other stressing conditions could influence Gab2 response to anchorage-independent growth, cells were starved by removing EGF and serum for 48h, then cell lysates were obtained from both adherent and suspended cells and added to the analysis. As shown in Figure 21, the levels of both activated AKT and ERK in adherent cells were lower in Gab2 overexpressing cells than in WT or LIB SEL cells. This unexpected result contrasts previous data in which high levels of Gab2 protein led to a marked amplification of intracellular signaling, as indicated by the enhanced Erk activation in unstimulated cells as well as in cells treated with EGF. One possible explanation for

this discrepancy could be that in our model we could not reach such high levels of Gab2 protein that could account for a constitutive activation of ERK or that possible feed back loops could exist to shut down Gab2-mediated ERK activation. Similar events could be expected to negatively regulate a basal hyperactivation of Akt. Indeed, recent in vivo data showed that Gab2-deficient primary tumor cells, derived from homologous deletion of GAB2 gene in mice, displayed similar levels of activated AKT compared to control cells which maintained an intact genomic locus (Ke Y et al 2007). From western blot analysis we also noticed that AKT was not activated during anoikis of MCF10A cells, rather it was suppressed in WT cells. Only starvation could restore AKT levels in WT and LIB SEL cells or activate AKT in Gab2-overexpressing cells respectively.

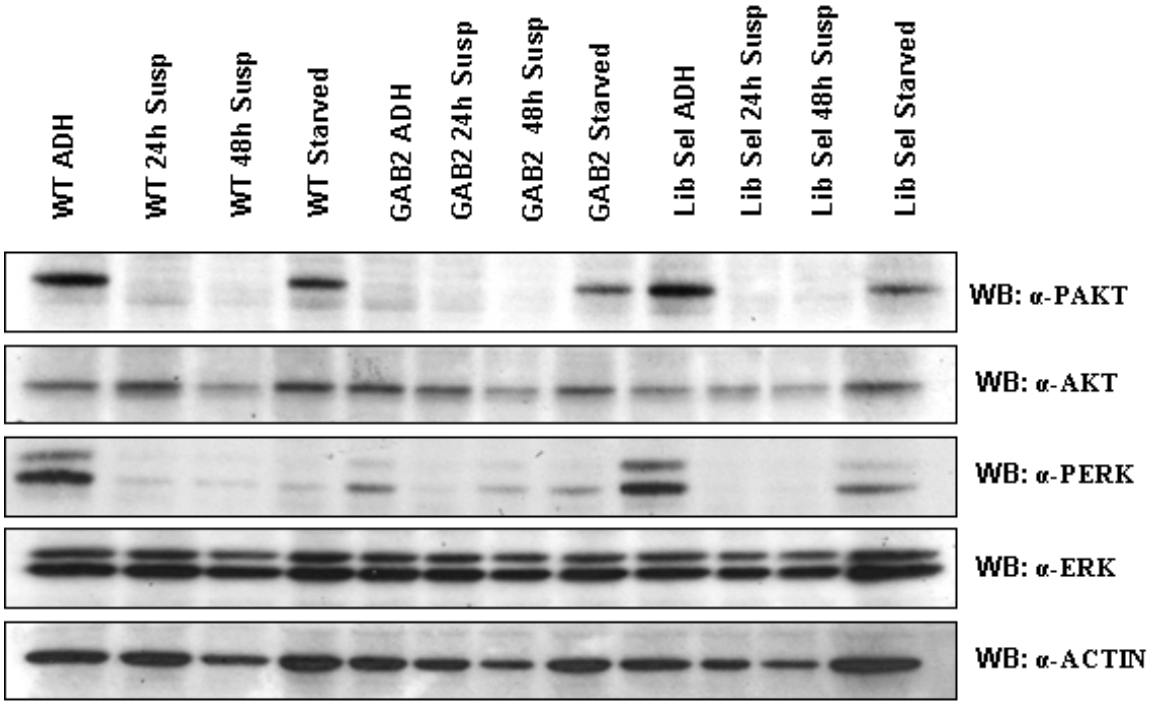

Figure 21.Western blot analysis on wild-type, Gab2-transduced and library selected cells in adhesion or after 24 and 48h in suspension, either in complete medium or starving medium. Specific antibodies for the activated (phosphorylated) forms of AKT and ERK as well as non activated forms (total) were used.

Similarly, ERK phosphorylation was abrogated during suspension of both WT and Gab2-overexpressing cells; however although lower levels of activated ERK were

detected in attached Gab2 overexpressing cells, prolonged detachment (48h) rescued ERK activation only in Gab2 overexpressing cells. This result suggests that Gab2 could promote anchorage-independent growth through the MAPK/ERK pathway, by overcoming negative feedback loops and by sustaining ERK activation. Interestingly, this effect was highlighted only at 48h from the detachment; raising the possibility that maybe Gab2 function could be fully executed and therefore observed upon long-term anoikis, which could mimic somehow the anchorage-independent growth selection that we applied. In this case, it would be reasonable to expect a further activation of the ERK pathway.

On the contrary, LIB SEL cells did not show a similar ERK activation dynamic as Gab2 overexpressing cells; this could be explained by the high heterogeneity of these cells and by the fact that they express other genes enriched after selection which could mediate anchorage-independent growth by alternative pathways.

Noticeably, no previous evidence showed direct link between GAB2, ERK and anchorage-independent growth.

Overexpression of Gab2 induces basal SRC and paxillin activation which is maintained during long-term suspension

Integrins can alter cellular behavior through the recruitment and activation of signaling proteins such as non-receptor tyrosine kinases like the focal adhesion kinase (FAK) and c-Src. The FAK-Src complex binds to and can phosphorylate various adaptor proteins such as p130Cas and paxillin (Mitra SK et al 2006). To verify the effective implication of SRC pathway in the anchorage-independent growth of Gab2 overexpressing cells, we examined the activation of SRC and of one of its downstream targets (paxillin) in regard of the adhesion status of the cells.

Western blot analysis on cell lysates from WT and Gab2 overexpressing cells which were kept in adhesion or suspension for 24 or 48h in growth medium was performed as before. To focus only on Gab2 effect we decided to exclude the LIB SEL cells from this analysis (Figure 22). Specific antibodies able to detect the activated form of SRC (phosphorylated at tyrosine 416) and of paxillin (phosphorylated at tyrosine 31) were used.

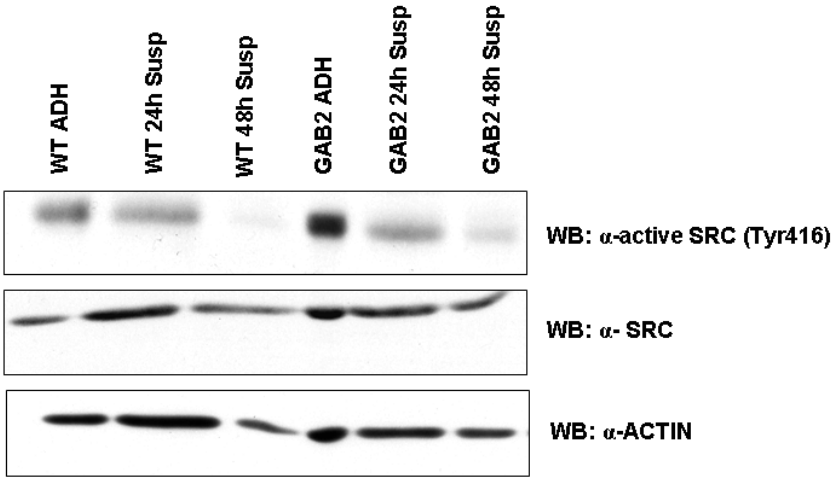

Figure 22. Western blot analysis on wild-type and Gab2-transduced cells in adhesion or after 24 and 48h in suspension, grown in complete medium. Antibodies directed against the activated form of SRC (phosphorylated at tyrosine 416) or total SRC were used.

The site of phosphorylation corresponding to Y416 in c-Src, which is not phosphorylated in inactive wild type Src, is constitutively phosphorylated in activated oncogenic Src mutants (Cooper et al 1986, Parsons & Weber 1989). As shown in Figure 22, Gab2 overexpressing cells displayed a basal phosphorylation of SRC which was greater compared to wild-type cells. Interestingly, SRC activity followed a kinetic of downregulation during anoikis of both cells. Clearly, activation of SRC was reduced as cells were placed in suspension, an effect which became particularly evident after 48h. In fact, in both cellular types and to the same extent, SRC activation was reduced after 24h of suspension culture. However, Gab2 overexpressing cells maintained SRC phosphorylation also at 48h in suspension, while in WT cells, SRC activation was completely abolished, highlighting a specific effect of Gab2 in promoting and sustaining SRC activation respectively during anchorage-dependent and anchorage-independent growth.

To evaluate whether Gab2 could act through integrin signaling and effectively impinge on SRC downstream effectors, we checked the phosphorylation status of paxillin. Paxillin is a focal adhesion associated signaling molecule that functions as an adaptor protein to recruit diverse cytoskeleton and signaling proteins into a complex, presumably to coordinate the transmission of down-stream signals (Mitra SK et al

2006). Tyrosine phosphorylation of paxillin was observed following integrin-dependent cell adhesion to extracellular matrix proteins, thus implicating paxillin in integrin-mediated signaling (Burridge et al., 1992). Y31 in paxillin is a target of SRC or FAK family kinases and represents a docking site for SH2-containing molecules such as CRK and CRKL; therefore we used a specific antibody able to recognize this phosphorylation site. As shown in Figure 23, compared to wild type cells in adhesion, Gab2 overexpression induced a greater tyrosine phosphorylation of paxillin and similarly to SRC kinetics; it was decreased following cell detachment.

Most importantly, paxillin activation was still detectable after 48h of suspension cultures only in Gab2 overexpressing cells, indicating the capacity of Gab2 to sustain the activation of paxillin also in the absence of a substratum consensus.

All together these data suggest that Gab2 effect on anchorage-independent growth relies on the activation of SRC pathway, possibly after integrin engagement, as demonstrated by a concordant kinetic of activation of SRC kinase and a well known downstream target such as paxillin. The capacity to proliferate of non adherent cells could therefore be provided by a Gab2-dependent mechanism which impinges on SRC signaling pathways.

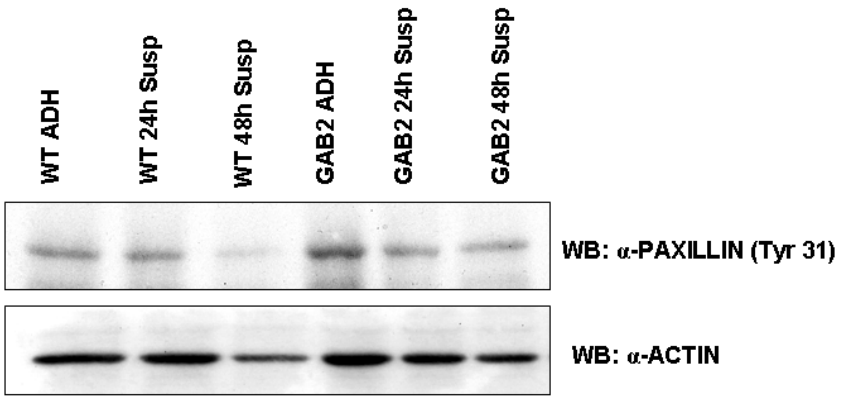

Figure 23. Western blot analysis on wild-type and Gab2-transduced cells in adhesion or after 24 and 48h in suspension, grown in complete medium. An antibody directed against the activated form of paxillin (phosphorylated at tyrosine 31) was used.

Gab2 overexpression and polyhema selection induce the expression of genes involved in cell cycle progression, cell adhesion and cell motility

Looking for further insight of the role of Gab2 in anchorage-independent growth, we designed to assess the changes in gene expression following Gab2 overexpression, using Illumina human gene chips which cover around 46.000 transcripts. For this experiment, we decided to compare the gene profiling of Gab2 overexpressing cells, before (GAB2 UNS) and after an anchorage-independent growth selection (GAB2 SEL) and GFP selected cells (GFP SEL), all against a common reference which was set on GFP-non-selected cells (GFP UNS). The anchorage-independent growth selection was performed as for the LIB cells described before, by repeating a cyclic culturing of Gab2 overexpressing cells on polyhema coated plates followed by a short recovery on regular plates. The RNA corresponding to GFP UNS and GFP SEL cells was the same used for the Xenoarray. Statistical analysis was applied to derive the most significant genes differentially expressed among the conditions considered. Briefly, a multiple comparison analysis, based on Dunnett's T test, was performed with a log(2)ratio threshold equal to 1, and a MSE (mean square error) multiplier equal to 2. FDR (false discovery rate) was estimated to be <5% according to the median distribution of the test, on 5000 randomly simulated data.

This analysis allowed us to recover a list of 758 genes, herein named "Gab2 Signature", GS. Functional analysis on the gene list obtained was performed using the annotation function of DAVID (http://david.abcc.ncifcrf.gov/home.jsp), a web-accessible program that integrates functional genomic annotations with intuitive graphical summaries. Lists of gene or protein identifiers are rapidly annotated and summarized according to shared categorical data for Gene Ontology, protein domain, and biochemical pathway membership. This enables to evaluate the enrichment for a particular term, biological process, molecular function or cellular component in the gene list. For instance, we used DAVID annotation chart tool to estimate the most enriched functional classes present in our dataset. As shown in Table 3, we observed a very significant enrichment of genes involved in cell cycle and its regulation, in particular, in the M phase of the cell cycle. Interestingly, also genes belonging to the category "cell motility" were represented, although with a lower p-value.

Table 3. Functional analysis of the GS based on DAVID annotation tool.

Functional Class	p-value
Mitotic cell cycle	$1,9 \times 10^{-18}$
M phase	$2,8 \times 10^{-14}$
Regulation of cell cycle	$6,8 \times 10^{-9}$
Sister chromatid segregation	$3,7 \times 10^{-6}$
Epidermis development	$2,5 \times 10^{-6}$
Organelle organization and biogenesis	$2,5 \times 10^{-5}$
Cell motility and wound healing	$3,9 \times 10^{-4}$
Acute phase response	$3,5 \times 10^{-4}$

Then, we performed a FLAME clustering of the GS to see if we could find gene patterns associated to the biological processes listed above and we retrieved a total of 23 clusters (Figure 24). Notably, the overall trend of gene regulation among the various comparisons was mainly a downregulation, although a number of clusters were clearly indicating a specific effect of Gab2 in gene upregulation compared to GFP cells. This was consistent for both Gab2 non selected and selected cells, with a particular enhancement for selected cells.

Generally, we could deduce some aspects of gene regulation by Gab2: firstly, genes upregulated by Gab2 upon adherent growth (GAB2 UNS) were further upregulated upon anchorage-independent growth selection (GAB2 SEL) while only for some downregulated genes by Gab2 in adhesion we could observe the same trend after selection; secondly, the anchorage-independent growth selection preferentially upregulated genes in Gab2 overexpressing cells while preferentially downregulated genes in GFP cells, with only few genes in common and some genes showing an opposite behavior. Finally, most of the Gab2-upregulated genes under adhesion were also upregulated by selection on GFP cells.

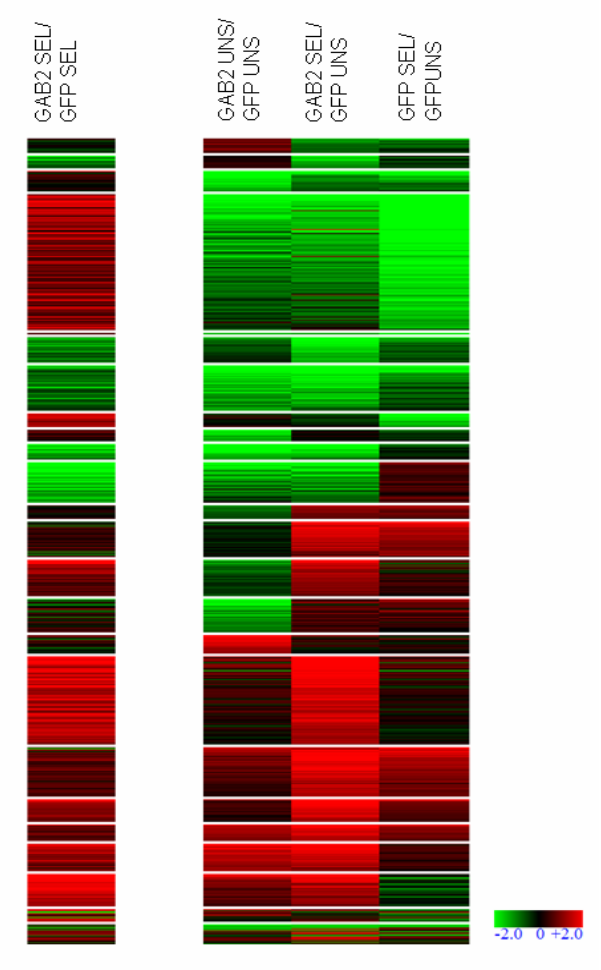

Figure 24. Gene expression clustering of GS (758 genes), subdivided in 23 clusters according to FLAME clustering algorithm and cosine distance metric on log 2 ratio values calculated in respect to reference control (GFP UNS) and resulting in three comparisons analysis (GAB2 UNS/GFP UNS; GAB2 SEL/GFP UNS; GFP SEL/GFP UNS). Comparison analysis of only selected cells (GAB2 SEL/GFP SEL) was also included to highlight the contribution of Gab2 overexpression in anchorage-independent growth.

Notably, we were able to find a cluster representative of genes involved in cell cycle or cell proliferation (Figure 25) with a p-value $<10^{-20}$ in respect of the 758 genes. Surprisingly, we detected a predominant downregulation of genes associated with cell cycle and cell cycle progression also in Gab2 overexpressing cells.

However, comparisons of selected cells revealed that the balance of gene regulation resulted to be an overall upregulation, indicating that Gab2 cells, which underwent a selection for anchorage-independency, were less sensible to negative gene regulations and could maintain higher levels of transcripts involved in G1/S and G2/M phase

transitions of cell cycle. Inputs from both growth factor receptors and integrins are required to stimulate progression through the G1 phase of the cell cycle, via induction of G1 cyclins and suppression of inhibitors of the G1 cyclin-dependent kinases (Aplin AE et al 1999). Among these genes we found several cyclins (cyclin B1, cyclin B2, cyclin A2, and cyclin D1) and cell cycle regulators such as CDK2, CDC2, E2F and CDC25C, which are essential for G1/S and G2/M phase transitions of cell cycle.

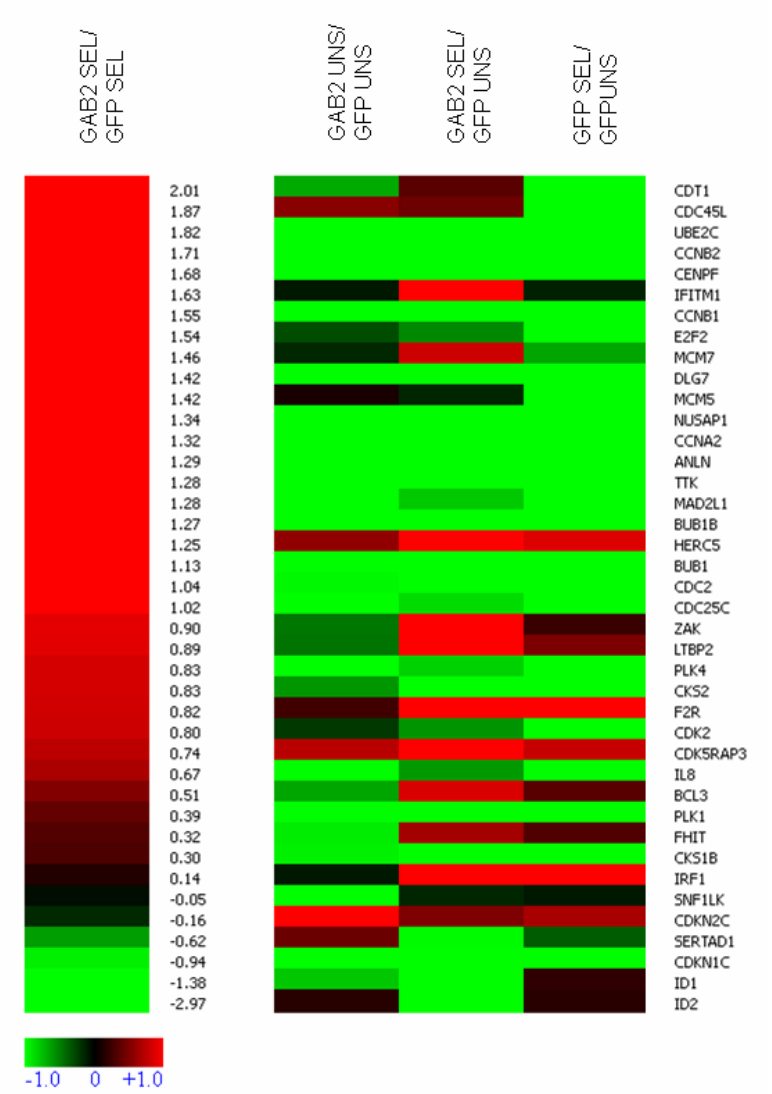

Figure 25. Gene expression profiling of genes associated with cell proliferation (cell cycle and cell cycle progression) represented as a cluster. Same comparisons as Figure 24 are shown. Log 2 ratio values resulting from comparisons between selected cells only (GAB2 SEL/GFP SEL) show Gab2 induction of positive regulators and downregulation of negative regulators in anchorage-independent growth.

For instance, CDC2 is a catalytic subunit of the highly conserved protein kinase complex known as M-phase promoting factor (MPF), which is essential for G1/S and G2/M phase transitions. Mitotic cyclins stably associate with this protein and function as regulatory subunits. Interestingly, we found an upregulation of cyclins B which form complexes with CDC2 to form the maturation-promoting factor (MPF). In addition, cyclin A2 is known to bind and activate CDC2 or CDK2 kinases, and thus promotes both cell cycle G1/S and G2/M transitions. Moreover, induction of CDK2 indicates that also G1 phase of cell cycle could be regulated transcriptionally. In fact, CDK2 is a catalytic subunit of the cyclin-dependent protein kinase complex, whose activity is restricted to the G1-S phase, and essential for cell cycle G1/S phase transition. This protein associates with cyclin A or E, and is regulated by CDK inhibitor p21Cip1 (CDKN1A) and p27Kip1 (CDKN1B). Not surprisingly, we found that in our cluster, negative regulators of cell cycle progression such as CDKN1C and CDKN2C were not induced by Gab2, but rather they were downregulated in respect of GFP SEL cells (log2 Ratio -0.94 and -0.2 respectively).

Furthermore, we were also able to define a cluster (56 genes) representing the upregulated genes by Gab2 in all conditions, both unselected and selected cells (Figure 26), although functional analysis did not enrich for a statistically significant category (only genes involved in regulation of cell proliferation but with a p-value $<10^{-4}$). Interestingly, among the most upregulated genes there were genes involved in adhesion and motility.

For instance, in Gab2 overexpressing cells, the cell-matrix adhesion molecule integrin β2 (ITGB2), showed a 4.5-fold increase in unselected and a 9-fold increase in selected cells compared to GFP UNS cells. Similarly, the chemokine receptor CXCR4 was upregulated by 5 times in both unselected and selected cells.

Moreover, PAK3 was another transcript which showed a strong regulation in Gab2 overexpressing cells. PAK3 belongs to the family of p21-activated kinases which are well-known regulators of cytoskeletal remodelling and cell motility, but have recently also been shown to promote cell proliferation, regulate apoptosis and accelerate mitotic abnormalities, which results in tumor formation and cell invasiveness

(Vadlamudi RK et al 2003). Interestingly, PAK1 has been described to rescue MCF10A cells from undergoing anoikis (Menard RE et al 2005).

Figure 26. GS expression clustering of 56 Gab2 upregulated genes in any condition. Same comparisons as Figure 24 are shown. Genes associated to cell adhesion (ITGB2, CXCR4) and motility (PAK3) were upregulated by Gab2 overexpression.

Besides, clustering analysis also highlighted a signature of 142 genes which discriminated Gab2 overexpressing selected cells from control cells. This gene list contained both genes upregulated only upon selection or genes whose expression was further enhanced following the anchorage-independent growth selection (Figure 27).

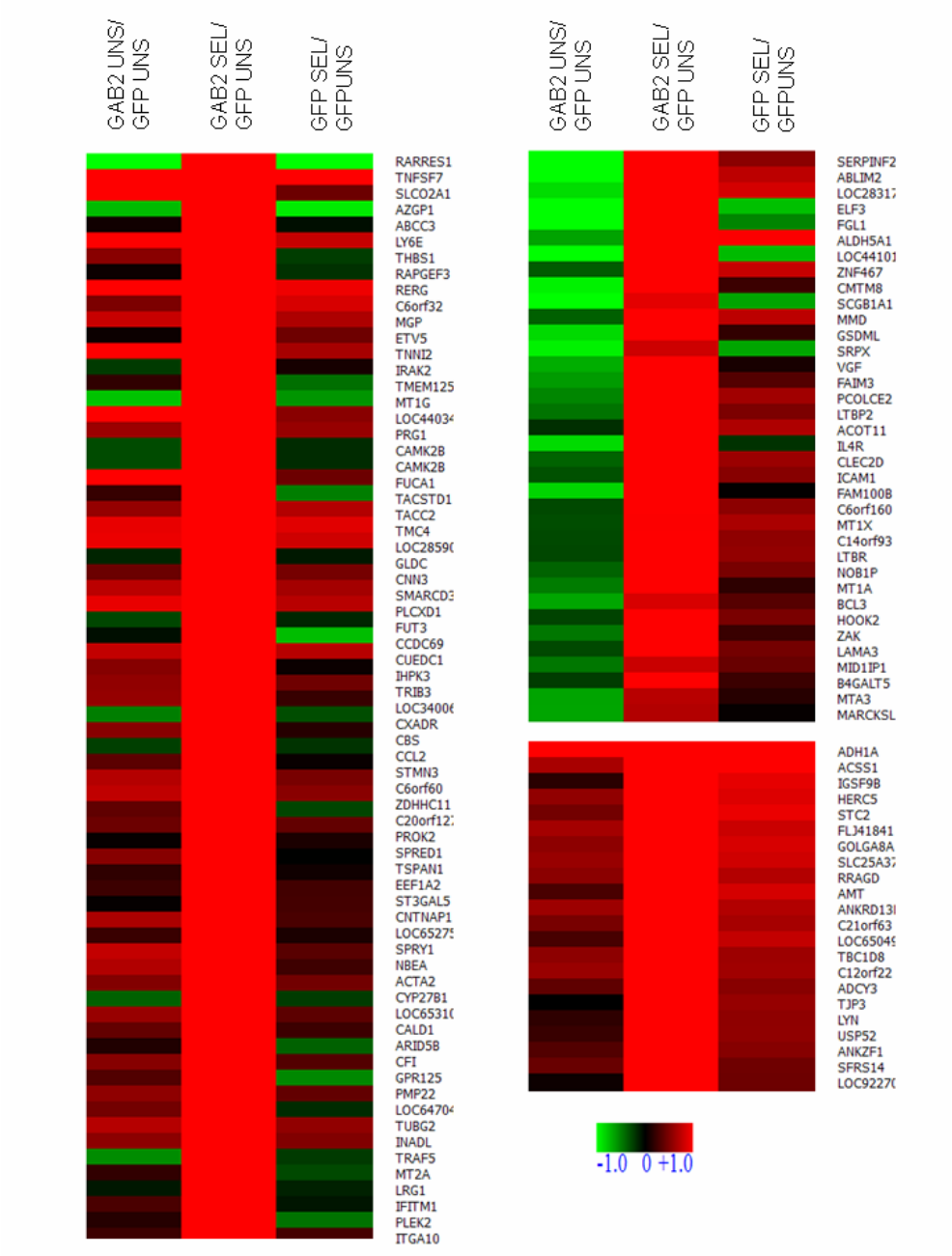

Figure 27. GS expression clustering of 142 Gab2 upregulated genes in anchorage-independent growth. Same comparisons as Figure 24 are shown. Genes associated to intracellular signaling (RAPGEF3) were enriched in the cluster and upregulated by Gab2 overexpression in selected cells.

Functional analysis of the corresponding gene list showed a significant enrichment for the intracellular signaling cascade category (p-value $<10^{-4}$). Notably, the most upregulated gene by Gab2 in these conditions was RAPGEF3, with a fold change increase of 9.5 compared to GFP SEL cells. RAPGEF3, also known as EPAC (Bos JL et al 2006), is a Rap1 guanine exchange factor, involved in Rap1 activation. Rap1 has been involved in several aspects of cell adhesion, including integrin-mediated cell adhesion and cadherin mediated cell junction formation (Kooistra MR et al 2007, Retta SF et al 2006). Rap1 controls both integrin activity (affinity) and integrin clustering (avidity), playing important roles in both "outside-in signaling" as well as "inside-out signaling". Transient overexpression of activated Rap1 stimulates strong αLβ2-dependent adhesion in human T-cell and mouse pre-B cell leukaemic cell lines and EPAC-Rap1 signaling regulates leukocyte trans-endothelial migration (Wittchen ES et al 2005). Noteworthy, our microarray data pointed to the upregulation of several genes participating in leukocyte trans-endothelial migration, such as ITGB2, CXCR4 and RAPGEF3. This suggests that Gab2 overexpression could have a key role in cell adhesion and motility.

Interestingly, we noticed that other transcripts corresponding to adhesion molecules or correlated were significantly induced by Gab2 in selected cells (Figure 28).

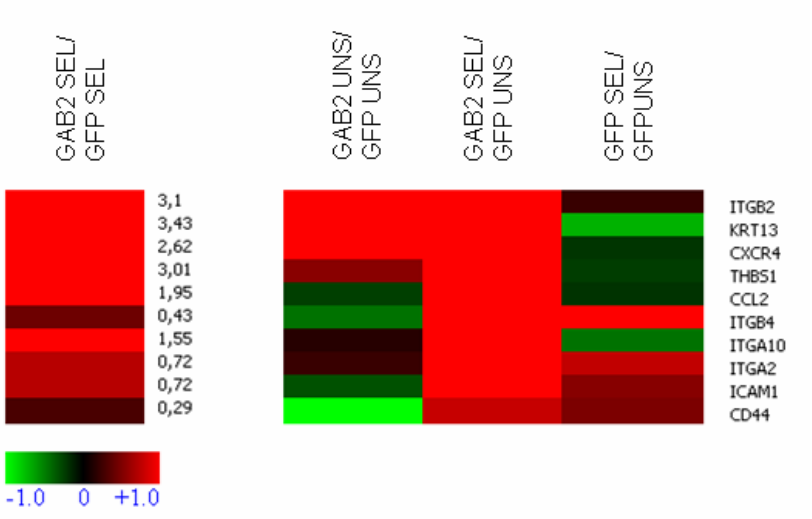

Figure 28. Gene expression profiling of genes associated with cell adhesion (cell-matrix, cell-cell adhesion and motility). Same comparisons as Figure 24 are shown. Log 2 ratio values resulting from comparisons between selected cells only (GAB2 SEL/GFP SEL) show a Gab2 specific gene induction in anchorage-independent growth.

These genes corresponded to THBS1 (thrombospondin 1), KRT13 (keratin 13), CCL2 (chemokine C-C motif ligand 2), ICAM1 (intercellular adhesion molecule 1), ITGA10 (integrin α10), ITGA2 (integrin α2) and ITGB4 (integrin β4).

Taken together these data suggest that Gab2 overexpression induces genes involved in cell-matrix adhesion and eventually in tumor progression such as thrombospondin (Varner JA et al 2006), CD44 (Suzuki M et al 2007) and ITGB4 (Guo W, et al 2006).

Gab2 overexpression promotes cell motility

Based on gene expression data, which showed that Gab2 overexpression induced a set of genes linked to cell migration, we decided to assess cell motility in a wound-healing assay. As depicted in Figure 29, Gab2 expressing cells were more motile than wild-type cells. This difference was evident already at 16h hours from the wounding and was maintained during time. For instance, Gab2 overexpression induced a 3-fold and 2-fold increase in cell motility compared to wild-type cells respectively 16h and 72h post-wounding. Together these results support a role of Gab2 also in cell motility.

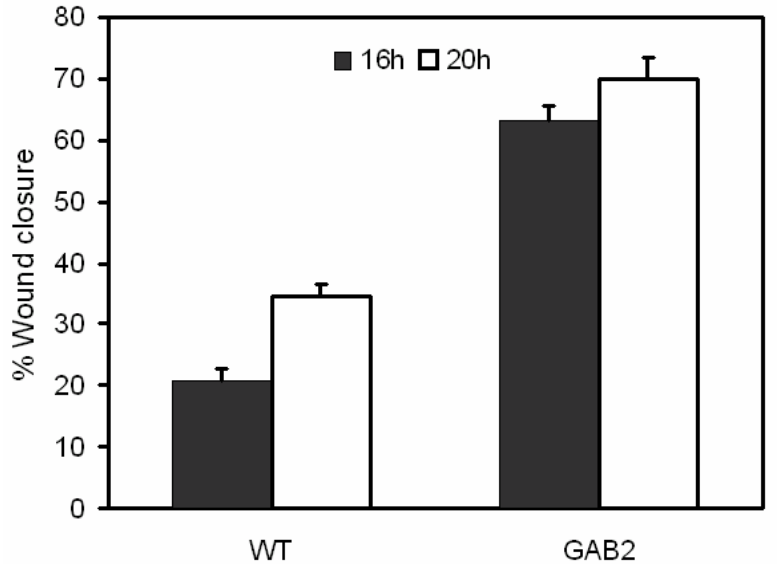

Figure 29. Wound healing assay. Cells were allowed to reach confluence on plastic dishes before a wound was generated by scratching the well monolayer with a plastic pipette tip. Cells were washed with PBS to remove cell debris, supplemented with culture medium. Images were captured by phase microscopy at 0, 16, and 20 from the wounding, and the wound area was quantified using the BD Pathway Attovision software. To quantify the wounded area, we measured the area of the wound at the time of wounding (0 hours) and

at the indicated times from the wounding. This area is represented as a percentage of the initial wound from triplicate values.

CONCLUSIONS

In multicellular organisms, a key role in formation and maintenance of tissues is played by the interactions between cells and the surrounding extracellular matrix (Berrier AL et al 2007). In particular, fibroblastic, epithelial, and endothelial cells must be attached to appropriate ECM components in order to survive and grow, a phenomenon termed "anchorage dependence"(Assoian RK et al 1997, Bottazzi ME et al 1997, Danen EH et al 2001). Normal cells integrate cues from soluble signaling ligands, like hormones, growth factors and cytokines, with signals derived from the ECM to ensure that they only proliferate in the 'correct' social context (Katz E et al 2007). Integrins are the principle cell surface adhesion receptors responsible for cell–matrix adhesion and for the consequent activation of signal transduction driving cell survival and growth (Alam N et al 2007). In this way they contribute to the maintenance of tissue homeostasis by assisting cells in sensing their appropriate developmental environment. In cancer, tumor cells evade these control mechanisms and display anchorage-independent growth (Ramsay AG et al 2007). In fact, the capacity of cells to survive and proliferate in the absence of integrin-mediated adhesion *in vitro* strongly correlates with tumorigenesis *in vivo* and may enable tumor cells to metastasize and grow at inappropriate sites in the body. Notably, many oncogenes mimic integrin-dependent signals, thereby allowing cancer cells to survive under conditions in which normal cells would undergo growth arrest and/or apoptosis (Srinivasan D et al 2007, Wu M et al 2007). The identification and characterization of novel genes or proteins promoting anchorage-independent growth, and thereby potentially driving a malignant phenotype, will likely provide candidate targets for innovative anticancer therapies.

Among the possible approaches developed for the identification of genes with a specific biological property are functional screenings based on "gain" and "loss-of function" (Brummelkamp TR et al 2003). The "gain-of function" screening, also named expression cloning, usually relies on transducing an expression library containing full length cDNAs into a given target cell line. Then a selective stress capable of strongly reducing cell viability and proliferative potential is applied. Only

cells expressing exogenous cDNAs conferring resistance to the selection will grow and form resistant colonies. This “gain-of-function” approach proved extremely valuable in the identification of novel genes involved in, e.g., neoplastic transformation, resistance to apoptosis, or escape from senescence. However, a huge amount of work is typically required to identify the integrated cDNAs in the resistant colonies, and to verify that they mediate the selective advantage. On the other hand, “loss-of-function” genetic screens exploit inactivation of genes by RNA interference. Recently, the generation of collections of thousands of short hairpin RNA (shRNA)-encoding viral vectors has provided one of the most powerful tools for genome-wide loss-of-function screens in mammalian cells. These screenings are based on the possibility of tracking the abundance of each shRNA before and after the selection using DNA microarrays recognizing specific “barcode” sequences included in each different construct. Compared to gain-of-function screens, the hits obtained with the shRNA-based approaches are less directly exploitable as new molecular targets: they are genes whose loss, and not gain, provides a selective advantage to the cells, and no obvious therapeutical approach can be directed at reactivating a gene whose function is lost.

We considered that improving gain-of-function, cancer-oriented functional screenings could streamline the identification of new potential druggable targets, and set-up a procedure for high-throughput, microarray-assisted expression cloning. The screening is based on transduction of mammalian cells with a retroviral cDNA expression library, and with specific tracing of the abundance of transcripts derived from the library, before and after selection, using DNA microarrays. To enable specific detection of exogenous cDNAs, a procedure has been conceived and optimized to avoid or otherwise exclude microarray signals derived from the endogenous transcripts of the host cell. The screening is then carried out by applying a selective pressure on the transduced cell population, so that cells transduced with genes conferring a selective advantage are enriched. Comparing microarray signal intensities of the exogenous genes before and after selection allows simultaneous detection of all advantageous ones. We called this procedure “Xenoarray” analysis, and validated it by transducing a mouse testis expression library into the MCF10A

cell line, immortalized from normal human breast tissue. Transduced MCF10A cells were then selected by cultivation in the absence of anchorage, to highlight library-derived genes conferring anchorage-independent growth. To strengthen the effectiveness of the screening, two independent infections and selections were designed. In both cases, biological assays on the selected cells showed their increased proliferation and anchorage-independent growth in soft agar. Using Xenoarray analysis, before and after selection, we were able to detect enrichment of 20 exogenous transcripts in both selections. The analysis revealed a strong a reproducible enrichment for the GAB2, but also for well-known transforming genes such as NTRK3 (Geiger TR et al 2007) and for uncharacterized genes such as CYP11A1. In the last case, however, recent evidence pointed to an association between CYP11A1 and breast cancer (Yaspan BL et al 2007). A significant allelic association of a simple tandem repeat (STR) polymorphism upstream of the CYP11A1 gene with breast cancer risk has been demonstrated, supporting the identification of such gene in our screen. The CYP11A1 gene encodes the cholesterol side chain cleavage enzyme that catalyzes the initial and rate-limiting step of the biosynthesis of sex hormones, including estrogen, progesterone, and androgens, known to profoundly affect cell metabolism and growth and to play a role in breast cancer. As before said, the gene which showed the higher and reproducible enrichment in both selections resulted to be GAB2, a member of the GRB2-associated binding protein (GAB) gene family (Gu H et al 1998). The Gab gene family, including mammalian Gab1, Gab2, Gab3, the Drosophila homolog DOS (Daughter Of Sevenless), and the c. Elegans homolog Soc1 (Suppressor-Of Clear), encodes scaffolding proteins closely related to insulin receptor substrates (IRS-1, IRS-2, IRS-3), fibroblast growth factor substrate (FRS2), linker of T cell (LAT) and downstream of kinase (Dok) (Nishida K et al 1999, Hibi M et al 2000). They are so called scaffolding or docking proteins because of the presence of multiple functional motifs mediating interactions with many other signaling molecules. All Gab family proteins share a common architecture consisting of a highly conserved N-terminal Pleckstrin homology (PH) domain, a central proline-rich domain (PRD) and multiple tyrosines, within potential binding motifs favored by various Src homology 2 (SH2) domain-containing proteins. Thanks to the PH domain, they can be targeted to specific membrane lipids, where they can serve as signal

‘amplifiers’ by binding multiple signaling molecules (including adaptors and enzymes) and assembling multimeric “transductosomes” (Edmead CE et al 2006). They are involved in signaling events triggered by a variety of stimuli, including growth factors, cytokines, G coupled receptors and T and B cell antigens, ultimately regulating cell growth and differentiation (Bouscary D et al 2001, Liu Y et al 2001, Wickrema A et al 1999, Nishida K et al 2003). Upon receptor stimulation, Gab proteins undergo rapid tyrosine phosphorylation, creating a number of docking sites to mediate interactions with SH2 domain-containing proteins (Crouin C et al 2001) such as the tyrosine phosphatase SHP2, p85 subunit of PI3K, Shc, PLCγ and Crk/CrkL which respectively activate MAP kinase, PI3 kinase and JNK pathways. Specific association with these molecules was found to be critical for the function of Gabs in mediating intracellular signaling pathways from the receptors. For instance, interaction between Gab2 and PI3K is important for Fc receptor signaling in mast cells and macrophages (Yu M et al 2006). Another important signaling function of Gab proteins is to bind and activate the protein-tyrosine phosphatase Shp2 (Besset V et al 2000, Craddock BL et al 2001), resulting in enhanced and/or more sustained Ras/Erk signaling: for example, Shp2 can positively regulate Ras activation by dephosphorylating Ras GTPase-activating protein recruitment sites on the EGFR or by indirectly activating Src family tyrosine kinases. Recent studies of Gab1 and Gab2 knockout mice have delineated distinct functions for individual Gab proteins. Although Gab3-deficient mice lack an obvious phenotype, suggesting functional redundancy with the other family members, Gab1 and Gab2 perform unique functions in normal and pathological states (Zhang Y et al 2007). Gab1 gene knockouts display embryonic lethality, probably due to the key role played by Gab1 in Met signaling. In contrast, mice lacking Gab2, which has a more tissue-specific expression pattern, display impaired mast cell-mediated allergic responses and osteoclast differentiation due to a requirement for Gab2 downstream of Fc receptor and RANK (Wada T et al 2005), respectively.

Gab2 has also been linked to tumorigenesis, since some oncogenes may depend on its function. It was demonstrated that Gab2 is required for transformation by Bcr-Abl (Ulivieri C et al 2005, Sattler M et al 2002) via activation of Akt and Erk, whereas the association of Gab2 to PI3K and Shp2 was essential for transformation by v-Sea

(Ischenko I et al 2003). Among the Gab2 direct interactors are proteins with key roles in human cancers when mutated, such as PI3K and the tyrosine phosphatase PTPN11 (also known as SHP2). The latter is mutated in several types of leukemias and solid tumors, and recent work suggested that the oncogenic properties of Shp2 mutant proteins require signal enhancement by Gab2 (Zatkova A et al 2006). Although not yet detected in human samples, mutation at serine 159 of Gab2, which uncouples from negative feedback phosphorylation by Akt, displays potent transforming properties in fibroblasts (Lynch DK et al 2002), identifying Gab2 as a proto-oncogene product. In addition, it was found that Gab2 maps to a chromosomal region amplified in 10-15% of breast cancers and its overexpression was also confirmed in several breast cancer cell lines (Daly RJ et al 2002). Brummer and coworkers showed that overexpression of Gab2 in MCF10A cells increases proliferation and depending on its expression level, is capable of conferring increased acinar size, resistance to proliferative suppression within acinar structures, independence of the morphogenetic program from exogenous EGF, and defective luminal clearance, supporting a role for Gab2 in breast cancer development (Brummer T et al 2006). Additionally, it was found that Gab2 acting through Shp2-Erk pathway cooperated with activated Neu in mammary carcinogenesis in mice (Bentires-Alj M et al 2006). Finally, it was demonstrated a requirement for Gab2 in promoting mammary tumor metastasis since homozygous deletion of Gab2 in mice severely suppressed lung metastasis while had only a modest effect on the initiation of Neu-induced mammary tumors (Ke Y et al 2007).

In this study, we show that Gab2 resulted to be the most enriched transcript in cells which underwent an anchorage-independent growth selection. Interestingly, no previous evidence reported a specific role for Gab2 in anchorage-independent growth. We validated library-specific Gab2 enrichment by Real time PCR and by western blot analysis on control cells (GFP) and library-transduced cells (LIB SEL) before and after the selection. These analyses demonstrated that in two independent selections Gab2 was reproducibly enriched at the mRNA and protein level, supporting the effectiveness of the screening. Knock-down by RNA interference of exogenous Gab2 in the transduced-selected cells abrogated their growth advantage in all conditions, confirming that library-derived Gab2 expression was the driving force in the selected phenotype.

The exogenous transduction of MCF10A cells with a Gab2-expression vector induced increased proliferation of adherent MCF10A cells, but also, and more robustly, increased growth in suspension cultures and in soft agar. Interestingly, Gab2-sustained growth advantage was almost totally lost when cells were cultured in the absence of EGF and with low serum, further demonstrating the role of this gene as a signal amplifier, rater than generator. Gab2 overexpressing cells also gained a motile phenotype as demonstrated by the wound healing assay. Knock down of Gab2 in overexpressing cells by shRNAs, although incomplete (70%), could still revert the growth advantage. A complete proliferative block was observed in wild-type MCF10A cells, which basally express very low Gab2, upon transduction with the Gab2 shRNA. Most importantly, Gab2 loss of function in breast cancer cells which endogenously overexpressed Gab2 protein completely abrogated anchorage-independent growth in soft agar, reflecting the importance of this adaptor in the maintenance of the transforming capacity of different cancer cell lines. For instance, it was reported that in MCF-7 cells, Gab2 was markedly tyrosine phosphorylated in response to heregulin and also following EGF, insulin or FGF administration, indicating that a variety of RTKs implicated in breast cancer development or progression coupled to this docking protein (Daly RJ et al 2002).

Analysis of cell cycle and apoptosis revealed that Gab2-driven anchorage-independent growth was not due to a protection from anoikis, since the percent of apoptotic cells was not significantly lower than wild-type cells after 48h of suspension. This result is in line with previous reports indicating that Gab2 did not prevent apoptosis of luminal cells during morphogenesis of MCF10A cells (Bentires-Alj M et al 2006); rather Gab2 could collaborate with anti-apoptotic oncogenes to promote luminal filling. Probably, Gab2 overexpression alone could enhance cell proliferation, but additional genetic lesions that impair proapoptotic pathways are necessary to allow mammary epithelial cells to become independent from ECM-derived signals and escape apoptosis. Alternatively, it could be that the levels of tyrosine-phosphorylated Gab2 must be increased above a particular threshold for oncogenic transformation to occur, as pointed by Brummer and coworkers, which showed that inhibition of luminal

clearance in MCF-10A acini occurred only upon further elevation of Gab2 expression. This is consistent also with the biological activity of the Gab2 S159A mutant, which is transforming in fibroblasts and exhibits enhanced tyrosine phosphorylation due to loss of negative feedback regulation. However, a potential role of Gab2 in regulating apoptosis during anchorage-independent growth was highlighted by mRNA expression analysis of the proapoptotic Bim gene. In MCF10A cells, Bim is upregulated upon detachment from the ECM, as a consequence of impaired Erk signaling, and drives anoikis (Reginato MJ et al 2003). Oncogenes responsible for the progression of glandular epithelial tumors suppress Bim transcription (Debnath J et al 2002, Reginato MJ et al 2005). We observed that Gab2-overexpressing cells fail to upregulate Bim mRNA expression upon detachment, thereby linking a new scaffold to the detachment-dependent Bim suppression.

Cell cycle analysis of Gab2 overexpressing cells reveled that Gab2 increases the fraction of proliferating cells, both under adhesion and suspension, reflecting Gab2 involvement in the sustenance of cell cycle progression independently from the adhesive consensus. When deprived of attachment to a solid substrate, even in the presence of growth factors, normal cells are unable to replicate their DNA, and they arrest in the G1 phase of the cell cycle. Here we show that Gab2 is able to maintain active proliferating cells even in the absence of anchorage, particularly by promoting progression through the G1/S phase of the cell cycle, thereby justifying the anchorage-independent growth observed and stressing Gab2 role in tumorigenesis.

Analysis of the molecular mechanism underlying such biological effect revealed a contribution of specific pathways to Gab2-driven anchorage-independent growth. While inhibition of PI3K pathway by pharmacological treatment resulted in severe reduction of cell vitality in both wild-type and Gab2 overexpressing cells, inhibition of Erk and Src pathways had a specific effect on Gab2 overexpressing cells, with substantial difference between adhesion or suspension. In particular, Src inhibition preferentially affected the growth of Gab2 overexpressing cells in suspension, with no effects on wild type cells and minor effects on adherent overexpressing cells. A similar pattern of efficacy, but less pronounced, was observed upon Erk inhibition. The SRC inhibitor specific effect provided a strong rationale for SRC pathway involvement in Gab2-driven anchorage-independent growth. This is reasonable, since

one of the earliest steps in transducing extracellular cues through integrins to the cytoskeleton is the activation of the tyrosine kinases Src and FAK (Satyajit K et al 2006, Mitra SK et al 2006). Besides, it has been demonstrated the important role of Src activation in EGF-induced hepatic mitogenesis, through the phosphorylation of Gab2 and the activation of the PI3-kinase cascade (Kong M et al 2000) and Yu et al identified Gab2 as an important regulator of β1-integrin signaling pathway during hematopoietic cell adhesion by interaction with Syk kinase, a non receptor tyrosine kinase of the Src family (Yu WM et al 2002). However, no evidence has ever linked Gab2 and SRC in the promotion of anchorage-independent growth. Biochemical analysis confirmed the contribution of the MAPK/ERK pathway and SRC pathway in Gab2-mediated anchorage-independent growth, although with different kinetics of activation. Gab2 overexpression did not induce a basal hyperactivation of ERK, but rescued ERK activation during prolonged detachment. Notably, ERK role in anchorage dependent and independent growth has been extensively documented (Mizutani K et al 2007, Howe AK et al 2002, Aplin AE et al 2002, Pullikuth AK et al 2007). Contrarily, Src activation was already detectable in adherent cells and, interestingly, Gab2 overexpression allowed the maintenance of Src phosphorylation during prolonged supension culture. This is consistent with the fact that SHP2, a major interactor of Gab2, activates Src by dephosphorylation of its inhibitory tyrosine (tyrosine 527) (Roskoski et al, 2005). Moreover, it was demonstrated that Shp2 promotes Src family kinase activation by phosphorylation of the Csk regulator PAG/Cbp, thereby controlling Csk access to Src family kinases (Zhang, S, et al 2004).
This result suggests that the higher proliferation rate of Gab2 overexpressing cells could be in part due to basal Src hyperactivation sufficient to drive DNA synthesis and that their oncogenic properties (anchorage-independent growth) could rely on continuous Src signaling. In several cancer types, abnormal events lead to elevated Src kinase activity and cause pleiotropic cellular responses including transformation and metastasis (Playford MP et al 2004, Frame MC et al 2004); furthermore expression of v-Src has been shown to rescue several cell types from apoptotic death induced by disruption of cellular interactions with extracellular matrix proteins (Diaz-Montero CM et al 2006, Wei L et al 2004, Hisano C et al 2003). Src involvement is consistent also with the observed increase in tyrosine phosphorylation of paxillin, an

SRC kinase substrate involved in focal adhesion signaling events (Brown MC et al 2005), by Gab2 overexpression. Indeed, it was argued that Shp2 could activate Src family kinases by regulating other Csk binding proteins such as paxillin, which is hyperphosphorylated on its proposed Csk binding site in Shp2 defective cells (Zhang SQ et al 2004). The finding of a specific role of the Src pathway in Gab2-driven anchorage independence opens interesting perspectives for future therapeutical approaches relying on Src family kinases inhibitors such as Dasatinib. Also, it should be noted that high expression of Gab2 may promote SRC activation via either Shp2 activation or direct association and that Gab2 may amplify integrin derived signals.

To gain further insight on the molecular events associated with Gab2-driven anchorage-independent growth, we performed a microarray analysis comparing the transcriptional profiling of control cells with that of Gab2-overexpressing cells, before or after selection by growth in suspension. Interestingly, most genes upregulated by basic Gab2 overexpression were further upregulated upon anchorage-independent growth selection of the transduced cells. We found that Gab2 induces genes involved in cell-matrix and cell-cell adhesion and, in particular, genes involved in leukocyte trans-endothelial migration, such as ITGB2 (Powner DJ et al 2007), CXCR4 (Huang YC et al 2007), ICAM1 (Koizumi K et al 2007) and RAPGEF3 (Lorenowicz MJ et al 2006). Regulated adhesion of leukocytes to the extracellular matrix is essential for transmigration across blood vessels and subsequent migration into the stroma of inflamed tissues. This process has been extensively studied because it is evoked during metastatic spread of cancer cells. For instance, the capacity of tumor cells to form metastatic foci correlates with their ability to interact with - and migrate through - endothelial cell layers, a step which involves multiple adhesive interactions between tumor cells and the endothelium. In this view, Gab2 overexpression could have a role in the extravasation process thus facilitating transendothelial migration. Moreover, Gab2 overexpression followed by anchorage-independent growth selection, induced genes of the extracellular matrix such as thrombospondin (THBS1) which has been positively associated with adhesion, motility, invasion, proliferation and angiogenesis, thus stressing its implication in tumor metastasis (Paydas S et al 2007, Varner JA et al 2006, Ioachim E et al 2006). Most importantly, microarray data highlighted the induction of PAK3 by Gab2 both in selected and unselected cells, thus representing a

robust Gab2 target gene. PAK3 belongs to the p21 serine/threonine kinase family, that activate downstream signaling cascades regulating several cellular responses including cell cycle progression, apoptosis, motility, and angiogenesis (Jaffer ZM et al 2002). Interestingly they are also involved in integrin signaling (Slack-Davis JK et al 2003, Schober M, Raghavan S et al 2007). Overexpression of Paks in epithelial cancer cells has been shown to increase migration potential, anchorage independent growth, and cause abnormalities in mitosis (Vadlamudi RK et al 2003). Deregulation of Paks has been reported in several human tumors and neurodegenerative diseases (Kumar R et al 2006). For instance, it was shown that antiapoptotic functions of Paks derive, in part, by inhibition of Bim functions (Vadlamudi RK et al 2004), suggesting that Bim downregulation and PAK3 upregulation in Gab2 overexpressing cells could have a causal role in anchorage-independent growth. Furthermore, it has been shown that Pak3 phosphorylates paxillin at Cdc42- and Rac1-driven focal complexes (Hashimoto S et al 2001), thereby participating in actin-based morphogenetic events. Finally, a recent study showed a correlation between expression of Gab2 and Pak1 in human breast tumors (Garau X et al 2005) and overexpression of Pak1 mutant promoted mammary hyperplasia in transgenic mice (Bekri S et al 1997, Balasenthil s et al 2004). Gab2 is located on 11q13.5-14.1, a region amplified in 10-15% of human breast cancers and it was found that PAK1 also maps within the core 1 amplicon where it resulted to be amplified and overexpressed in some breast tumors (Reyal et al 2005). This data, together with our microarray results, suggest that Gab2 could collaborate with PAK genes in order to promote tumorigenesis and it would be interesting to investigate whether they could be coamplified in human breast cancers.

To ensure successful metastatic dissemination, malignant cells undergo an adaptive process that relies mainly on two strategies. One is the abnormal and unpolarized secretion of basement membrane molecules, which aberrantly reconstitute the original histological niche, thus imparting surrogate proliferative and survival signals. Parallel (or alternative) to this is the hyperactivation of oncogenic pathways, which produce a global amplification of the cell signaling activity with consequent elusion of the external adhesive consensus. Whatever the mechanism used, the phenotypic outcome of this process is that neoplastic cells acquire the ability to grow in the absence of their environment of origin.

In this study, we show that anchorage-independent growth conferred by Gab2 could be mediated by both mechanisms: firstly by the transcriptional regulation of genes important for cell adhesion (chemokines and their receptors, integrins, ECM proteins) and cytoskeletal remodeling (PAK3), secondly by the biochemical amplification of oncogenic signaling pathways impinging in particular on Src activation and downstream associated events.

In summary, the ability of Gab2 to stimulate cell cycle progression, to override adhesion consensus, to inhibit the anchorage-independent growth of breast cancer cells and to induce cell motility is consistent with a role for this protein in mammary tumorigenesis. Notably, several receptor and non-receptor tyrosine kinases that couple to Gab2 exhibit enhanced expression and/or activity in breast cancer, including c-Src. Therefore, the cooperation between such kinases and Gab2 in malignant transformation of breast epithelial cells will be an important area for future study.

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Material and Methods

Cell Culture and Reagents

MCF10A cells were obtained from ATCC and cultured in "Growth Medium" containing DMEM/F12 (Invitrogen) supplemented with 5% horse serum (Gibco), 20ng/ml epidermal growth factor (Peprotech), 10ug/mL insulin (Sigma), 100ug/ml hydrocortisone (Sigma), 1ng/ml cholera toxin (Sigma), 50U/mL penicillin and 50 mg/ml streptomycin. MCF7, MDA-MB-231 AND MDA-MB-435 breast cancer cells were obtained from ATCC and cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Sigma).

The following inhibitors were used to stimulate both adherent and non adherent MCF10A cells for 24h: MEK inhibitor (PD098059, 40μM, Calbiochem), SRC inhibitor (PP2, 10μM Calbiochem), PI3Kinase inhibitor (LY294002, 50μM Calbiochem), JNK inhibitor (SP600125, 10μM Calbiochem). The antibody used were: polyclonal anti-GAB2 (Upstate Biotechnology), polyclonal phosphorylated Erk-specific (Cell Signalling), polyclonal phosphorylated PKB/Akt-specific (Cell Signalling), polyclonal phosphorylated active SRC (Tyrosine 416) (Cell Signaling), polyclonal anti-total SRC (Cell Signaling), polyclonal phosphorylated paxillin (Tyrosine 31) (Cell Signaling), polyclonal anti-total Erk (Cell Signalling), polyclonal anti-total PKB/AKT (Cell Signalling), anti-actin (Santa Cruz). Retroviral expression vector for GAB2 in pMIG (also known as pMSCV-IRES-GFP) was kindly provided by R. Daly (Cancer Research Program, Garvan Institute of Medical Research, Sydney, New South Wales 2010, Australia).

Retrovirus production was obtained by transfection of the amphotrophic packaging cell line (Phoenix) with 10μg of plasmid DNA using Lipofectamin (Invitrogen). Retroviral supernatants were collected 48 h post-transfection, passed through a 0.22μm filter, supplemented with Polybrene at a final concentration of 8μg/ml, and diluted with fresh MCF-10A growth medium. This solution was then used to infect a subconfluent culture of MCF-10A cells, as described (Debnath, J et al 2003). GFP-positive cells expressing GAB2 were then sorted to homogeneity by flow cytometry.

RNA interference and shRNA vectors

Lentiviral expression constructs carrying the shRNA sequence targeting murine (RefSeq NM_010248) and human GAB2 (RefSeq NM_012296) respectively were purchased from Sigma (MISSION™ TRC shRNA Target Set). pLKO.1-puro Control Vector, which does not contain an shRNA insert, was used to infect control cells. This allows to examine the effect of transfection on gene expression and to interpret the knockdown effect seen with shRNA clones. Infected cells were selected by adding puromycin (2ug/mL) to growth medium and efficiency of knock-down was evaluated by western blotting with Gab2 specific antibodies.

The specific shRNA sequences were respectively:

Murine shRNA (TRCN0000100127):

CCGGCCGACACAATACAGAATTCAACTCGAGTTGAATTCTGTATTGTGTCG GTTTTTG

Human shRNA (TRCN0000155921):

CCGGCAGCCAACTCTGTTCACGTTTCTCGAGAAACGTGAACAGAGTTGGC TGTTTTTTG

Western Blot

Cell lysates from 2–5 × 10^6 MCF-10A cells either attached or in suspension for the indicated times were prepared in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 50 mM TrisHCL at pH 8, 0.1% SDS, 10% glycerol, 5 Mm EDTA, 20 mM NaF and 1 mM Na3VO4) supplemented with 1 μg ml−1 each of pepstatin, leupeptin, aprotinin, and 200 μg ml−1 phenyl methylsulphonyl fluoride (PMSF). Lysates were cleared by centrifugation at 12,000 rpm for 20 min at 4°C and normalized with the BCA Protein Assay Reagent Kit (Pierce). Extracts were electrophoresed on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (Hybond; GE Healthcare) and incubated with different Abs overnight at 4°C Nitrocellulose-bound antibodies were detected by the ECL system (GE Healthcare).

Transductions for the functional screening

The mouse testis retroviral expression library, packaged in the VSV envelope was purchased from Stratagene (ViraPort, Cat n. 972300). The library is constructed in

pFB, a replication-defective Mouse Moloney murine leukemia (MMLV)-based vector.

As control vector we used a VSV-G pseudotyped pFB-hrGFP retroviral supernatant containing the coding sequence for the humanized recombinant green fluorescent protein provided by Stratagene. For the transduction of MCF10A cells 1.5 x 105 cells were seeded in each of 2 x 60mm tissue culture plates. The following day the medium was removed and 1 ml of undiluted supernatant (corresponding to an MOI = 1), supplemented with DEAE-

dextran at final concentration of 10 μg/ml was added to one of the 2 plates. Growth medium with undiluted pFB-hrGFP retroviral supernatant, corresponding to the same MOI, was added to the other plate for an uninfected control. The plates were incubated at 37° for 3 hours then additional 1.7 ml of medium was added. These transductions were repeated in duplicate by independent infections.

Anchorage-independent growth selection

Polyhema-coated 100mm Petri dishes were prepared by applying 4ml of a 12mg/ml solution of poly-hydroxy-ethyl-methacrylate (polyhema; Sigma) in ethanol, drying under tissue culture hood, repeating the application once and incubating the plates overnight at 37°C. For the selection, cells were trypsinized, counted and 3 x 10^6 cells were plated onto 100 mm poly-HEMA coated plates. Cells were cultured in suspension for 48h then cell debris was removed by spinning the suspension at low speed (400 rpm) and resuspending the pellet in fresh medium. In this way viable cells growing in clumps were recovered, while dead cells were discarded. Cells were let to recover on regular plates for 24h before performing additional 48h of poly-HEMA selection. After 3 weeks of selection pressure, cells were expanded on regular plates for one week before RNA was extracted for microarray analysis and further biological assays.

RNA extraction and processing for microarray analysis

RNA was extracted using the TRIzol reagent (Invitrogen), according to the manufacturer's protocol, and then further purified using the RNeasy Mini kit from Qiagen. The quantification and quality analysis of RNA was performed on a Bioanalyzer 2100 (Agilent).

Synthesis of cDNA and biotinylated cRNA was performed using the Illumina TotalPrep RNA Amplification Kit (Ambion Cat. n. IL1791), according to the manufacturer's protocol, with the only variation to optimize xenoarray analysis being the use of 20 micrograms of total RNA for cDNA synthesis with 4 picomoles of a primer specific for the pFB vector, with standard reaction conditions. Quality assessment and quantification of cRNAs were performed on Bioanalyzer 2100.
Hybridization of MCF10A-derived cRNAs was carried out using Illumina Mouse 48k gene chips (Cat. N.BD-26-101), with 1.5 micrograms of T7-pFB-derived cRNA. Array washing was performed using Illumina High-stringency wash buffer for 30 min at 55°C, and followed by staining and scanning according to standard Illumina protocols. Probe intensity data were obtained using the Illumina BeadStudio software, and further processed with Excel software.

Xenoarray analysis, Cross-matching probes filtering and Statistical analysis

The Xenoarray analysis pipeline was performed with Illumina BeadStudio software and subdivided in 3 main tasks: (i) Removal of probes cross-hybridizing to endogenous transcripts. For this task, the "Detection" value provided by the Illumina Beadstudio software was used. This value ranges from 0 (non-detected) to 1 (detected), with 0.99 or higher as the usual thresholds for significant detection. All probes with a Detection value higher than 0.99 in at least one sample of GFP-transduced cells are excluded from the subsequent steps. (ii) Data preprocessing to improve reproducibility, and reduce technical noise. The data were scaled according to the tenth percentile of the average distribution of all the chips. A background reduction was then performed by subtracting 2/3 of the minimum signal value. (iii) Identification of probes giving higher signal after selection. The software dot-plot package was used to compare probe signals from transduced cells before and after the selection, and to calculate for each probe the $\log_2$ of the ratio between the signals before and after selection.

MTT assay for cell vitality measurement in adhesion or suspension

For cell vitality assay, 10^3 cells of each cell line were seeded in triplicate in regular 96-well plates or Poly-Hema coated plates. Cells were cultured in growth medium

containing all supplements (5% horse serum, cholera toxin, insulin, EGF and hydrocortisone), or in "starved" medium lacking EGF, serum and cholera toxin. Where indicated cell vitality was estimated in the presence of inhibitors (PD098059, LY294002, PP2 and SP600125) after 24h of adherent or supsension culture. At the indicated times (24h, 48h or 72h after plating), a tetrazolium salt–based reagent (CellTiter96 Aqueous One Solution, Promega, Milan, Italy) was added to each well according to the instructions provided by the manufacturer. After an incubation of 2 h, absorbance was read at 490 nm on a Beckman Coulter (Milan, Italy) DTX 880 plate reader. To avoid assay variability, a control of cell plating was included by estimating cell vitality after 4h from plating the day of the assay. This value was used as a reference to adjust subsequent acquisitions of each cell line and cell vitality was expressed as a percentage of plated cells at 48h or 72h post-plating. The mean of the triplicate values were calculated and plotted with error bars representing the SD of triplicate samples from three independent experiments.

Soft agar assay

$2x10^4$ cells were resuspended in 1 ml of 0.5% top agar (SeaPlaque Agarose from Cambrex) in growth medium and seeded in 6 well plates previously filled with 2 ml of 1% basal agar in growth medium. The assay was performed in duplicate. After 3 weeks, phase-contrast pictures were captured and analyzed with a BD Pathway Workstation.

Growth curve analysis of adherent cells

For growth curve analysis, cells were trypsinized, counted and 2×10^4 cells were plated in triplicate in 24-well costar. Cell number was evaluated respectively 24h, 48h, 72h, 96h, and 120h after plating using a cell counter. The mean of the triplicate values were calculated and plotted with error bars representing the SD of triplicate samples from three independent experiments.

Detachment-induced apoptosis assay and flow cytometry analysis of apoptosis induction

MCF-10A cells were cultured in growth medium containing all supplements (5% horse serum, cholera toxin, insulin, EGF and hydrocortisone), then the day of the

assay 300,000 cells ml−1 were placed in suspension on poly-HEMA-coated 35-mm plates or on regular plates as control cells. After 48h, suspended and adherent cells were washed with PBS and counted. Cell death was measured by assessing the number of hypodiploid nuclei with the DNAcon3 kit (ConsulTS, Rivalta, Italy). Briefly, cells were gently lysed with 0.1% Triton X 100 in the presence of RNAse and of a chromatin stabilizer, and DNA was stained with 50 μg/ml propidium iodide. Samples were kept for 1 h in the dark at room temperature and the DNA index was then measured by cytofluorimetric analysis using a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA). Data acquisition was performed using CellQuest software and data analysis with WinMDI software. Debris staining was eliminated on a PI area vs. PI width diagram. PI (FL2) fluorescent signals were displayed as histograms and shown as units of fluorescence on a logarithmic scale The PI signal was calculated on the histogram window of WinMDI after discriminating apoptotic and non apoptotic subpopulations with a logical gate tool. Hypodiploid, subG0/G1 nuclei were defined as those displaying a PI staining value lower than that of cells in the G0/G1 cell cycle phase (diploid DNA peak).

Real-Time PCR for validation of enriched genes after selection

In order to validate the enrichment of some genes in the 2 Poly-Hema selected populations, Quantitative Real-Time PCR with Sybr Green assay (Applied Biosystems) was used to measure the relative amount of the cDNA of interest respect to the amount of a housekeeping gene in different samples (unselected and selected cells). Total RNA was extracted from unselected and selected cells using the TRIzol reagent (Invitrogen) and 2μg were used as template for first-strand synthesis in a 20μL volume reaction. The resulting first-strand cDNA was diluted and used as template in the PCR reaction. Analysis of gene expression was performed with ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Oak Brook, IL). Reactions for each sample were performed in triplicate using equal amounts of template cDNA, and each triplicate. The expression of the housekeeping gene, PGK, was used to normalize for variances in input cDNA.

The primers used for Real-Time PCR were were designed with Primer Express software (Applied Biosystems) and were murine (except for the housekeeping) in order to detect only library-derived transcripts:

hPGK: sense 5'-CTTATGAGCCACCTAGGCCG-3'; antisense 5'-CATCCTTGCCCAGCAGAGAT-3'

mNTRK3: sense 5'- TGGCAACTACACCCTCATTGC-3'; antisense 5'-GAAATCTGTGCTCTCTGGAAAGG

mCYP11A1: sense 5'- GGACTTAAGGCAGAAGCGAGACT-3': antisense 5'-AATGTTGGCCTGGATGTTCTTG

mETS1: sense 5'-GGATATCCTGTGGGAGCATCTAGA-3': antisense 5'-GGTGTAACAGGATTCTGGGTAGGT

mGAB2: sense 5'- CTGCTGAACCTCCAGGAAAGA-3'; antisense 5'-GCCAGCAGGGTAGAAGAACCT-3'

Real-Time PCR for Bim expression

Total RNA was extracted from either attached or suspended cells using the TRIzol reagent (Invitrogen), according to the manufacturer's protocol, and 2μg were used as template for first-strand synthesis with Sybr Green assay (Applied Biosystems) as described before. Reactions for each sample were performed in triplicate using equal amounts of template cDNA, and each triplicate. The expression of the housekeeping gene, PGK, was used to normalize for variances in input cDNA. The primers were designed with Primer Express software (Applied Biosystems) and the sequences of the PCR primer pairs are as follows:

hPGK: sense 5'-CTTATGAGCCACCTAGGCCG-3'; antisense 5'-CATCCTTGCCCAGCAGAGAT-3'

hBIM: sense 5'- GCACCCATGAGTTGTGACAAA-3'; antisense 5'-GTTCAGCCTGCCTCATGGAA-3'

Wound healing Assay

For wound-healing migration assay, cells were seeded on 24-well plates at a density of 5×10^4 cells/well in culture medium. A single scratch wound was created using a micropipette tip on confluent monolayers. Cells were then washed with PBS to remove cell debris and supplemented with culture growth medium. Cells were then fixed in glutaraldeyde 11%, washed and stained with crystal violet and images were captured by phase microscopy using a 2x objective. A 3x3 montage (BD Pathway Workstation) was then performed for each well. To quantify the wounded area, we measured the

area of the wound at the time of wounding (0 hours) and at the indicated times from the wounding using the BP pathway Attovision software. This area was represented as a percentage of the initial wound. The percentage of wound healing for each cell lines was averaged over three experiments and statistical significance was calculated as standard error within triplicates.

RNA extraction and processing for microarray analysis of Gab2 unselected and selected cells

RNA from GFP transduced cells, GAB2 transduced cells before and after the anchorage independent-growth selection was extracted using the TRIzol reagent (Invitrogen), according to the manufacturer's protocol, and then further purified using the RNeasy Mini kit from Qiagen. The quantification and quality analysis of RNA was performed on a Bioanalyzer 2100 (Agilent). Synthesis of cDNA and biotinylated cRNA was performed using the Illumina TotalPrep RNA Amplification Kit (Ambion Cat. n. IL1791), according to the manufacturer's protocol. Quality assessment and quantification of cRNAs were performed on Bioanalyzer 2100. Hybridization of cRNAs (20 micrograms) was carried out using Illumina Human 48k gene chips (Human_Ref8_<V2). Array washing was performed using Illumina High-stringency wash buffer for 30 min at 55°C, and followed by staining and scanning according to standard Illumina protocols. Probe intensity data were obtained using the Illumina BeadStudio software, and further processed with Excel software. Probe intensity data were obtained using Illumina BeadStudio 1.5.1.3 software, and further processed with R-Bioconductor and Excel software. Data was normalized according to Rank-Invariant and a filter for detection was applied in order to discard genes with a Detection Score beyond 0.99 in at least one condition. Filtered data were scaled to remove negative expression values from the analysis. Dunnett's T-test metric was exploited to compare the following groups: GAB2-UNS, GFP-SEL, and GAB2-SEL versus the common reference control set on the GFP-UNS sample. A comparison analysis only for selected cells was performed by comparing GAB2-SEL expression profile with GFP-SEL in order to highlight GAB2 target genes specific of anchorage-independent growth. A minimum log(2)ratio of 1 was defined with MSE multiplier. FDR (false discovery rate) was estimated to be <5% according to the median distribution of the test, on 5000 randomly simulated data.