A Role for PVRL4-Driven Cell-Cell Interactions in Tumorigenesis

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Abstract

Deciphering genetic determinants of tumorigenesis is the greatest challenge and promise of the present-day era of biomedical research. As extensive tumor genome characterization efforts of the past decade had revealed, tumor genomes harbor multiple point mutations and gene copy number alterations. This exquisite complexity brings forth the challenge of distinguishing numerous incidental alterations from those that are functionally relevant to tumorigenesis. During the past decade, functional genetic screens have shown their utility in identifying genetic changes that functionally contribute to tumor-specific hallmarks and thus hold a great potential for identifying promising new targets for the rational design of successful anticancer therapies.

A key hallmark of cancer cells is their ability to escape signals that govern homeostasis of normal tissue. In normal epithelia, growth and survival of cells is dictated by their physical anchorage to the extracellular matrix, and disruption of proper cell-matrix anchorage triggers cell death. Tumors of epithelial origin develop ways to subvert anoikis signals, which enables both their uncontrollable expansion at the primary site as well as metastatic colonization of distant organs. Understanding the genetic determinants of matrix-independent growth of cancer cells is a promising approach to identify potent and selective anticancer targets.

In the work presented in this dissertation, we use an unbiased functional genetic screening approach to test a large set of eight thousand human genes to identify those that are involved in inducing and maintaining resistance of mammary epithelial cells to matrix

detachment-induced cell death. We show that a cell adhesion molecule PVRL4 promotes cell survival in the absence of matrix anchorage in normal epithelial cells and in cancer cells. Our work reveals that PVRL4 promotes anchorage-independent growth by promoting cell-to-cell attachment and matrix-independent c-Src activation. PVRL4 is focally and frequently amplified in several types of solid tumors. Growth of orthotopically implanted tumors *in vivo* is inhibited by blocking PVRL4-driven cell-to-cell attachment with monoclonal antibodies, demonstrating a novel strategy for targeted therapy of cancer.

Table of Contents

List of Figures

List of Tables

Credits and Attributions

All experiments were designed by Natalya Pavlova[1] and Stephen J. Elledge[1], and performed by Natalya Pavlova, with the following exceptions:

1. The ORFeome screen for drivers of anchorage independence in Chapter II was designed by Thomas F. Westbrook[2] and performed by Thomas F. Westbrook and Natalya Pavlova.

2. *In vivo* experiments in Chapter IV were performed by Christian Pallasch[3] and Natalya Pavlova.

3. Mass spectrometry analysis in Chapter V was performed by Andrew E.H. Elia[4].

4. A whole-genome loss-of-function genetic screen for suppressors of anchorage independence described in Appendix V was performed by Natalya Pavlova and Laura Sack[1] with the assistance from Hongjung Qu[1] and Anthony Liang[1].

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Chapter I: Introduction

Part 1: The blueprint of an enemy

Cancer is a unique type of a genetic disease

Cancer is a complex and heterogeneous group of proliferative abnormalities which is capable of striking virtually any tissue. The uncontrollable cellular expansion characteristic for cancer occurs in disregard of those mechanisms which normally regiment tissue proliferation, attrition and differentiation. Altogether constituting the malignant phenotype, the hallmarks of cancer are driven by numerous genetic and epigenetic alterations carried by cancer cells. The critical role that these alterations play in tumor initiation and persistence warrants the classification of cancer as a genetic disease.

The unique complexity of cancer as a genetic disease is that it is fueled by **a combination of somatic alterations** which are acquired in a sequential manner over the long course of tumor genesis. This combinatorial nature of cancer's genetic determinants distinguishes it from the other known genetic diseases. Indeed, a genetic disease typically arises as a result of a single genetic aberration. Such aberration may be conveyed by a germline genetic alteration (for example, mutation of CFTR gene in cystic fibrosis [1]), or a somatic genetic alteration (for example, mutation of GNAS1 gene in McCune-Albright syndrome [2]). Furthermore, genetic aberration responsible for the disease pathology phenotype may affect not one gene but a contiguous stretch of genes - such as in case of Prader-Willi syndrome [3] or various trisomy disorders, e.g. Down syndrome [4].

The pattern of genetic alterations seen in cancer is orders of magnitude more complex in that not a single alteration, but a **combination of alterations** scattered throughout the genome of

a somatic cell is causatively involved in tumorigenesis. These alterations include several types such as mutations, gene copy number aberrations and chromosomal translocations, and are being acquired by affected cells over the long course of tumor genesis, punctuated by bursts of clonal expansion as a consequence to the acquisition of a genetic alteration that incites unchecked survival and proliferation. Alterations to a cell's genome that are deemed responsible for promoting malignant behavior are referred to as **driver alterations** or drivers. Driver alterations are further subdivided into two major classes with regard to their effect on gene function: alterations that result in a gain-of-function phenotype, represented by activation of oncogenes, and those that trigger a loss-of-function, represented by inactivation of tumor suppressors.

A do-it-yourself manual or a Voynich manuscript?

The understanding that acquired mutations are the driving force of tumorigenesis predates the era of molecular biology. The presence of **profoundly abnormal genetic material** in form of aberrant mitotic figures and of individual chromosomal breakages was first noted and characterized by Theodor Boveri in 1914 [5], who was first to suggest that genetic abnormalities are the driving force of tumorigenesis first to predict the clinical utility of DNA-damaging treatments in eradicating cancer. In 1960, the discovery that two patients with chronic myelogenous leukemia (CML) shared the identical, abnormally small chromosome - became known as a **Philadelphia chromosome** - was the first demonstration that malignancies from different individuals can share, and potentially be driven by, the same genetic aberration [6].

Further supporting a causative link between mutagenesis and tumorigenesis, a wealth of evidence linking the exposure to **mutagenic agents** to increased cancer incidence has been accumulated by the 1950s. Despite the observed correlation, it was nonetheless clear that mutagenesis by itself could not be equated to carcinogenesis. As has been first demonstrated by

Katsusabiro Yamagiva in 1915, repeated application of coal tar to ears of rabbits caused squamous cell carcinoma with a 100% penetrance [7]. At the same time, a single event of an exposure to a strong mutagen failed to initiate carcinogenesis in animals no matter the dose [8]. Similarly, there was a noted delay between the carcinogen exposure and onset of cancer in humans, such as a 5-year delay in the onset of leukemias and a 10-year delay in the onset of solid tumors seen in the aftermath of atomic bombings of Hiroshima and Nagasaki [9].

Even in cases that could not be clearly traced to carcinogen exposure, a marked increase of cancer incidence with age was observed. In 1956, Carl O. Nordling estimated from the rate of increase in cancer diagnoses with age that it may require, on average, seven mutations in order for normal cell to develop into a malignant tumor [10]. More recent estimates suggested that for a solid tumor, five to ten genetic 'hits' may be needed in order for a normal cell to progress to clinically detectable cancer [11]. In agreement with these estimates, a pattern of a progression through a series of four to six, histopathologically distinct, stages has been documented - first, for colorectal carcinoma [12] and later, for other tumor types. Taken together, epidemiology and histopathology of human cancers provided further support to a multistep theory of tumor progression.

Supported by sound correlative evidence, the idea of cancer as a genetic disease, however, lacked veritable experimental proof. The discovery of first viral oncogene, *v-src* from Rous sarcoma virus (RSV), followed by its cellular analog, *c-src*, had all but revolutionized the understanding of tumorigenesis. For decades, the RSV tumor isolate was known of turning normal chicken fibroblasts into cells with characteristic sarcomatous morphology and behavior, thus inducing a readily identifiable phenotypic change, aptly named **transformation** [13]. In 1976, aided by the discovery of reverse transcriptase and development of subtractive

hybridization technique, the cDNA copies of RSV-encoded sequences responsible for transforming normal fibroblasts into sarcoma-like cells has been, unexpectedly, found to hybridize with the normal genomic DNA of several bird species [14]. As Peter Vogt reminisces, the publication describing the finding of a human gene inside a viral genome has been, at first, ridiculed by the research community [15] – and yet soon the cancer-inducing power of other tumor viruses was similarly found to be attributed to sequences that were 'borrowed' from the genomes of their respective hosts. Up to a 40 other viral oncogenes as well as their cellular counterparts were discovered from a variety of transmissible avian and rodent tumors [16, 17], as well as through transfection of genomic DNA of human tumors of non-viral genesis [18, 19]. Taken together, these discoveries provided strong empirical proof of not only cancers harboring aberrant versions of human genes, but also that non-cancerous cells can become transformed by introducing such aberrant genes exogenously.

Importantly, introduction of a single oncogene, such as Ras, led to transformation of immortalized fibroblasts, such as the NIH-3T3 line, but insufficient for transformation of primary cells [20], whereas **a combination of two oncogenic events**, namely, Ras and Myc, readily transformed primary embryo fibroblasts [21]. Similarly, in genetically engineered mouse models of cancer, germline expression of Ras only rarely gave rise to tumors, but did so with a markedly increased penetrance when combined with Myc [22]. Aided by the development of targeted gene deletion technology, a variety of mouse models of tumorigenesis have been created (reviewed in [23]). Importantly, the timing of tumor onset, clonality of tumors as well as recapitulation of a multistep character of cancer progression indicates that for a tumor to form, sporadic acquisition of extra genetic perturbations is necessary.

In addition to oncogene discovery via fibroblast transformation assay, methods of cytogenetic analysis and comparative genomic hybridization were instrumental for identification of recurrent genetic deletions, amplifications and translocations in human tumors. In particular, profiling of allelic deletions in tumors and adjacent normal tissues via restriction fragment length polymorphism analysis has been instrumental for the discovery of frequent losses on chromosomes 5, 17 and 18 in colorectal cancer, paving the way to the discovery of tumor suppressors APC, p53 and DCC, respectively [24]. Furthermore, implementation of a PCR-based representational difference analysis assay made possible cloning of key tumor suppressors PTEN [25, 26] and BRCA2 [27, 28]. In 2004, a **census of cancer genes** has been compiled, into which 291 genes with casual implications in tumorigenesis have been included [29].

Two events prompted the explosion of knowledge about genomic alterations harbored by tumors: the completion of the human genome sequence in 2001 [30, 31] and technological advances in microarray hybridization and DNA sequencing, the latter including the development of automated Sanger sequencing, and more recently, invention of massively parallel ("next-generation") sequencing technology. With the first studies reporting on the systematic exome resequencing and studies performing high-resolution copy number variation analyses of tumor genomes allowed appreciating the extent of the alterations suffered by individual tumor genomes.

The data from cancer genome analyses has revealed that the load of genomic alterations carried by tumors is strikingly complex. In particular, numbers of somatic substitutions ranged from 1,000 to 10,000 per tumor. The somatic substitution count in those types of cancers that arise as a result of mutagen exposure, such as melanoma or smoking-related lung cancer, is an order of magnitude higher, reaching up to 100,000 substitutions [32]. Furthermore, catalogues of

tumor-specific copy number alterations reveal extensive amounts of deletions and amplifications of chromosomal regions of varying lengths. For example, across recently characterized lung cancer genomes, 26 out of 39 autosomal arms have been affected by large-scale copy number gains or losses, but only 6 out of 31 copy number alterations that were recurrently identified had genes in them that were known to be involved in driving tumorigenesis [33].

It is conceivable that the vast majority of genes altered in cancers likely bear no consequence on tumorigenesis as they are merely a byproduct of a decade-long shaping of a clinically detectable tumor and of catastrophically unstable genomes of cancer cells. A large fraction of alterations can therefore be regarded as **passenger alterations**. The task of separating drivers from passengers appears to be especially complex when copy number alterations are considered, as they more often than not span large chromosomal areas and encompass multiple of genes. To narrow down the spectrum of genes recurrently affected by copy number alterations, statistical methods have been developed. Thus, in a comprehensive study of 3,131 tumors of various lineages, 76 focal amplification peak regions and 82 focal deletion peak regions, containing a median of 6.5 and 7 genes, respectively, have been identified through a statistical approach termed GISTIC [34].

Importantly, recent insights into functional significance of individual genes within chromosomal regions recurrently affected by copy number gain or loss in tumors reveal that copy number alterations may benefit tumor growth in a manner that is driven not by a single gene embedded within an amplified or hemizygously deleted region, but rather, by **a cumulative effect of multiple genes**. For example, functional interrogation of a frequently amplified in hepatocellular carcinoma 11q13.3 chromosomal region revealed that in addition to a known oncogene CCND1, another gene within that region, FGF19, also strongly contributed to

transformation [35]. Furthermore, genes that negatively affected proliferation rates of immortalized human mammary epithelial cells were found to be statistically overrepresented inside chromosomal regions which are recurrently deleted in cancer, elegantly demonstrating that hemizygous deletions are likely to be targeting not one gene, but rather, blocks of aptly named "STOP" genes, thus having a cumulative effect on the cancer cell's fitness [36]. As a result, such polygenic alterations are likely to be selected for due to their cumulative effect on a proliferative potential of cancer cells. In further agreement with this demonstration, four genes within a recurrently deleted 8p22 were found to have an additive tumor-promoting effect [37]. Taken together, this evidence strongly suggests that genetic alterations selected during the process of tumorigenesis may have a higher order of complexity than has been previously appreciated.

Cancer in time and space – new dimensions to genetic complexity

As a consequence of a high proliferative capacity of cancer cells and their marked genome instability, novel genomic alterations are continuously acquired and those of functionally advantageous consequence to a tumor are being selected for. An important complication in isolating driver alterations that stems from this is that a profile of genomic alterations harbored by a tumor at the time when a biopsy is performed reflects a history of all alterations suffered by it. Thus, some alterations that once were essential for tumor initiation may have become less important at a later stage of tumorigenesis. In other words, a driver alteration responsible for placing the cell on the road to eventual cancer development could become later substituted by **alterations that are analogous in function** and yet convey a pro-tumorigenic advantage more strongly. In support of that, tumors often harbor multiple alterations converging upon the same pathway, such as those exemplified by the co-existence of PTEN loss or mutations in PIK3CA with the amplification of HER2 in breast cancer. Another such example is

overexpression of EGFR in early stages of pancreatic interstitial neoplasia, which has been shown to be required for a $KRAS^{V12}$-dependent progression to PDAC but is dispensable at the invasive stage, which is when pancreatic tumors are diagnosed [38, 39]. Thus, the information about how and when particular alterations are acquired and which ones are actively contributing to the tumor as a clinically dangerous entity may be missed by whole-genome characterizations of clinically advanced tumors; however, attempts such as ultra-deep sequencing of cancer genomes to trace the clonal evolution of a tumor demonstrate ways of how this problem may be circumvented [40].

In-depth characterization of individual tumor genomes has brought the issue of **tumor heterogeneity** into a sharp focus, revealing that within the confines of a single tumor, multiple subpopulations may be coexisting, each harboring distinct genetic alterations. A prominent demonstration of such spatial heterogeneity is a recent analysis of twelve biopsies of the same primary pancreatic tumor which were sequenced along with seven metastases of various organ tropisms, all spawned by the primary tumor. The comparison of mutation spectra harbored by these samples revealed that even though metastases contained some mutations that were not uniformly present throughout the bulk of a primary tumor, they still existed within a primary tumor as minor subpopulations [41]. In another striking example, a brain tumor composed of three subpopulations derived from the common ancestor – each one bearing a high grade amplification of a distinct receptor tyrosine kinase EGFR, MET and PDGFRA – has been identified [42]. Since most of the cancer genome characterization studies are performed using a single biopsy specimen as a source of genetic information, such studies may fall short of capturing the full spectrum of tumor-specific driver alterations, especially those involved in enabling metastatic colonization.

In conclusion, whole-genome characterizations of cancer-specific alterations reveal that large and complex sets of gain- and loss-of-function alterations are accumulated by cancer genomes over the course of disease progression. Some of these alterations are purely incidental; while others act in concert to bestow the malignant phenotype upon the affected cell. In addition to functional consequences of alterations to gene-containing regions of the genome, which only account for 1% of the human genome, massively parallel sequencing will undoubtedly reveal tumor-specific alterations in non-coding RNAs, some which had been already demonstrated to profoundly affect tumorigenesis. Furthermore, tumor-specific alterations of the epigenetic landscape of a cell add a whole other layer of complexity to the picture. The altered genomic milieu of cancer cells elicits profound changes in transcriptional and translational profiles, posttranslational modifications and rates of protein degradation, altogether contributing to tumor expansion. Finally, germline polymorphisms, as well as aspects of the whole-body physiology, such as obesity and chronic inflammation, have a likely strong and as of now underappreciated influence on induction and maintenance of the malignant state as well.

With deeper and more extensive sequencing and copy number analyses underway, the exhaustive catalogue of all alterations seen in human cancer will be completed in the foreseeable future. In addition to providing extensive characterization of cancer landscapes, tumor sequencing has revealed that the spectra of cancer-driving alterations are more complex than has been previously appreciated. All things considered, the task of interpreting cancer genomic data, deciphering molecular networks which fuel tumorigenesis and isolating its weaknesses, which could be exploited for a new generation of cancer therapeutics, remains far from being completed.

Precision warfare

The goal of anticancer therapies is to selectively eradicate malignant cells while sparing the healthy tissue. Prior to the discovery of oncogenes, successful cancer therapies were guided by **clinically evident aspects of cancer pathology**. For instance, use of classic cytotoxic treatments, such as gamma-irradiation, DNA alkylating agents and mitotic poisons, was based on the difference in the extent of the deleterious effect they had on rapidly dividing cell types compared to slowly dividing or non-dividing majority of cells of the body. In another instance, the discovery of sex steroids and their role in driving proliferation of reproductive tissues led to the development of hormone-ablation as a therapy for breast cancer – first by oophroectomy, and since 1970s, by specific estrogen receptor antagonists [43]. In yet another classical example of pathophysiology guiding therapy, low levels of folic acid in children with acute lymphoblastic leukemia led Sidney Farber to propose that leukemia cells may be exquisitely reliant on folic acid [44]. The design and implementation of antifolate therapy led to remarkable remissions, predating the discovery of its target, dihydrofolate reductase (DHFR) and the role played in producing intermediates for nucleotide synthesis, in high demand for leukemic blasts [45].

The discovery of the genetic basis of tumorigenesis had revolutionized the understanding of the tumorigenic process on a molecular level. Cancer-specific genetic alterations, while influencing activities of individual proteins, were demonstrated to profoundly affect pathways and networks which are implicated in fueling various aspects of cancer cell's aberrant physiology, as well as the physiology of its surrounding microenvironment. The demonstration that malignant behavior is driven by alterations to individual components of growth and survival pathways via mutation or a change in a level of expression has made targeting these pathways a priority. Multiple **oncogene-targeted drugs** have shown their efficacy in a clinical setting. Thus,

with less than a two-decade delay after the discovery of the genetic identity of the Philadelphia chromosome [46], a staggering success of imatinib (Gleevec, Novartis), the inhibitor of c-Abl kinase, in producing stable remissions in 90% of CML patients with BCR-ABL fusions has been achieved [47]. In the same year that the report of a remarkable efficacy of Gleevec in CML was released, use of a humanized monoclonal antibody trastuzumab (Herceptin, Genentech) in HER2-positive metastatic breast cancer patients became another targeted therapy success story [48].

These clinical successes described above provided a proof-of-principle to the concept of **oncogene addiction** [49]. This principle states that tumors become exquisitely dependent on driver alterations that fuel their hyperactive pro-growth and pro-survival signaling. Such oncogene dependencies therefore allow a unique window of opportunity for a rationally designed therapeutic which is exclusively targeted against cancer cells. This principle has been validated by studies in genetically engineered animals, where oncogene-driven tumors (*c-Myc* [50, 51], BCR-ABL [52] and Ras [53]) were all but extinguished by an acute genetic shutdown of oncogene expression. In addition, restoration of p53 in a genetically engineered mouse model of lung cancer provided yet another dramatic demonstration of the addiction principle [54], thus extending the addiction principle to the loss-of-function alterations as well.

However, even when a dominant oncogene is clearly present, incomplete responses, as well as development of drug resistance, is often seen in clinic. One explanation of the difference between animal models of oncogene addiction and cancer in humans is that in animal models, introduction of potent driver alterations - such as activated Ras allele and loss of p53 – bypasses the tortuous process of shaping tumor-specific networks, accumulation of layers of redundancies among network activators and allowing fine-tuning of negative and positive feedback loops,

driving a remarkable resilience of tumors to chemicals that target individual prosurvival and proliferative signaling pathways. Furthermore, presence of substantial intratumoral heterogeneity allows the outgrowth of drug-resistant subclones, and a variety of molecular mechanisms for development of resistance to specific therapies has been described [55].

Another challenge to the targeted therapy paradigm is that dominant driver alterations have only been identified for a fraction of tumor subtypes, while for some others **no clear driver oncogenes** have been assigned. For example, in the most recent whole-genome characterization of 183 cases of lung adenocarcinoma, only 47% of tumors contained mutations in a known driver oncogene, and only 55% had amplifications of known proliferation-sustaining genes [56]. Therefore, for half of the cases a potential therapeutic target cannot be readily identified. Moreover, whole classes of tumors, such as triple-negative breast cancers, pancreatic cancers and glioblastomas, essentially lack known targets that are easily druggable. Additional tumor genomes may need to be sequenced to increase the statistical power of discovery of recurrent driver alterations. In addition, the lack of recurrently identified oncogenic perturbations in some cancers also suggests that whereas some cancers may be fueled by clearly identifiable dominant oncogenes, such as HER2 amplification in 30% of breast cancers, others may be instead driven by highly redundant networks of alterations - and may be, ostensibly, harder to treat.

In conclusion, besides rare exceptions, targeted therapies in cancer are not curative and more often than not produce only transient remissions in patients, with the inevitable emergence of a drug-resistant tumor. Thus, a new anticancer drug that receive FDA approval may be prolonging the patient median survival by a measure of two months [57]. Alarmingly, phase II trial success rates for novel therapeutics dropped from 28% in 2006-2007 to 18% in 2008-2010 [58]. A more comprehensive understanding of how cancer-specific genetic alterations are fueling

various aspects of aberrant cellular physiology and a search for novel therapeutic targets, as well as the development of novel therapeutic strategies against presently "non-druggable" classes of targets, are urgently needed.

How to spot a perpetrator in a genetic lineup

The expected benefit of a comprehensive characterization of cancer genomes lies in providing a complete map of gain- and loss-of-function alterations that are responsible for cancer being cancer. Identifying those genetic elements that bear functional significance to a cancer cell can then be used to develop novel targeted therapeutics and combination therapeutic strategies. However, as has been discussed above, the problem of separating driver events from passenger alterations severely complicates this task. Moreover, up to 40% of genes in the human genome have no known function and many more have been only cursorily described [59]. As a result, assessment of their potential for functional contribution to cancer is challenging. Finally, some clinically successful treatments, such as proteasome inhibitor bortezomib (Velcade) are targeted towards proteins and pathways that are not altered in cancer themselves, instead being components of stress-response networks upon which cancer cells become dependent as a consequence of harboring certain oncogenic alterations. These potential targets will be altogether missed by mining cancer genome alone.

In support of these concerns, even with significant efforts directed towards tumor genome characterization, the discovery of clinically-validated therapeutic targets through this approach alone, has been infrequent. Notable exceptions include a discovery of transforming EML4-ALK fusions in a subset of lung carcinomas [60], which led to a rapid adoption and successful application in clinic of an ALK-specific inhibitor crizotinib (Xalkori) [61-63]. Another prominent example of exome-sequencing-led discovery was identification of gain-of-function

mutations in catalytic domains of IDH1 and 2 proteins (isocitrate dehydrogenase) in a large subset of glioblastomas, and the development of specific small molecule inhibitors is currently underway [64].

To address the functional validation problem, several function discovery-oriented approaches have been developed and displayed their utility in isolating critical genetic alterations that directly contribute to the various aspects of tumor biology. Two of the most prominent among such approaches are (a) **comparative oncogenomics** in genetically engineered mouse models of cancer and (b) *in vitro* and *in vivo* **genetic screening** approaches.

Comparative oncogenomics takes advantage of genetically engineered mouse models of cancer, profiling tumor-specific copy number alterations to identify those that foster the formation of either a primary tumor [65] or acquisition of metastatic ability [66] in the context of specific driver oncogenes. Some drawbacks of this approach are that it does not faithfully model the sequential mode of acquisition of individual oncogenic lesions and the extent of genomic instability seen in human cancer. Engineering mice which replicate the extent of genomic instability which is seen in human tumors has shown a potential to overcome this problem [67].

Genetic screening approaches, on the other hand, allows a direct assessment of hyper- (or hypo-) active states of individual gene products in a setting that models cancer-specific phenotypes either *in vitro* or *in vivo.* Two prominent screening technologies that are applied to identify causative perturbations in cancer include (i) **insertional mutagenesis screens** and (ii) **library-based screens**. As an example, insertional mutagenesis screens, such as one based on the action of Sleeping Beauty transposase has been successfully used to identify novel genes that, when disrupted, contribute to the colorectal cancer initiation [68].

Among functional genomics approaches aimed at profiling cancer-relevant genes, library-based screens had arguably been the most widely used, having been adapted to the assessment of a wide range of cancer-specific phenotypes. It was the advent of new methods of constructing large-scale libraries of genetic elements, which include gain-of-function libraries of open reading frames, and RNAi-based loss-of-function libraries, that opened new opportunities to address what just two decades ago seemed to be an insurmountable task in cancer biology.

Functional genetics in cultured cells becomes a reality

Until recently, functional genetics has been the realm of researchers who worked with model organisms in which sexual reproduction was possible - and feasible - such as *S. cerevisiae*, *D. melanogaster* and *C. elegans*. In *S. cerevisiae* in particular, performing targeted gene deletions has made reverse genetics a tool of great utility. Cultured mammalian cells were challenging for use as a geneticist's tool due to their somatic nature, which complicated the task of positional cloning of genes.

When the first oncogenes were discovered, not only the conceptual understanding of cancer was enriched, but so was **the arsenal of cancer biology methods**. First, studies of avian and rodent transmissible oncogenes provided a set of assays by which to identify and measure cancer phenotypes in cultured tissue. Most notable of these assays were **methylcellulose colony formation assay** [69] and **contact inhibition assay** [70]. In the latter, chicken fibroblasts infected with a single copy of RSV were found to acquire a capability of increasing cell numbers even when grown in a fully confluent monolayer culture, forming macroscopically visible foci of multiple layers of small rounded cells. Use of contact inhibition assay in NIH-3T3 line further led to the phenotype-driven discovery of other oncogenes, including *neu* and *ras* [18, 19] from the genomic DNA of human tumors of non-viral origin.

Another important contribution of cancer virology to the emerging field of mammalian cell genetics was that oncogene-carrying viruses became prototype for designing **retrovirus-based cDNA expression vectors** [71]. Similarly to transfectable plasmids, viral genome sequences were encoded on a plasmid that contained a bacterial origin of replication and therefore could be propagated in *E. coli*. However, retrovirus-based vectors had several **advantages over transfectable plasmids** as they (a) allowed introducing a gene of choice into virtually any cell line expressing a suitable receptor, (b) could be stably integrated into mammalian cell genome, which allowed stable expression of genes and assessment of a potential phenotype over an extended period of time, (c) could be integrated into the host genome at a single copy per cell, allowing gene expression levels to be closer to those seen in the context of normal cell physiology.

In addition to studying the action of individual genes at a level of expression which more closely replicate the physiological expression levels than a plasmid transfection, another advantage of a single-copy mode of integration of virus into cell genome was that they permitted introduction of **complex expression libraries** of cDNAs into cultured cells [72]. Infections with such libraries can be performed in a manner so that a complex mix of clonal cell populations – in which a single cell carried just one copy of a virus and therefore just one cDNA - are created. The resulting library-infected populations of cells can then be assayed in a pooled, massively parallel format, with a potential of using a wide variety of biological phenotypes as a potential readout. Genetic screens with tumor-derived cDNA libraries had soon proven their superiority in discovery of novel cancer-relevant genes. For example, a NIH-3T3 focus formation assay screen with a neuroblastoma cDNA library yielded three previously described oncogenes as well as nine previously unknown ones [73]. In a set of two further examples, screens for genes conferring

resistance to *c-myc*-induced apoptosis and to TGFβ-induced growth arrest identified Twist1 [74] and Mdm2 [75], respectively.

A drawback of using cDNA libraries as a screening tool is that they only represent a snapshot of a cell's transcriptome at a time point at which cDNA is obtained. Since any given cell has only a fraction of its mRNAs expressed, and the relative abundance of cDNAs in the library is reflective of the levels of expression in the cell from which the library is obtained, the coverage of such type of library is far from comprehensive and highly expressed transcripts are overrepresented in it. The sequencing of human and mouse genome has simplified the task of cloning of individual genes, making possible the creation of a new generation of expression libraries of open reading frames (ORFs). Thus, creation of the ORFeome library, encompassing 8,000 human open reading frames in 2004 [76] and expanded to over 16,000 ORFs in 2011 [77] became an important step forward towards creating a whole-genome tool for performing gain-of-function genetic screens in mammalian systems.

Last but not least, the discovery of RNA interference (RNAi) [78] fostered the creation of loss-of-function libraries – either in an arrayed format of transfectable siRNAs for performing well-by-well screens or in a format of retrovirus-encoded short hairpin RNA sequences (shRNAs) for stable expression in cell populations [79-81]. RNAi libraries allowed efficient downregulation of individual genes in genomes of mammalian cells, providing highly effective means of conducting loss-of-function screens in mammalian cells.

Logical frameworks are guiding the design of genetic screens

Newly available tools of functional genomics made possible a high-throughput, unbiased assessment of relative functional consequences of hyper- or hypoactivation of individual mammalian genes. In the past decade, mammalian genetic screens have proven their utility as a

tool of systematic interrogation of a wide range of phenotypes pertaining to cancer cells. The versatility of library-based genetic screening technology is arguably one of its key advantages. Indeed, virus-encoded libraries can be introduced into a wide spectrum of cell lines which can be pre-modified with additional genetic elements and exposed to a variety of conditions. Furthermore, diverse cancer-relevant phenotypes can be assayed in cultured cells *in vitro*. The variety of cancer-specific hallmarks and the obvious complexity of networks that contribute to the establishment and maintenance of the tumorigenic state highlight the importance of using rigorous logic when designing genetic screens. Several of such frameworks could be isolated among those emerged in the past decade, all successfully adapted to use with the genetic screening technology.

A majority of recurrent perturbations seen in tumors drive the aberrant flux though pathways that stimulate proliferation and most of currently approved targeted therapeutics are inhibitors of such pathways. Genetic screens can be readily used for a systematic profiling of genes for their relative contribution to **cell proliferation** rates. Both pooled [36, 82-84] and well-by-well approaches [85] have been implemented to systematically assess cell proliferation. *In vivo* screens for regulators of xenograft growth in immunocompromised mice have also been carried out. The limitation faced with *in vivo* screens is the sizes of libraries that are needed to interrogate whole genomes - especially sizes of shRNA libraries. However, separation of libraries into sub-pools [86], use of focused libraries that target genes of specific function [87] or genes that are frequently altered in cancer [88] can be used to circumvent this problem.

Another important application of *in vitro* genetic screens is **modeling the emergence of resistant states** of tumors to targeted therapies. Screens that address the problem of resistance of cancer cells to therapeutics such as anti-HER2 antibody trastuzumab (Herceptin) [89] and BRAF

inhibitor vemurafenib (Zelboraf) [90] are examples of this approach. The limitation of genetic screening as a tool for the discovery of drivers of drug resistance is that only those resistance mechanisms driven by a single genetic change can be discovered. Nevertheless, insights gained from these types of screens can help designing combinatorial therapy approaches for a preventative targeting of both the original oncogene as well as the likely resistance mechanism.

A majority of targeted therapies approved for use in clinic are designed to target drivers of abnormally fast cellular proliferation. However, alternative approaches to target selection are gaining ground as well. Such alternative approaches address the existence of cancer-specific phenotypes other than abnormal proliferative index and could be further subdivided into (a) those targeting unique tumor-specific **stress states**, created as a direct consequence to the genetic aberrations carried by tumors and (b) those designed to reinstate the **normal cellular/tissue homeostasis** through inhibiting tumor-specific adaptations developed to circumvent it or via recruiting existing homeostatic mechanisms.

Existence of a set of distinct **stress states** that tumors are burdened with to a greater extent than normal tissues is the foundation of the principle of **non-oncogene addiction** [91]. Several distinct types of stresses – including genomic instability, as well as mitotic, proteotoxic, metabolic and oxidative stresses – are experienced by cancer cells as a result of acquired genetic alterations and cell physiology changes stemming from these alterations. The presence of such vulnerabilities, or at a more likely scenario, the extent of such vulnerabilities creates a unique therapeutic window with a great potential for specificity, as targeted therapeutics designed to inhibit appropriate stress relief pathways will preferentially affect cancer cells over normal cells. Importantly, stress response pathways themselves may not themselves be genetically perturbed in tumors and therefore will be altogether missed by cancer genome data mining alone.

Examples of the therapeutics that are successfully employing this principle are a agents disrupting protein folding (HSP90 inhibitor geldanamycin [92]), protein degradation (proteasome inhibitor bortezomib [93]) and DNA repair in BRCA-deficient tumors (PARP inhibitor olaparib [94]). The principle of non-oncogene addiction is readily applicable to functional interrogation with library-based screens and several screens aimed at isolating vulnerability points that are specific to various oncogenes have been successful in identifying novel synthetically lethal interactions [95-97].

Cancer develops from normal cells which, via genetic and epigenetic alterations, lose sensitivity to **cellular and tissue homeostasis signals**. These include intrinsic signals, such as entry into a senescent state in response to oncogene activation, as well as diverse extrinsic signals. Extrinsic signals may be specific to the tissue of origin, such as differentiation-inducing action of retinoic acid in neurons or programmed cell death triggered by the detachment from the basement membrane in epithelia, which will be discussed in detail in the next section. A distinct set of hallmarks involves acquisition of privileges that are normally reserved for other cell types, or other developmental stages. These examples include (a) epithelial-to-mesenchymal transition, which correlates with metastatic phenotype in epithelial cancers and allows cells to develop abilities to modify and invade the surrounding environment, and (b) heightened ability to induce *de novo* growth of blood capillaries.

Genetic determinants of various aspects of cancer cells' faulty homeostasis can be readily interrogated via *in vitro* genetic screening. Successful genetic screens for determinants of phenotypes such as matrix anchorage-independent growth [98-101], changes in balance between stemness and differentiation [102, 103], bypass of cellular senescence [104, 105], as well as cell motility and invasiveness [106, 107] have been performed, representing examples of how

library-based screens can be used to interrogate the genetic basis of corrupt homeostasis mechanisms.

In conclusion, gain- and loss-of-function genetic screening approaches allow deciphering genetic determinants of a wide spectrum of abnormal behaviors characteristic for tumors. Furthermore, application of a target discovery-minded logic to the design of genetic screens allows not only evaluating contributions (or lack thereof) of individual gene products to cancer, but isolating potential drug targets. Combining genetic screening data with evidence from recurrent cancer-specific genetic alterations further fosters development of novel therapeutic ideas. Finally, the integration of knowledge produced by independent logical frameworks can assist the development of combinatorial therapies that approach the problem of eradicating cancer from different angles and may be have a greater success in clinic in the long run.

Part 2: Adherence to principles

The epithelium – matrix connection: the declaration of dependence

Epithelia are found in a wide variety of organs where they provide inner lining of hollow structures and carry out a variety of secretory, barrier or mixed functions specific to the demands of a site of residence. A distinct characteristic of epithelia is their unique two-dimensional pattern of growth in a form of organized monolayers. Even though epithelia account for a meager 1% of total mass of tissue in the human body, they give rise to a disproportionately large fraction of adult tumors. Thus, in 2012, 82% of all adult cancers diagnosed in the United States were of epithelial origin; furthermore, epithelial cancers accounted for 77% of cancer-related deaths [108]. Cancers that arise from epithelial tissues are termed **carcinomas**. Carcinomas are further subdivided into **adenocarcinomas** – those arising from cells involved in secretory functions, such those residing in lumens of breast, prostate and stomach, and **squamous cell carcinomas**, which originate from epithelia serving the barrier function, such as epithelial layers of skin, oral cavity and uterine cervix.

The functions served by epithelial tissues in the body require a strict maintenance of the geometrically constrained pattern of growth and a prompt removal of cells that fail to conform to that pattern. This, in turn, requires a set of exquisitely complex quality-control mechanisms which serve to maintain tissue homeostasis. Several important growth constraints are being imparted upon epithelia, altogether enforcing the maintenance of monolayer pattern. First, survival and proliferation of epithelial cells is dictated by their **physical anchorage on the basement membrane**, an organizing acellular substratum composed of a mix of proteglycans, such as heparan sulfate, and extracellular matrix (ECM) molecules, such as fibronectin, vitronectin, laminin and various types of collagen [109]. Physical attachment to ECM is carried

out through a family of integrin receptors, activation of which is dependent on their ligation to specific components of ECM. In addition to inducing activating conformational changes in individual integrin molecules, adhesion to a contiguous stretch of matrix induces clustering of individual activated integrins, thereby enhancing the avidity of binding and the strength of the intracellular signal [110]. When activated, integrins influence intracellular signaling [111]. Diverse intracellular signaling cascades downstream of integrins are triggered via ECM-dependent recruitment of a number of integrin-associated tyrosine kinases such as $p125^{FAK}$ [112], ILK [113], as well as members of Src family of kinases [114]. A variety of downstream pathways become activated upon integrin-ECM ligation (reviewed in [115]), including those affecting proliferation (such as MAPK [116]), survival (such as PI3K [117]) and cytoskeletal remodeling (such as Rho, Rac and Cdc42 [118]). In addition, some integrins directly associate with receptor tyrosine kinases and thereby enhance growth factor-dependent signaling as well [119]. Specific integrins display selectivity in their affinity to particular ECM molecules, which ensures that not only a complete ECM deprivation, but also alterations to the molecular composition of ECM can profoundly affect integrin-dependent signaling. Taken together, dependence of epithelial cells on ECM-mediated signaling ensures their growth as monolayers and prevents improper colonization of other microenvironments.

Second, the monolayer growth pattern is enforced by the requirement for individual epithelial cells for having a particular **geometric shape**. In a set of elegant experiments, this was demonstrated by seeding cells onto ECM-coated micropatterned islands. Remarkably, cellular fate was determined by the number of islands that each cell was stretched across, and not by the cumulative area of ECM that a cell was occupying [120]. Mechanistically, changes in geometric shape of cell were shown to affect activation of RhoA and modulate protein levels of $p27^{Kip1}$

CDK inhibitor, thus directly governing proliferation [121]. Thus, changes in cell shape following complete or partial loss of anchorage can affect cellular proliferation independently from integrin engagement.

Third, besides providing the physical substratum enabling cell spreading and specific integrin engagement, heparin and heparin sulfate proteoglycans within ECM bind and accumulate a variety of growth factors, making them available to epithelial cells, and as a result, stimulating pro-growth and survival signaling [122]. Such **capture and accumulation of growth factor molecules** further ensures a close association of epithelial cells with the underlying matrix. In addition, epithelial cells are forced to stay in close proximity to the basement membrane due to the avascular nature of epithelia, which makes them dependent on **diffusion of nutrients**, oxygen and hormones across the basement membrane.

A stark sense of detachment

As summarized above, growth and survival of epithelial cells is governed by interactions with the ECM. The requirement for *in vitro* growth of epithelium-derived cells on glass or treated plastic substratum has been described in 1968 and given the term **anchorage dependence** [69]. Importantly, tumor-derived cell lines or cell lines transformed *in vitro* by oncogenic viruses or chemical carcinogens[123] did not have a similar substratum attachment dependency, thus they were characterized as having had acquired **anchorage independence**. Solidifying the functional link between anchorage independence and tumorigenesis, a cell line's ability to proliferate in the absence of anchorage was demonstrated to be the best predictor of an ability to grow in a form of xenografts in immunocompromised mice when compared to other cancer-specific phenotypes – among these, resistance to serum deprivation, contact inhibition or

presence of chromosomal aberrations [124, 125]. In addition, anchorage independence correlated with the metastatic character of cancer cell lines derived from patients [126].

However, it was not until 1994 when two groups independently discovered that cell death in the absence of attachment is not merely a process of passive cell attrition, but is, in fact, a type of a programmed cell death [127, 128]. Indeed, in a manner similar to apoptosis, substratum-deprived cells were shown to display the characteristic nuclear morphology and fragmentation of genomic DNA. The apoptosis-like response to substratum deprivation in cultured normal epithelial cells was termed ***anoikis***, or "homelessness", derived from the Greek *an-* (lack of) and *oikos* (home). Furthermore, ectopic expression of a known anti-apoptotic protein Bcl-2 potently inhibited the execution of anoikis. Similarly, ectopic expression of viral oncogenes such as *v-Src* and *v-Ha-Ras*, prevented anoikis, providing a direct demonstration of how anoikis can be circumvented by cancer cells.

Further insights into signaling events that are associated with the execution of the anoikis program have demonstrated that it consists of intrinsic and extrinsic components (reviewed in [129]). Thus, the **intrinsic anoikis pathway** is driven by the pro-apoptotic protein Bim, which triggers cytochrome *c* release from mitochondria and activates the caspase cascade. As a consequence of ECM detachment and concomitant cytoskeletal remodeling, Bim becomes displaced from the cytoskeleton, where it is normally sequestered. In addition, disengagement from ECM results in accumulation of total Bim consequential to the reduced flux through PI3K and MAPK pathways, which normally phosphorylate Bim and target it for proteasomal degradation. **Extrinsic anoikis pathway** is carried out through increased expression of Fas and TNF receptor as well as their respective ligands, also contributing to the initiation of the caspase cascade.

The **Hippo/YAP pathway** has recently emerged as a novel sensor of cell anchorage. Specifically, loss of ECM attachment and concomitant changes in cytoskeletal arrangement has been shown to trigger YAP protein inactivation and cytoplasmic retention, thus inhibiting Hippo pathway-driven transcription of a number of pro-growth and survival genes [130].

Another prominent consequence of ECM deprivation, in addition to abrogation of proliferative and survival signaling and initiation of cell death program is a profound **defect in cellular metabolism** [131]. In particular, ECM-deprived mammary epithelial cells had profound defects in glucose uptake, fatty acid oxidation and production of antioxidants through pentose phosphate pathway, all of which were rescued by expressing a breast cancer oncogene HER2 or by exogenous antioxidant supplementation. As a result, metabolic deficiencies contribute to cessation of cell proliferation, cooperating with other anoikis mechanisms. Taken together, the cell death response to ECM disengagement displayed by epithelial cells is not a passive phenomenon, but rather, a process of active sensing of specific microenvironmental cues and acting upon them.

Hematopoietic cells and fibroblasts share tips on staying protected

One way of getting a perspective on some of the possible ways of how a cell can avoid the consequences of anchorage loss is to consider cell types that are constitutively protected from anoikis and examine some of the possible ways of how (and why) their requirements for matrix anchorage are less stringent than those of epithelial cells. Cells of different lineages and specializations display varying degrees of dependence on ECM anchorage. On the end of the spectrum opposite from epithelial cells are cells of hematopoietic system, which are entirely anchorage-independent in their mature state. This is thought to be a consequence of expression of a diverse array of ITAM (immunoreceptor tyrosine-based activation motifs)-containing

transmembrane proteins, as well as various cytokine receptors [132]. As a result, a constitutively **high flux through pro-survival and pro-growth pathways** is maintained, which prevents the induction of pro-apoptotic proteins like Bim. Intriguingly, ectopic expression of ITAM-containing fusions is sufficient for the induction of anchorage-independence in mouse mammary epithelial cells [133].

In the middle of the anchorage requirement spectrum are fibroblasts, which populate stromal compartments of various organs. Fibroblasts specialize in ***de novo* synthesis of matrix** and express a **wide spectrum of integrins**, which allows a degree of flexibility in modifying and using the surrounding ECM environment. Furthermore, motility and invasiveness of fibroblasts, reflected in their key role in tissue repair, is associated with a greater sensitivity to **growth factor signaling pathways** [134]. On the far end of the anchorage dependency spectrum are epithelial cells - they express only a limited set of integrins and therefore require a matrix composed of specific ECM proteins, plus growth factor signaling in them are strictly regulated.

A *de novo* acquisition of changes reminiscent of those that underlie partial (displayed by fibroblasts) or complete (displayed by hematopoietic cells) autonomy from matrix anchorage can similarly enable anchorage-independent growth of cancer cells of epithelial origin. One well-described way that cancer cells use to withstand the lack of matrix-dependent signaling is through acquisition of genetic changes which result in permanently **increased flux through survival and growth signaling pathways** (reviewed in [135]). In carcinomas, such changes may be represented by amplification or mutation of receptor tyrosine kinases, such as EGFR, HER2 or c-Met, or activation of key downstream effectors, such as K- and H-Ras and PI-3 kinase. Alternatively, increases in pro-growth and pro-survival signals may be achieved via inactivation of various tumor suppressors, such as PTEN, INPP4B or NF1. A second set of means through

which cancer cells bypass the anchorage requirement is through integrin-dependent mechanisms, such as the **integrin switch**. For instance, whereas normal mammary epithelial cells express integrins specific for laminin and fibronectin, the molecules that basement membranes within mammary gland are composed of, breast cancer metastasis to the bone is facilitated via the *de novo* expression of αVβ5 integrin, which allows survival on bone-specific ECM sialoproteins [136]. Similarly, in melanoma, inappropriate expression of αVβ3 integrin permits cell survival on dermal collagen, which is abundantly present in the stroma, thus facilitating melanoma transdermal invasion [137].

A switch to a fibroblastoid cellular state, such as what is seen in **epithelial-to-mesenchymal transition (EMT)** further allows cells to survive in the absence of proper ECM. Governed by TGF-β signaling and transcriptional regulators such as Twist, Snail and Slug, EMT initiates a profound change in cellular state, which involves driving pro-survival signaling through upregulation of c-Met and NF-κB, increasing cellular motility and upregulating *de novo* synthesis of extracellular matrix.

Finally, upregulation of ***de novo* synthesis of individual ECM molecules** can lead to anoikis protection outside of the context of EMT phenomenon. For example, secretion of laminin-5, a ligand of α6β4 integrin, by cancer cells was shown to mediate their survival in the absence of anchorage [138]. Similarly, collagen XXIII production was shown to be essential for metastasis and anoikis protection in another breast cancer cell line as well [139].

Different tissues, different signals

The precise nature of a cellular **response to attachment deprivation is tissue-specific**. For example, normal mammary epithelial cells undergo apoptosis-like death when cultured in the absence of attachment [140]. At the same time, keratinocytes that become deprived of ECM

attachment enter a state of terminal differentiation, or cornification [141]. Interestingly, some interchangeability is seen between various responses to ECM deprivation. For example, mammary epithelial cells that are deficient in pro-apoptotic protein Bim, instead of cell death undergo growth arrest and begin expressing markers characteristic for terminal differentiation of keratinocytes [142].

Anoikis and related cellular responses play a pivotal **role in growth and remodeling of normal tissue**. In the context of the mammary gland, anoikis allows fast and precise molding of the glandular architecture of trees of milk-producing alveolae and ducts, triggered by pregnancy and lactation [143]. In skin regeneration, a time-delayed onset of detachment-induced terminal differentiation maintains the stratified organization of skin epidermis. Thus, the suprabasal layers, by virtue of losing the attachment to the basement membrane are subjected to a gradual onset of differentiation, which culminates with cornification and a non-apoptotic cell death of the outermost layer of the skin [144]. Thus, anoikis does not only maintain the *status quo* but is actively involved in tissue and organ morphogenesis.

Increased anoikis plays a key role in a number of pathologic conditions. For example, in cardiovascular disease, matrix-degrading enzyme elastase, secreted by macrophages, triggers the degradation of the basement membrane and anoikis of cardiomyocytes and smooth muscle cells, thereby exacerbating cardiac damage [145]. In diabetes, chronic hyperglycemia promotes excessive glycation of extracellular matrix proteins, thus disrupting the integrity of extracellular matrix and triggering anoikis in endothelial cells, which results in a widespread vasculopathy as a consequence [146]. Finally, a variety of snake venoms, such as acurhagin [147] and jararhagin [148] act as integrin inhibitors and induce hemorrhage by triggering massive levels of anoikis in the endothelium.

In summary, anoikis and related responses represent a set of critical quality-control mechanisms which are operational in epithelial tissues throughout an organism. Importantly, such mechanisms play a role in both normal and pathological histology contexts.

Once a drifter, always a drifter – resistance to anoikis in a context of multistep tumorigenesis

While excessive anoikis underlies pathologic states seen in cardiovascular disease and diabetes, **insufficient anoikis** is a hallmark state of epithelial tumorigenesis. The histopathological progression of ductal breast carcinoma is a classical example of how, at all stages of tumor genesis, insufficient anoikis contributes to the uncontrollable expansion of the abnormal tissue. Breast glandular tissue is shaped in a form of a tree, multiple branched ducts of which end with terminal ductal-lobular units (TDLUs), where, in their differentiated state, milk is produced. TDLUs are composed of the two monolayers of distinctly specialized cell types - (a) **luminal**, which are lining the lumens of ducts and lobules, and (b) **myoepithelial**, which are facing the basement membrane, which, in turn, separates the mammary tree from the surrounding stroma (reviewed in [149]). The myoepithelial layer is fenestrated so that even though luminal cells are not resting on the basement membrane directly, they nevertheless maintain physical contacts with it via specialized protrusions (Figure 1, adapted from [149]).

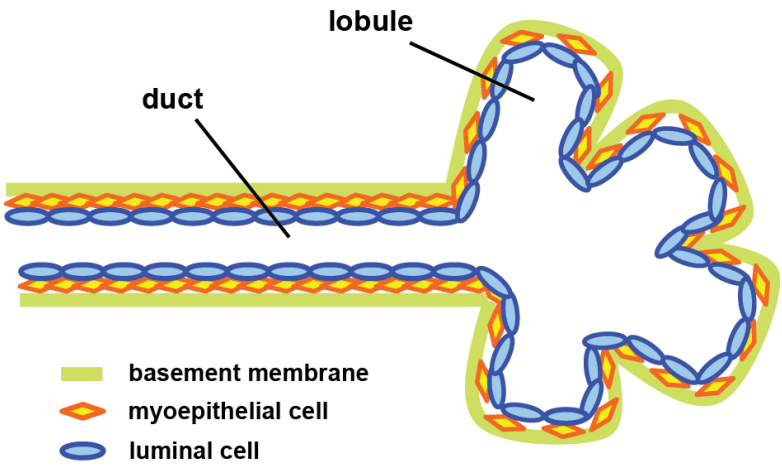


Figure 1. Cellular architecture of a terminal ductal-lobular unit.

Breast cancer originates predominantly not from myoepithelial but from luminal cells, of which 80% are of ductal subtype and up to 10% of lobular, while the rest are of the mixed origin (reviewed in [150]). The earliest described (but not universal) precursor of breast ductal carcinoma is **FEA (flat epithelial atypia)**, represented by an enlarged lobule and atypically small columnar epithelial cells lining the lumen. **ADH (atypical ductal hyperplasia)** is a first stage at which the lining of a lobular unit becomes thickened due to a local cellular hyperproliferation distinctly shaped into such structures as micropapillae, trabeculae and Roman arches. Thus, the emergence of ADH in breast is the first stage where three-dimensional manner of transformed cell proliferation becomes evident. Atypical ductal hyperplasia further proceeds to becoming a **DCIS lesion (ductal carcinoma *in situ*)**, which is characterized by (a) a complete filling of the lumen with cells (solid DCIS), (b) formation of multiple lumens at a site of lesion (cribiform DCIS) or (c) a maintenance of ADH-like multilayered luminal cell outgrowth in which cells remain attached to each other while becoming progressively necrotic in the center (comedo DCIS). Thus, being acquired early, defective luminal clearance is a prominent hallmark of the preinvasive stage of breast cancer evolution.

Furthermore, as cancer progresses towards becoming **invasive ductal carcinoma**, tumor cells begin to traverse through the stroma located underneath the basement membrane in a form of sheets and cords of interconnected cells or as individual cells [151]. Even though stroma itself is rich in extracellular matrix, the molecular composition of it is markedly different of that in the basement membrane, composed mostly of collagen for which epithelial cells lack integrin receptors. Next, to disseminate and form metastases, cells must **travel through blood and lymphatic circulation**, thus having to withstand the state of a complete matrix deprivation. At last, seeding and expansion of cancer cells within lymph nodes and in distant organs such as

brain, lung or bone requires not only tolerance for a distinct matrix composition but also ability for clonogenic growth. In conclusion, anchorage-independent growth ability plays critical adaptive role in all stages of cancer progression, making possible both the uncontrollable tissue expansion at a site of a primary tumor as well as metastatic colonization of distant tissues.

Foci, colonies and spheroids - anchorage-independence in a dish

First experimental observations of oncogene-induced disturbances in cellular monolayer pattern were obtained from studies of Rous sarcoma virus-transformed cells. Having been independently described by Adams and Groupe [70] and by Temin and Rubin [13], infection of cultured chicken fibroblasts with RSV infection triggered formation of distinct cellular foci which consisted of small, round, tightly-packed cells that did not cease proliferating even when cellular confluency was reached. When limiting dilutions of the virus were applied to fibroblasts, the number of resulting foci was reduced proportionally, suggesting that cells within such foci represented the progeny of a cell infected with a single viral particle. Facilitating the discovery of the first viral oncogene *v-src*, as well as its human homologue *c-src*, **the focus formation assay** was further utilized for identification of dozens of other viral oncogenes as well as of cellular oncogenes derived directly from human tumors.

The focus-forming phenomenon has been regarded as a manifestation of a so-called **loss of contact inhibition**. As discussed in the previous section, not only the degree of integrin engagement, but also the degree of cell stretch on the substratum, together comprising a set of cues that epithelial cells receive from their contact (or absence thereof) with ECM. Thus, even though cells in a focus-formation assay remain attached to the substratum, they nevertheless experience changes in cell shape as a result of local cell crowding and progressive reduction of the surface area of cell-substratum interaction. A focus formation assay provides demonstration

that for transformed cells, a much lesser amount of cell attachment to the substratum can be tolerated without halting cell proliferation, so that a focus can be formed.

Another important *in vitro* assay of cellular transformation is **colony formation in semi-solid medium**. Adoption of this assay was based on the observation that tumor-derived, but not normal, cells could be subjected to a cloning technique borrowed from the study of hematopoietic cells. In this method, cells are seeded at a clonal density into a so-called semi-solid medium to which an inert thickening agent, such as soft agar [152] or methylcellulose [69], has been added. The presence of a thickening agent prevents attachment of cells to the plastic substratum as well as associations of cells with one another. Resulting clonally-derived colonies are later isolated. Curiously, it was found that only tumor-derived cells, but not cells obtained from normal tissues, were amenable to this method of cell cloning. In addition, normal cells that were transformed in culture with either oncogenic viruses or chemical carcinogens were found to acquire the colony-forming ability *de novo*. Importantly, a colony-formation assay is a measure of not only an ability of a cell to sustain anchorage-independent growth, but also its ability to generate a multicellular colony from a single cell when seeded at a clonal density. Thus, colony formation in the semi-solid matrix can be also regarded as recapitulating the metastatic outgrowth or of stem-cell like pluripotency. The latter property forms a rationale for a **mammosphere formation assay**, which bears similarities to colony formation assay [153]. In a mammosphere assay, which is used to measure the percentage of cells with stem-like characteristics, cells are introduced into suspension conditions at a clonal density and assayed for a number of resulting spheric organoids which replicate the *in vivo* mammary architecture. The mammosphere formation assays brings forth an important connection between a cellular state of pluripotency and insensitivity to anchorage deprivation.

A three-dimensional morphogenesis assay is another *in vitro* assay that has been developed to study the phenomenon of anchorage dependence in mammary epithelial cells. In this assay, cells are seeded into a three-dimensional matrix composed of a complex mixture of extracellular matrix molecules (such as Matrigel, bovine collagen or a combination of the two), which serves to recapitulate aspects of breast microenvironment that orchestrate glandular expansion and differentiation [154, 155]. When introduced into three-dimensional matrix, normal epithelial cells undergo morphological differentiation and form so-called acini, which are hollow spheroids morphologically reminiscent of lobular units within a mammary gland. Importantly, cells that become displaced into the lumen in the process of acinar formation undergo anoikis, and the hollow architecture is maintained. Furthermore, in dramatic contrast to normal mammary epithelial cells, various cancer cells as well as cells ectopically induced to express oncogenes, display cancer-like aberrations of acinar growth, including filling of lumens, formation of multilobular structures or disordered, invasive growth in the matrix.

In summary, detachment from proper ECM triggers downregulation of matrix-dependent integrin signaling. Besides compromised flux through c-Src, PI3K-AKT and MAPK pathways, stresses of anchorage loss include defects nutrient utilization and antioxidant production and defects in maintaining the proper cytoskeletal tension. To tolerate absent or inadequate anchorage, cancer cells must undergo adaptive changes that allow them to proliferate and survive independently of anchorage. The multifaceted nature of stresses associated with anchorage loss highlights it as an important node of therapeutic intervention. As the histopathologic progression of breast ductal adenocarcinoma illustrates, some degree of ability to tolerate ECM deprivation often must be acquired early in the course of cancer development. Even a localized hyperplasia within an epithelial surface theoretically cannot arise without cells developing at least some

degree of resistance to anoikis. At the same time, anchorage independence phenotype continues to be important and, ostensibly, increases in its importance, throughout all the stages of multistep tumorigenesis. Therefore, regardless the disease stage, cancer cells should display sensitivity to therapies that are designed to target the mechanism of anchorage-independence. As has been reviewed above, ways of circumventing the anchorage requirement are remarkably complex – and many more likely remain to be discovered.

Chapter II: A genetic screen for drivers of anchorage-independence in human mammary epithelial cells

Background and Rationale

In vitro transformation of normal human cells

The first cancer genes were isolated from oncogenic viruses and from genomic DNA derived from human tumors or chemically mutagenized mouse cells. When introduced into recipient cell lines, such as cultured chicken fibroblasts or mouse embryonic fibroblasts (MEFs), oncogenes like *v-src* and *Ha-ras* induced a distinct phenotype described as transformation, or ability to form foci. At the same time, fibroblasts that were freshly derived from animal tissues failed to undergo transformation upon introduction of an oncogene, entering a distinct state of growth arrest instead. Contrary to established fibroblast cell lines, achieving transformation in primary fibroblasts required introducing additional oncogenic elements, such as adenoviral E1A [20, 156]. The difference between freshly isolated and culture-adapted fibroblasts was found to be due to a spontaneous immortalization that fibroblasts underwent upon continued passaging, through, most often, a spontaneous inactivation of the p53 gene [157].

Human cultured cells posed an even bigger problem with regard to transformation in culture. In a stark difference to mouse cells, human cells failed to immortalize spontaneously and entered a state of irreversible growth arrest after a number of passages in culture. For example, human fetal fibroblasts enter a state of irreversible "degeneration", or "senescence" by the 50^{th} passage [158], whereas normal adult cells sustained even fewer passages. Human mammary epithelial cells (HMECs), in particular, were able to sustain proliferation for only 10-20 passages

[159]. Replicative senescence in both fibroblasts and epithelial cells could be bypassed through exogenous inactivation of Rb and p53 tumor suppressors by expressing Large T antigen of SV40 or a combination of E7 and E6 antigens of human papillomavirus; however, it did not prevent them from entering an eventual state of "crisis" [160], in which massive genome instability, fusion of chromosomal ends and cell death was observed.

For human cells, the bypass of replicative senescence appeared to have fewer requirements in epithelial cells than it did in fibroblasts. Indeed, human fibroblasts required both E6 and E7 HPV proteins for immortalization, whereas E7 protein appeared to be dispensable for immortalization of HMECs [161]. Furthermore, breast epithelial cells, but not breast stromal fibroblasts, isolated from a patient with a germline p53 mutation due to Li-Fraumeni syndrome, were able to spontaneously bypass senescence in culture [162]. The bypass of Rb inactivation requirement in HMECs was found to occur via outgrowth of rare clones (up to 0.01% of the HMEC population) that silenced $p16^{INK4A}$ gene expression [163] as a consequence of p16 promoter methylation [164]. HMECs that lost p16 expression continued proliferating past the initial "stasis" stage, eventually reaching the second state of growth arrest, or "agonescence". Interestingly, culturing HMECs on feeder cells [165] or on treated plastic that mimicked the natural ECM [166] permitted cells to avoid the induction of p16 altogether, suggesting that the state of "stasis" may be a consequence of stress associated with the absence of proper microenvironmental factors in culture. Similarly to human fibroblasts, "agonescence" could be bypassed with the exogenous inactivation of p53 [167], only to reach the third state which involved massive cell death from a so-called "crisis". This third barrier to HMEC immortalization could not be spontaneously bypassed.

The death of cells from "crisis" in human fibroblasts and epithelial cells was discovered to be the consequence of massive DNA damage response to critical shortening of telomeres [168]. In a stark contrast to mouse cells, which had a sustained expression of telomerase enzyme component TERT, normal human cells lacked its expression [169]. When human TERT was ectopically introduced, the "crisis" state in human fibroblasts and epithelial cells has been avoided, so that cells became fully immortal [170, 171].

Exogenous immortalization with hTERT made it possible to introduce oncogenes into cells of human origin and thus model the emergence of the transformed state. For example, a combination of three ectopically introduced elements: (i) hTERT, (ii) the early region of SV40 and (iii) Ha-Ras^{V12} transformed a variety of human cells - fibroblasts, embryonic kidney cells, small airway epithelial cells and HMECs without apparent need for further clonal selection [172, 173]. Even though the early region of SV40 was originally thought to contain only the Large T antigen, subsequent studies had shown that it also contained a small t antigen, an inhibitor of a widely-acting serine/threonine phosphatase PP2A, and that both Large T and small t were required for transformation [174, 175].

Extended passaging of hTERT-immortalized HMECs selects for subpopulations of cells with increased levels of *c-myc* [176]. In addition, the outgrowth of cell populations with the genomic amplification of *c-myc* locus, through events such as trisomy of chromosome 8 or unbalanced translocation of the region of chromosome 8 that contained *c-myc*, was seen following the transforming combination of SV40 and Ha-Ras^{V12} transgenes. This brought forth an important caveat that both prolonged passaging and transformation may be selecting not only for *c-myc*, but for additional genes in the vicinity of *c-myc* locus as well [177]. Importantly, *c-myc* activity itself is positively regulated by Ras. In agreement with this, in late-passage hTERT-

HMECs or in early-passage hTERT-HMECs in which *c-myc* was ectopically expressed, Ha-Ras^{V12} was no longer required for *in vitro* anchorage independence and Large T and small t expression was sufficient. However, *c-myc* could not substitute for all actions downstream of Ha-Ras^{V12}, because Ras was still required for *in vivo* xenograft growth [178].

PP2A phosphatase, a target of a small t antigen, acts upon a wide range of proteins within growth and survival pathways, some of which overlap with those stimulated by *c-myc* [179]. Further dissection of the requirements for *in vitro* anchorage-independence of hTERT-HMECs revealed that myristoylated catalytic subunit of PI-3 kinase (myr-p110-PI3K) or a combination of its constitutively active effector mutants, myr-Akt and Rac1^{V12}, can substitute for small t in both late-passage and early-passage/*c-myc*-expressing hTERT-HMECs [178].

In addition to potentiating anchorage-independent colony formation, ectopic expression of a small t antigen, or myr-p110-PI3K, or a combination of myr-Akt and Rac1^{V12} also enabled HMEC proliferation in conditions where growth factor concentration was reduced to 0.5% of standard amount, yet these oncogenic perturbations had no effect on HMEC proliferation rates in regular media [178]. Thus, a constitutively active PI-3 kinase pathway is sufficient to mitigate the proliferation-suppressing effects of both anchorage deprivation as well as growth factor deprivation. This illustrates the concept that the flux through PI-3 kinase pathway in adherent cells is potentiated through a combined effect of growth factor signaling pathways and integrin signaling. The added effect on PI-3 kinase flux is that reducing the stimulation through either of the two inputs – growth factor starvation in the first instance and culturing in the absence of attachment in the second – compromises cellular growth. Along these lines, it is conceivable that increasing growth factor stimulation in conditions of anchorage deprivation may partially bypass the integrin requirement and promote colony formation.

In vitro screens for determinants of anchorage-independence phenotype

Anchorage-independence is a hallmark phenotype of epithelial cancers that has been found to be a best predictor for xenograft growth *in vivo*, and it has been used extensively as a tool of oncogene discovery. An important technical challenge of testing of an ability of a gene-of-interest to induce anchorage-independence *in vitro* is that it involves formation of either a macroscopic focus or an anchorage-independent colony by a progeny of an individual cell, a recipient of a tested gene. Therefore, unless the expression is only necessary for the survival of a founding cell, a stable expression of the gene in question is required.

First functional screens for oncogenes involved transfecting fragmented genomic DNA from human cancers, or from cells mutagenized with chemical carcinogens [19, 180]. However, stable genomic integration events are extremely rare with this method of gene transfer. The development of retrovirus-based expression vectors allowed, for the first time, increasing the number of successful stable integration events into genomes of recipient cells by orders of magnitude and, in addition, allowed easy recovery of integrated sequences. Thus, a retroviral vector-mediated introduction of a neuroblastoma-derived cDNA library into NIH-3T3 MEFs yielded 19 independent cDNAs that induced foci formation, which included *raf*, *lck* and *ect2* - previously known oncogenes, and 16 that were novel, including a cDNA for β-catenin [73]. This screening method was further applied to identification of genes capable of suppressing anoikis in anoikis-sensitive rat intestinal epithelial cells (RIE cells). Screening a mouse embryo-derived cDNA library for sequences that were able to induce anchorage-independence in RIE cells identified NTRK3/TrkB receptor tyrosine kinase as a novel transforming gene with strong ties to cancer [181].

The demonstration that cells derived from normal human tissues could be transformed though introducing a small number of defined genetic elements had opened new possibilities for functional genomics-based oncogene discovery - this time, in the context of cultured human cells. In addition, creating libraries of shRNAs targeted against thousands of human genes has made loss-of-function screens a reality, facilitating the discovery of not only potential oncogenes but tumor suppressors as well.

Thus, taking advantage of the modular nature of **late-passage hTERT/Large T HMEC model (TL-HMEC model)**, a library of shRNAs sequences has been screened for those shRNAs that could substitute for the small t as enablers of anchorage-independent colony growth. This screen identified a novel tumor suppressor REST, a frequent target of mutation in colon cancers [98]. A similar loss-of-function screen was performed with a human BJ fibroblast cell line, in which a combination of hTERT, small t, RAS^{V12} and shRNAs against p53 and $p16^{INK4A}$ triggered transformation. Screening an shRNA library of 4,000 shRNAs targeting human genes in this model identified that an shRNA against PITX1, a putative suppressor of RAS, was able to induce anchorage-independent growth in the absence of RAS [100].

Genetic screens aimed at the discovery of regulators of anchorage-independence had also been performed with focused libraries of ORFs and shRNAs which were designed to cover functionally-related groups of genes. Thus, screening a library of shRNAs targeting human kinases and phosphatases in TL-HMEC model allowed identification of a tumor suppressive function of PTPN12 phosphatase, which is a target of frequent deletion in triple-negative breast cancer [99]. Also, a gain-of-function screen in human embryonic kidney cells which expressed hTERT and dominant-negative p53 with a library of 354 myristoylated kinase ORFs identified a

transforming activity of IKBKE kinase, an upstream activator of NF-κB pathway and a target of focal amplification in breast cancer [83].

To summarize, genetic screens in human epithelial cell lines have been successfully performed with both loss-of-function RNAi libraries or with focused libraries of ORFs encoding genes of specific function. Large-scale screens for positive regulators of anchorage independence in systems like TL-HMEC have not yet been performed. An effort aimed at cloning of human ORFs led to a creation of a human ORFeome library which consisted of 8,000 genes and could be readily shuttled between different types of expression vectors using Gateway recombination [76]. Taking advantage of this library, we set out to perform a screen with the ORFeome library to identify novel transformation-inducing ORFs in TL-HMEC system, without the inherent limitation of using libraries of genes with certain function only. In addition, we performed key modifications to TL-HMEC system and the anchorage-independence protocol to improve the signal-to-noise ratio to facilitate discovery of novel oncogenes and tumor suppressors as well as their subsequent study.

Results

Anchorage-independence screen with the ORFeome library

To identify novel genes that promote transformation of epithelial cells, we performed a genetic screen for anchorage-independent colony formation in methylcellulose, using a TL-HMEC system in which late passage hTERT-immortalized human mammary epithelial cells induced to express Large T antigen of SV40. TL-HMECs fail to form colonies in semi-solid medium, such as methylcellulose, when deprived of attachment. However, ectopic expression of oncogenic H-RAS^{V12}, myristoylated catalytic subunit of PI3K [178] or shRNA against PTEN [98] readily induces formation of macroscopic colonies. We introduced a pROLES retroviral vector-encoded library of 8,000 human open reading frames (ORFeome v1.1) [76] into TL-HMECs and selected successful integrants with puromycin (for the workflow schematic, see Figure 2). Expression of ORFs was driven by a CMV promoter. To avoid potential positional effects of provirus integration on proliferation or transformation of individual clones, the ORFeome library infection and the subsequent screening was performed in two independent replicates. In addition, we took care to avoid introducing multiple ORFs into the same cell by performing the viral infection at a multiplicity of infection of 0.2. This way, the number of recipient cells exceeded the number of viral particles by a factor of 5, ensuring that viruses were not present in excess. Furthermore, we continued to maintain the representation of the library at a 200-fold magnitude throughout the process of a pre-screen cell culture as well as during seeding the cells for the anchorage-independence screen. This was done to ensure that each ORF is sufficiently represented in the resulting polyclonal population of TL-HMECs to further reduce stochastic effects on cell viability and growth.

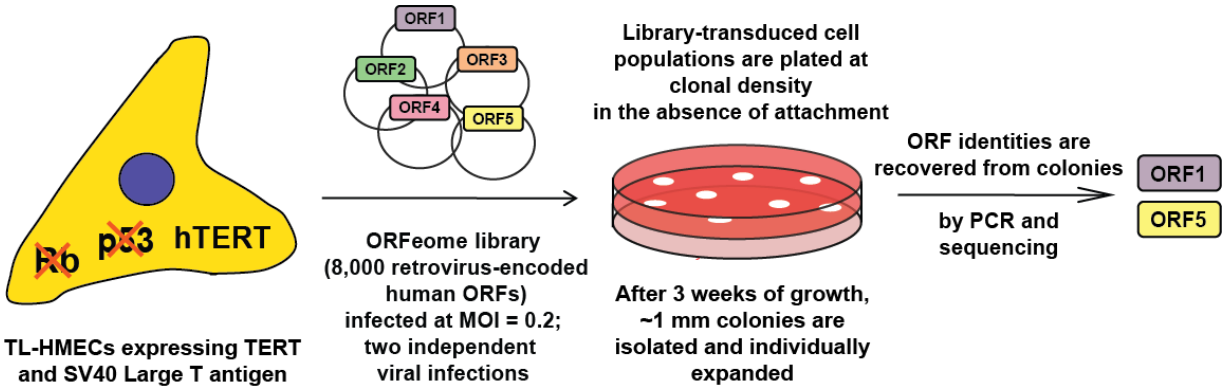


Figure 2. A genetic screen for drivers of anchorage-independent growth in human mammary epithelial cells. A total of 200x8,000=1,600,000 cells was plated per replicate as to maintain the 200-fold library coverage.

Library-transduced cell populations were seeded into methylcellulose medium at a clonal density and formation of macroscopic colonies was observed three weeks post-seeding. Methylcellulose is superior to soft agar for performing genetic screens in that it does not solidify like agar does and therefore allows easy recovery of anchorage-independent clones. We have isolated individual colonies from methylcellulose and re-adapted them to adherent growth by culturing each one individually in wells of 96-well plates. A total of 732 colonies have been isolated from the screen with a 100% recovery rate. Once all the clones reached confluency, we proceeded to isolate the genomic DNA and recover proviral integrations by PCR amplification. A pair of primers used for PCR was designed to target common sequences outside the ORF insert, and successful PCR amplification has been verified by agarose gel electrophoresis.

By this method, we were able to successfully recover ORF inserts from 83% of colonies. From 68% of total colonies, a unique ORF has been recovered from each colony, 15% of colonies contained two ORFs integrated into them, and two clones had each three integrated ORFs. This shows that even with a 5-fold excess of recipient cells over viral particles, double

integrations of a provirus do occur with a substantial frequency, which could be reflective of aggregation of individual viral particles or of selection of cells containing two copies of a puromycin resistance gene during the process of antibiotic selection. This does not rule out the possibility that in some double integrants, both ORFs contribute to anchorage-independence phenotype; therefore, it would be of further interest to compare the rate of double integrations among isolated colonies to that of the starting cell population.

The identity of ORFs recovered by PCR was determined by sequencing, yielding the total of 646 unique ORFs. Since we have performed our screen in two independent replicates, we reasoned that ORFs identified in both replicates will be enriched for those with *bona fide* transforming sequences. A total of 40 ORFs were identified in both screen replicates and were selected for individual cloning into pROLES retroviral vector for further validation of the phenotype (see Appendix II for the list of candidate ORFs).

Modifications to TL-HMECs system to facilitate further studies of transformation

We saw that with the increasing passage number of TL-HMEC cells, the number of anchorage independent colonies in an empty vector-transduced population, that is, the background level of colony formation, was increasing. This phenomenon caused the reduction of the dynamic range of TL-HMEC transformation assay and made the system less effective for studying moderate transformation phenotypes.

Thus, we set out to improve the dynamic range of TL-HMEC transformation system and employed two strategies for it: (1) isolation of individual clones from TL-HMEC line and characterization of them with respect to a fold change in a number of anchorage-independent colonies induced by an oncogenic perturbation compared to control population; (2) modifications to the culture medium composition to increase the assay stringency.

We reasoned that the observed reduction in signal-to-noise ratio may be due to a polyclonal nature of the TL-HMEC line. One may hypothesize that, as a result of specific genetic and epigenetic differences, which could be both original or accumulated in culture, some of the clones may have a higher proliferation index than others. If this is the case, the fraction of such clones in the total population is bound to increase with each passage. Furthermore, if these hyperproliferative clones are also spontaneously transformed, then the presence of such clones may explain the decreasing dynamic range of transformation assay that we consistently observed.

To ask directly if this is indeed the case and to obtain clones of TL-HMECs with a dynamic range superior of that of the parental population, we have isolated six TL-HMEC clones, expanded them and transduced with an shRNA against PTEN tumor suppressor or a control shRNA which targeted firefly luciferase gene (FF2). Resulting clones were then seeded into methylcellulose medium and anchorage-independent colony formation was monitored.

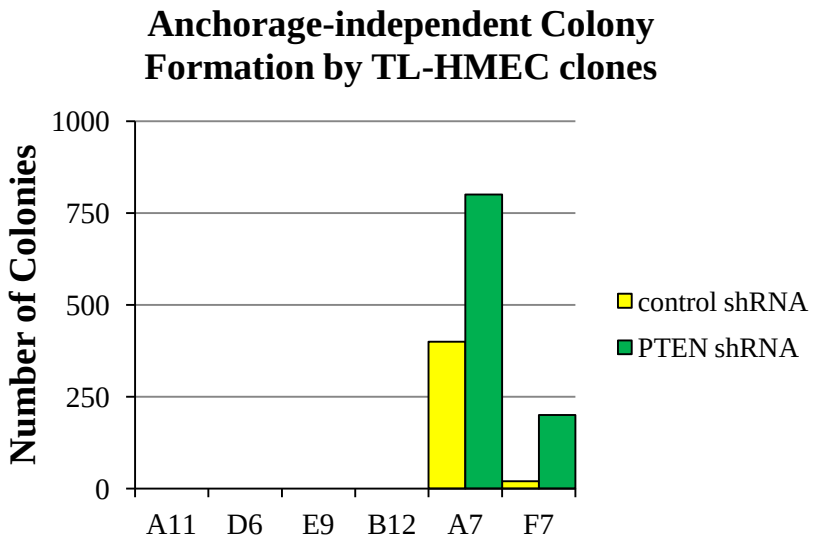


Figure 3. Spontaneous and PTEN shRNA-induced anchorage-independent colony formation by individual TL-HMEC clones.

Strikingly, we found that four out of six clones failed to form colonies regardless of whether they expressed a control FF2 shRNA or an shRNA against PTEN. Furthermore, one clone (clone A7) yielded a massive number of colonies when transduced by FF2 shRNA, and only a two-fold increase in colony number was seen with PTEN shRNA in it. Finally, one clone (clone F7) had a low background rate of colony formation and had a 10-fold increase in colony formation in presence of PTEN shRNA (Figure 3). Thus, a great heterogeneity with respect to clones' ability to form anchorage-independent colonies was seen across individual clones of TL-HMEC line. First of all, only two out of six clones became transformed by PTEN shRNA. Strongly supporting our original hypothesis about the presence of hyperproliferative, spontaneously transformed clones, we indeed found that clone A7 was a spontaneously transformed clone. In addition, during the process of clone isolation, we noticed that A7 clone occupied an area on the dish approximately five times larger than the rest of the clones. Because all six clones were isolated at the same time and individual cell sizes did not vary across individual clones, this may be indicative of an increased proliferation rate of A7 clone, or, alternatively, of a decreased rate of cell death. We have decided to use F7 clone for our

future experiments, as it exhibited a superior dynamic range in colony numbers between positive and negative control.

The origins of the striking heterogeneity between individual clones from TL-HMEC line that we observed are not entirely clear. First of all, HMECs are isolated from human mammary tissue which is a polyclonal mix of cells of at least two lineages – luminal and myoepithelial cells, and may contain tissue stem cells as well. HMECs are further transduced with two separate viral constructs – hTERT and Large T, and positional effects from provirus integration events may influence expression of transduced genes and genes in the vicinity of the genomic integration as well. Finally, HMECs exhibit some degree of genomic instability – for example events such as a trisomy of chromosome 8, as well as presence of unbalanced translocations have been observed. The heterogeneity with regard to alterations to gene copy numbers could further contribute to differences in proliferation rates and anchorage-independence phenotype across individual TL-HMEC clones.

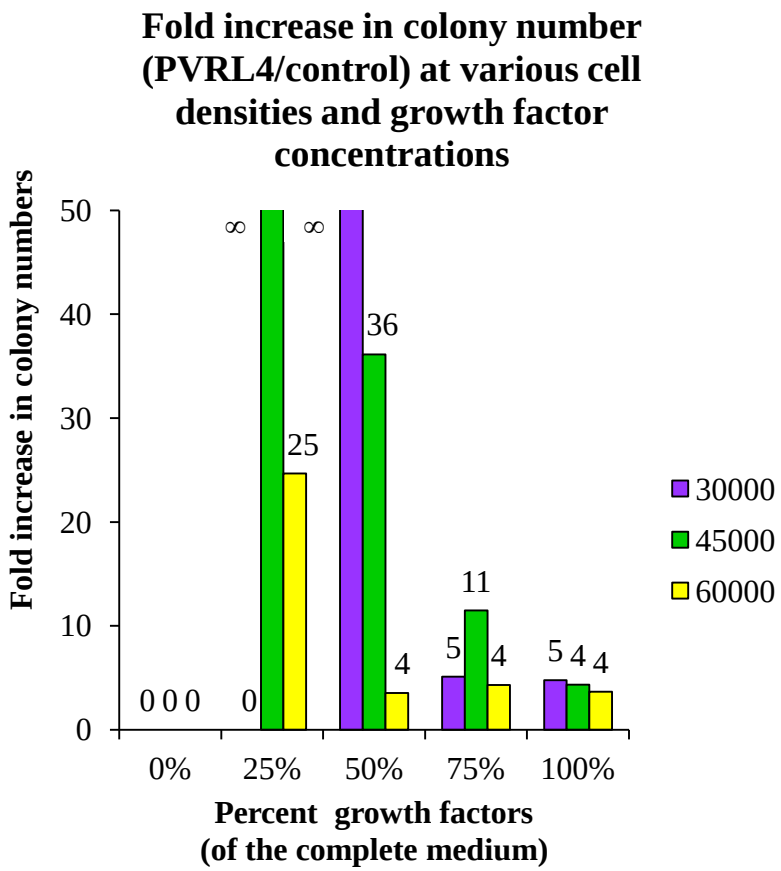


Figure 4. A ratio of anchorage-independent colony formation induced by a screen candidate PVRL4 ORF over vector control at various cell densities and growth factor concentrations. Numbers in the legend denotes various cells seeded into methylcellulose. Assays were performed in five different medium compositions in which concentrations of all four growth supplements varied from 0% to 100% of that in the complete medium. Y-axis values represent the magnitude of colony growth stimulation by a positive control (PVRL4 ORF) as compared to empty vector-transformed cells.

In addition to isolating a clonal population from TL-HMECs, we asked whether we can further improve the system by modifying the anchorage-independent growth conditions. As was previously shown, transducing TL-HMECs with a small t antigen, myr-PI3K or a combination of myr-Akt and $RAC1^{V12}$ not only facilitated anchorage independence, but also made cells less sensitive to reductions of growth factor concentrations in culture medium. Indeed, PI3K pathway activation in adherent cells is maintained via two crucial inputs: growth factor stimulation and signaling downstream of activated integrins. In agreement with this, ectopic hyperactivation of PI3K pathway makes cells insensitive to the absence of either of the two stimuli. We reasoned that TL-HMECs may be partially able to tolerate anchorage-independence conditions through receiving

the non-cell-autonomous prosurvival signaling input via growth factors. Therefore, we hypothesized that reducing growth factors concentration in methylcellulose medium may reduce the background colony formation and further improve the dynamic range, thereby facilitating the discovery of more moderate phenotypes. HMEC growth medium is a semi-defined culture medium, which contains two defined growth factors - insulin at 10 μg/mL and EGF at 5 μg/mL - as well as bovine pituitary extract at 2% v/v, which is a mixture of various growth factors, predominantly basic FGF. To test how changes in growth factor concentrations will affect anchorage-independence assay performance, we prepared methylcellulose medium containing 100%, 75%, 50%, 25% or 0% of normal growth factor concentration and seeded TL-HMECs expressing either empty vector or one of the validated candidate ORFs, PVRL4, in these conditions. In addition, we also plated cells at three different cell densities to simultaneously assess the paracrine stimulatory effects on transformation from cells themselves.

We have indeed observed that reducing the concentration of growth supplements in the culture medium produced a very strong effect on the dynamic range of our assay (Figure 4). Seeding cells in 100% and 75% growth factor medium resulted in an average of a five-fold increase in anchorage-independent colony formation of PVRL4 ORF-expressing cells over empty vector, but when cells were seeded in 50% or 25% growth factor medium, a dramatic increase in dynamic range was observed. Similar effects were further observed with other transforming sequences such as PTEN shRNA (not shown). Based on these onservations, we decided to modify our assay protocol and prepare methylcellulose medium with 50% growth factors for future studies.

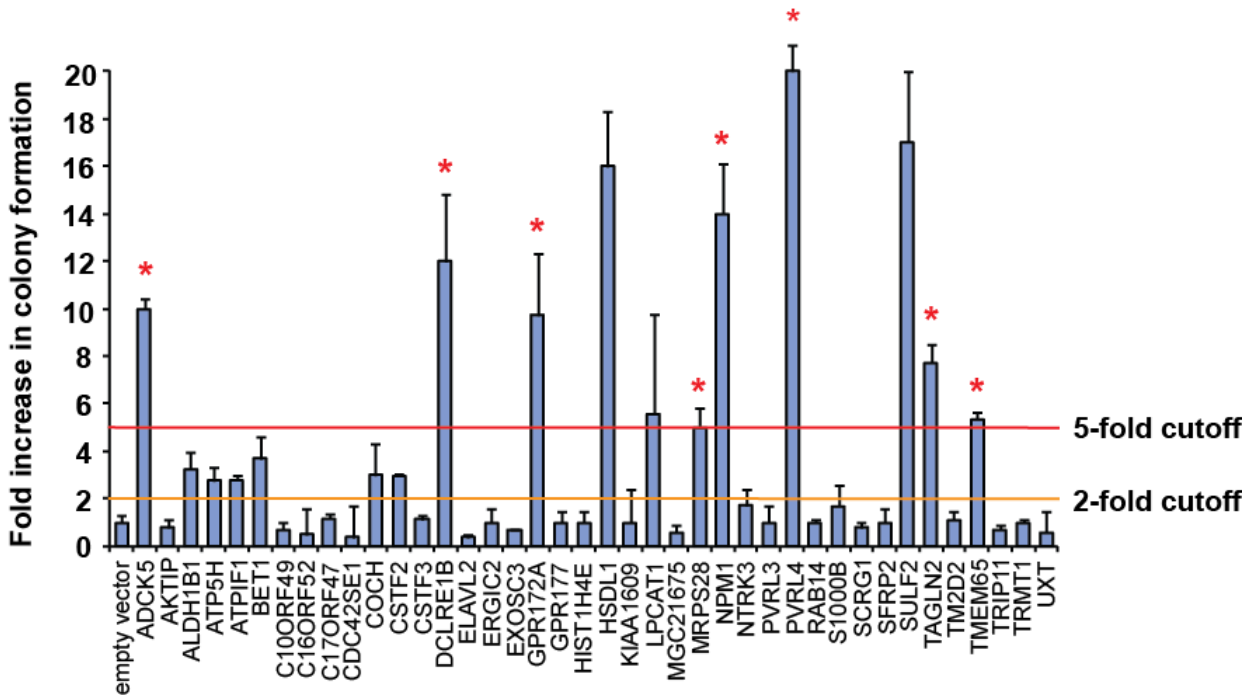


Figure 5. Validation of screen candidates. ORFs recovered from two independent screen replicates were individually transduced into TL-HMECs and plated into semi-solid medium. Colonies were counted and colony numbers were normalized to an empty vector-transduced sample. Asterisks denote strongly validated ORFs that localize to focal amplification peaks in at least one tumor subtype. Assays were performed in triplicate (error bars ± SD).

Validation of anchorage-independence screen candidates

We have introduced 40 ORFs that were indentified in both replicates of the transformation screen into F7 clone of TL-HMECs by viral transduction and assayed resulting lines for ability to form anchorage-independent colonies under reduced growth factor conditions (Figure 5). We have found that 11 out of 40 ORFs potently induced TL-HMECs colony formation (5-fold increase over background), and another 8 ORFs produced a moderate phenotype (2-5-fold increase over background).

Table 1. Strongly validated ORFs located in recurrently amplified genomic regions.

Gene Symbol	Genomic Location	Cancer Subset	Amplification Peak	Number of Genes in Peak	Q Value	Fraction Amplified	Fraction Focally Amplified
ADCK5	chr8:145568538-145589261	all_cancers	chr8:140480593-146264218	97	0.000	0.3548	0.0843
DCLRE1B	chr1:114249560-114258217	Lung SC	chr1:44531979-143798352	450	0.184	0.375	0.175
GPR172A	chr8:145553032-145555754	all_cancers	chr8:140480593-146264218	97	0.000	0.3548	0.0843
MRPS28	chr8:80993649-81105061	Lung NSC	chr8:80901900-82348486	7	0.000	0.4243	0.0955
NPM1	chr5:170747402-170770493	Lung NSC	chr5:165042046-180624927	126	0.006	0.2333	0.0928
PVRL4	chr1:159308119-159325966	all_cancers	chr1:158326896-159953449	52	0.000	0.3526	0.0782
TAGLN2	chr1:158154526-158161908	all_hemat. cancers	chr1:155498052-161098015	112	0.060	0.0801	0.0143
TMEM65	chr8:125392339-125454121	Lung NSC	chr8:116319084-125488036	37	0.000	0.5321	0.1501

Next, we searched publicly available datasets of recurrent copy number alterations in cancers to find whether transforming ORFs that we recovered were frequently targeted for amplification in human tumors. According to comprehensive copy number variation analysis across more than 3,000 cancers, encompassing a variety of tumor types [34], 8 out of 11 ORFs

that potently induced anchorage-independent colony formation in our system, were localized to statistically defined peaks of focal amplification in at least one tumor subtype ($p=0.009$, Fisher's exact test), further suggesting that our screen identified genes that are under positive selection in cancer (Table 1).

Among the list of positively validated ORFs identified by our screens were those corresponding to genes for which survival-promoting function in cancer cells has been previously described. One of such genes is SULF2 (sulfatase 2) [182], which functions by remodeling heparin sulfate matrix components. This, in turn, mobilizes various growth factors, making them available for binding to corresponding receptor tyrosine kinases. The second gene previously involved in prosurvival signaling is NPM1 (nucleophosmin 1). Indeed, in addition to its function in the nucleolus, the non-canonical role of nucleophosmin in potentiating PDGF receptor signaling has been described [183]. This suggests that our screen identified a number of transformation-inducing genes with a strong functional and genomic relevance to the process of tumorigenesis.

Materials and Methods

Constructs and virus production. For screen candidate validation, ORFs from isolated colonies were subcloned into pROLES retroviral vector (provided by W. Harper). For negative and positive transformation controls, TL-HMECs were transduced with pMSCV-mir30-FF2 or pMSCV-mir30-PTEN shRNA constructs. Retro- and lentiviral supernatants were generated by transient transfection of 293T cells following Mirus Bio's TransIT transfection protocol and harvested 48 hrs later.

Cell Culture. TL-HMECs expressing hTERT and SV40 Large T antigen [178] were cultured in MEGM (Lonza). 293T cells were cultured in DMEM (Invitrogen), supplemented with 10% FBS. Retroviral infections were performed in presence of 8 ug/mL of polybrene (Sigma). Successful viral integrants were selected with puromycin (2 ug/mL) or Geneticin (200 ug/mL).

Anchorage-independent colony formation assay. Anchorage-independent colony formation assays were performed as previously described[98] with minor modifications. Briefly, cells were suspended in reduced growth factor MEGM (containing 50% of kit-supplied BPA, Insulin, EGF and hydrocortisone) with 2% methylcellulose (Sigma) and plated on tissue culture dishes precoated with 0.6% Noble Agar (Sigma) in MEM (Invitrogen). For assays performed in 6 cm dishes, 4.5×10^4 cells per dish were plated. For assays performed in 6-well plates, 1.2×10^4 cells per well were plated. Colonies were counted after three weeks of growth. For each assay, except for those in Figures 3 and 4, an average of three replicates +/- SD is shown.

Chapter III: The investigation of PVRL4–driven anchorage-independence: the role of cell-cell contacts

Background and Rationale

Is cancer a sticky business?

Whereas the prosurvival role of cell-ECM contacts has been extensively described, the role of cell-cell contacts and individual cell adhesion molecules (CAMs) in driving epithelial cell survival and the process of tumorigenesis is considerably less well understood. On one hand, development of invasive tumors is associated with the loss of normal cell-cell adhesions and facilitation of cellular motility, yet other studies demonstrate that in many solid tumors, cell-cell contacts are preserved even at advanced stages of disease and may be serving a critical prosurvival role.

The study of how cell-cell adhesiveness contributes to tumorigenesis is complicated by a remarkable variety of molecular mechanisms that mediate cell-to-cell attachment. Indeed, mammalian genomes have over two hundred described cell adhesion molecules (CAMs), which are involved in stable as well as transient types of cell-cell interactions and serve a variety of physiological roles. Thus, various CAMs function in maintaining tissue integrity, governing tissue morphogenesis and relaying cell-to-cell signals between immune system components. Normal epithelial cells possess three types of cell-cell junctions which altogether constitute the junctional complex: adherens junctions and desmosomes, which are built by **E-cadherin** and other tissue-specific cadherins and tight junctions, built by **claudins**. In addition to cadherins and claudins, which promote stable intercellular interactions, three other classes of cell adhesion

molecules are involved in facilitating more transient cell-cell contacts. These include **integrins**, which can potentiate cell-to-cell adhesion in addition to their role in cell-to-ECM adhesion, **selectins**, which mediate adhesion of leukocytes to endothelial cells via various sialoproteins [184], and a diverse family of **immunoglobulin-like CAMs (IgCAMs)**. Extracellular regions of IgCAMs contain from 1 to 5 immunoglobulin-like folds and, sometimes, fibronectin-type III domains. IgCAMs form cell-cell contacts through *trans*-interacting with other IgCAMs in a homotypic or heterotypic manner, or with integrins, thus facilitating the cell-cell adhesive function of the latter. The role of IgCAMs in mediating cell-to-cell signaling in the immune system is best described [185], however various IgCAMs - such as nectins - are involved in carrying out other functions as well, such as guiding neural and epithelial tissue morphogenesis.

Histopathological analysis of invasive tumors often reveals a characteristic appearance of individualized cancer cells or small cell clusters at the invasive front of a tumor, a phenomenon termed **"cell budding"**, which is associated with a more adverse prognosis. Concomitantly, **loss of junctional complexes** characteristic for normal cells is a hallmark of a transition from a benign to a more aggressive disease stage. Both whole-genome tumor analysis and immunohistochemical analysis show that invasive carcinomas frequently attenuate the expression of E-cadherin. Loss of E-cadherin, a major structural component of normal cell-cell junctions, can occur via events such as loss-of-function mutations [186], epigenetic silencing [187] and transcriptional downregulation during the process of epithelial-to-mesenchymal transition (EMT) (reviewed in [188]). Importantly, loss of E-cadherin is an independent predictor of a poor disease outcome [189]. Functional studies support the significance of E-cadherin loss to the onset of invasion: indeed, ectopic re-expression of E-cadherin reduces *in vitro* invasiveness [190]. In addition, it blocks the transition from adenoma to invasive carcinoma in a

mouse model of pancreatic tumorigenesis, whereas a dominant-negative mutant of E-cadherin accelerates this transition [191]. On a signaling level, E-cadherin sequesters critical signaling modulators β-catenin and p120-catenin, preventing their participation in activating Wnt-dependent signaling [192] and small GTPase activation [193], respectively. In support of the critical role of the cytoplasmic region of E-cadherin in mediating its growth-suppressive functions, expression of a membrane-tethered cytoplasmic region of E-cadherin was sufficient for the inhibition of cell transformation, thus demonstrating that growth suppressive function of E-cadherin is functionally distinct from its role in mediating cell-cell adhesion [192].

The prevalence and even relevance of EMT as being an obligatory step towards metastatic disease has been hotly debated in the field [194, 195]. One possibility is that rather than being an irreversible transdifferentiation process, EMT is a transient phenomenon driven by the contact between tumor cells and the underlying stroma, whereas once seeded in a distant organ, cells revert back to the epithelial morphology [196]. This theory is supported by the immunohistochemical analysis of carcinoma metastases, the majority of which display epithelial differentiation [197]. Unexpectedly, cancer cells can seed inside the bone marrow as early as at the *in situ* stage of a primary breast carcinoma, and progression to invasive disease does little to affect the number of individual tumor cells that reach bone marrow [198]. Furthermore, rather than being a universal mechanism of metastasis, EMT may represent just one of the modes of cellular invasion. In support of that, an alternative mode of invasion, termed **collective invasion**, whereby cancer cells invade the surrounding stroma as large groups, shaped as cords or sheets, where individual cells remain connected to each other, has been observed both *in vitro* [199] and is seen routinely on histopathological examinations of various types of carcinomas as well.

While there is a tendency for the loss of tight and adherens junctions in tumors, some CAMs are on the contrary, upregulated in cancer and are functionally involved in promoting survival and invasiveness. Thus, N-cadherin, a less adhesive mesenchymal cadherin, becomes upregulated during the EMT process and acts to promote prosurvival signaling and invasiveness; furthermore, monoclonal antibodies against N-cadherin inhibit prostate cancer cell line growth *in vivo* [200]. Mechanistically, N-cadherin associates in *cis* with FGFR, enhancing its downstream signaling [201]. In addition, intracellular domain of N-cadherin can be cleaved and translocated to the nucleus where it enhances CREB-dependent gene expression [202]. Both of these signaling mechanisms appear to be independent of cell-to-cell adhesion. Some IgCAMs are upregulated in cancers and are involved in facilitating invasion and growth. These include L1CAM [203], EpCAM [204] and NrCAM [205]. The function of EpCAM is thought to be carried out though translocation of its intracellular domain to the nucleus, whereas NrCAM and L1CAM potentiate integrin activation via cleaved extracellular regions, binding to their respective integrin counter-receptors and promoting invasion. This evidence taken together, some of the functions of CAMs in tumors may occur independently from their role in cell-cell contact formation.

In addition, several lines of evidence support the notion that enhanced cell-to-cell adhesion may have a pro-survival role and be selected for during tumorigenesis. Cells in which junctions are preserved display resistance to anoikis [206]. Among cancer cell lines, aggregate formation in oral squamous carcinoma cultured under anchorage-independent conditions promotes their survival through an EGFR-dependent mechanism [207], whereas preventing cell-cell aggregation with antibodies against E-cadherin induces anoikis [208]. One caveat is that oral squamous carcinoma is a type of cancer where E-cadherin expression is not typically lost [209],

which may not be the case in other cancer types such as breast cancer, where loss of E-cadherin at the invasive stage is a frequent event.

Correlative evidence suggests that aggressive cancer cells display higher propensity for self-aggregation than their less aggressive counterparts or than normal cells. Thus, the multicellular aggregate-forming propensity of virus- and chemically-transformed cell lines correlates with anchorage-independent colony formation phenotype and xenograft growth *in vivo* [210]. Furthermore, renal carcinoma-derived subclones selected for greater metastatic capacity *in vivo* display increased self-aggregation *in vitro* [211]. Reciprocally, melanoma cell line-derived subclones selected for increased *in vitro* aggregate formation were shown to be more metastatic in mice [212].

Whereas early studies on correlations between cell-cell adhesiveness and tumor phenotypes were largely performed in established cell lines, the advent of technologies for isolation and characterization of circulating tumor cells (CTCs) from patients further hints at the pro-tumorigenic role of cell-cell aggregation. Indeed, among CTCs from the blood of patients with breast, colorectal, prostate and lung cancers, as well as in mouse tumor models, there is a noted preference for multicellular cluster formation [213-215], with one study detecting as many as 50% of all CTCs from the blood of breast and lung cancer patients to be residing in multicellular clusters [216]. Importantly, clusters of CTCs, named circulating tumor microemboli (CTMs) were shown to display reduced levels of apoptotic markers compared to isolated CTCs, although the possibility that isolated CTCs become separated from CTM clusters as a result of their apoptosis, cannot be ruled out [213]. Circulating tumor microemboli are associated with poor prognosis and are a frequent cause of death in patients with invasive cancers [217]. In inflammatory breast carcinoma, numerous microemboli tend to accumulate within the lymphatic

vasculature [218]. Tumor clumps released by a primary tumor are detected in venous outflow in a third of a cohort of patients with clear cell renal carcinoma [219]. In one striking demonstration, when mutant allele frequencies of 48 point mutations and small indels were assessed by deep sequencing in the primary tumor and the metastatic lesion from the same patient, the mutant allele frequency for 26 mutations was the same in the metastatic lesion and the primary tumor, while 20 mutations displayed an increase and yet another two – a decrease in the metastatic lesion, suggesting that the metastasis originated not from one but from at least three initiating cells [220]. Furthermore, an *ex vivo* microscopy study provided a direct demonstration that intravascular CTMs can initiate metastatic colonies directly inside micro capillary lumens, bypassing the extravasation step altogether [221].

Finally, the increase in adhesive behavior of cancer cells is not limited to self-aggregation, and interactions with a variety of other cell types were shown to be essential for dissemination of cancer cells and subsequent metastatic colonization. Thus, *de novo* expression of ligands for E- and P-selectins on the surface of cancer cells facilitates their adhesion to endothelial cells within target organ microvasculature and promotes intravasation and metastatic lesion formation, and are associated with the worse disease outcome [222]. Moreover, signaling elicited by physical association of cancer cells with platelets [223] and macrophages [224] have been demonstrated to be essential for successful seeding and metastatic outgrowth.

Taken together, several lines of evidence suggest that increased cell-to-cell adhesiveness may protect cells from anoikis and is under positive selection during tumorigenesis, and is a prominent phenomenon during cancer cell dissemination by through vasculature. Despite that, specific drivers of cell-cell aggregation exhibited by cancer cells and mechanisms of their action are not well understood.

PVRL (nectin) family of cell adhesion molecules

Having screened a library of 8,000 open reading frames (ORFs) to identify ORFs that are able to promote anchorage-independence in TL-HMECs, we recovered 11 potent inducers of this phenotype. In particular, one ORF that consistently tested as strongest in our dataset corresponded to a full-length PVRL4 gene (poliovirus receptor-like 4), also known as Nectin-4. Together with PVRL1, PVRL2 and PVRL3, it comprises a family of four calcium-independent cell adhesion molecules (reviewed in [225, 226]). Nectins belong to IgCAM superfamily and play a prominent role in morphogenesis of various tissues through the formation of heterotypic types of cell-cell contacts. All four family members have a sequence homology to the poliovirus receptor (PVR), and three of the nectin family members were demonstrated to act as viral receptors themselves. Thus, PVRL1 and PVRL2 have been originally cloned as receptors for herpesvirus α [227, 228], and PVRL4 is a receptor for measles virus [229, 230].

Nectins mediate cell-cell adhesion between a wide variety of cell types, including epithelial cells, fibroblasts and neurons. A prototypical nectin is a single-pass transmembrane protein with three immunoglobulin-like domains in the extracellular part, a transmembrane domain and a short cytoplasmic tail containing no known domains. The exception is a E/A-x-Y-V motif at the C-terminus of nectins, which has been characterized as a PDZ-domain-interacting sequence [231]. Notably, PVRL4 is the only nectin that does not contain this motif but is nevertheless capable of interacting with PDZ-containing proteins [232].

Nectin-mediated cell-cell attachments take place at early stages of adherens and tight junction formation, but nectins are also capable of mediating less stable cell-cell interactions in a manner that is autonomous from other junctional structures. For a contact to form, nectins first form a *cis*-homodimer on the surface of one cell, which then *trans*-interacts with a *cis*-

homodimer on a cell juxtaposed to it, thus forming a heterotetramer. Cell-cell contacts formed by nectins share two distinguishing characteristics. First, different nectins exhibit a specific preference for a *trans*-interacting partner. *In vitro* binding studies have demonstrated that PVRL1 preferentially interacts with PVRL3 and PVRL4 [232, 233], whereas PVRL2 interacts only with PVRL3. Some nectins also interact with other IgCAM classes. For example, a *trans*-interaction of PVRL3 with PVR in MDCK cells has been described [234], and PVRL2 was shown to interact with NK-cell specific receptor TIGIT, which inhibits NK cell activation [235].

Second, a *trans*-interaction of nectin dimers at a site of a cell-cell contact has been proposed to trigger the formation of nectin polymers at the site of contact. In particular, crystal structure analysis of individual PVRL1 and PVRL3 *cis*- and *trans*-dimers [236] suggests that the mode of interaction in which two *cis*-homodimers bind in *trans*- can triggers formation of long chains of alternating *cis*- homodimers at the extracellular interaction interface between two cells, thus increasing the avidity of nectin-based contacts dramatically.

The heterotypic nature of nectin-driven cell-cell interactions is reflected in the role various nectins play in facilitating interactions between two distinct cell types; in which cells of different types express two different nectins with preferential affinity for *trans*-interacting with one another. A wide variety of such inter-cell type interactions have been described, including the following: (a) in the hippocampus, between axon-containing neurons (PVRL1) and dendrite-containing neurons (PVRL3) [237] (b) in the hindbrain, between commissural axons (PVRL1) and floor plate cells (PVRL3) [238], (c) in the ciliary body of an eye, between pigment (PVRL1) and non-pigment epithelial cells (PVRL3) [239]; (d) in the testis, between Sertoli cells (PVRL2) and spermatid cells (PVRL3) [240] and (e) in the developing tooth, between ameloblasts and cells of stratum intermedium [241]. Furthermore, the *trans*-interacting nature of nectins drives

formation of some unique cellular arrangements, such as a checkerboard-like pattern of auditory epithelium within the organ of Corti [242].

PVRL4 and its function in mammalian tissue patterning remains the least studied. While other members of the family are expressed in wide spectra of tissues and cell types, PVRL4 is expressed in only a limited number of tissues [243], namely, skin epidermis and hair follicles, teeth, cornea, trachea and placenta. In particular, expression of PVRL4 in stratified epithelia of skin and hair follicles is restricted to intermediate layers of skin and hair epithelia, whereas both topmost, terminally differentiated (cornified) layer and the basal layer, which consists of cells directly anchored on the basement membrane are negative for PVRL4 staining [244].

Recently, mutations of PVRL4 and PVRL1 have been identified in patients with ectodermal dysplasia syndrome [244-247]. Ectodermal dysplasia can be caused by multiple genes and the severity of symptoms, which affect a variety of tissues, including skin, teeth, nails, sweat glands and hair, vary greatly [248]. Whereas patients with PVRL1 mutations that disrupt its ability to *trans*-interact with other nectins exhibit severe craniofacial abnormalities such as cleft palate, patients with loss-of-function PVRL4 mutations are characterized with milder symptoms, namely, sparse brittle hair and distinctly small teeth. These are consistent with some but not all sites where PVRL4 is known to be expressed, and suggest that other nectins or other functionally related CAMs may have some functions that are redundant with PVRL4.

In addition to their role as “intercellular glue”, nectins interact with various cytoplasmic proteins through their PDZ-binding C-terminal sequences and with other membrane proteins through a lateral *cis*-interaction. Among proteins shown to be recruited to C-termini of nectins are afadin (AF6) [249] and PAR-3 [250]. The functional consequences of afadin interaction with nectins in MDCK cells are well studied. Specifically, it triggers the assembly of

afadin/Rap1/p120^{ctn} complex, which positively regulates membrane retention of E-cadherin, further assisting in adherens junction (AJ) formation in MDCK cells [251].

In addition to interactions with PDZ-containing proteins via their C-termini, two instances in which nectins engage in lateral interactions with other transmembrane proteins have been described. In particular, through their extracellular regions, PVRL1 and PVRL3 recruit an activated form of αVβ3 integrin to sites of adherens junctions, which also serves to potentiate downstream signaling [252]. After an adherens junction is established, PVRL3 further acts to recruit a PTPμ phosphatase via C-terminal region. This phosphatase then inhibits interaction of αVβ3 integrin with talin, dampening the level of integrin-dependent downstream signaling [253]. Another known example of nectin *cis*-interacting with other membrane-localized proteins is a recruitment of PDGF receptor by PVRL3-PVR interaction to sites of cell-cell contact in embryoid bodies which results in the potentiation of PDGF ligand-stimulated downstream signaling of PRGF receptor, which was shown to be afadin-dependent [254].

To summarize, nectins play roles in promoting contact formation between cells of same as well as of distinct identity, which is made possible as a result of preferential heterotypic affinities between different nectins. Nectins are involved in guiding morphogenic processes in various tissues. Devoid of catalytic domains themselves, nectins recruit other signaling and scaffold proteins either through their cytoplasmic C-termini or laterally through their extracellular regions. In addition, it has been proposed that *trans*-interacting nectin *cis*-dimers self-organize into supramolecular structures at a site of cell-cell contact. Whereas other nectins are ubiquitously expressed, PVRL4 (nectin-4) has a limited pattern of tissue expression and is the least studied member of nectin family.

PVRL4 is overexpressed in several types of solid tumors

Our initial analysis of the list of validated candidates has shown that 8 out of 11 ORFs, including PVRL4, corresponded to genes that localized to regions of focal amplification in tumors, according to a publicly available dataset [34]. In breast tumors in particular, PVRL4 localizes to a statistically defined peak of focal amplification containing only 12 other genes, none of which known oncogenes, according to the Cancer Gene Census [29]. This peak of focal amplification was shared by 8% of all breast tumors in the dataset; furthermore, the PVRL4 locus was gained without being focally amplified in another 30% of breast tumors.

In addition to genomic evidence, expression of PVRL4 protein has been detected in large subsets of breast ductal adenocarcinoma [255], non-small cell lung adenocarcinoma [256] and ovarian serous carcinoma [257]. In particular, immunohistochemical analysis of breast ductal adenocarcinomas revealed that 62% of analyzed samples were positive for PVRL4, whereas its expression was absent in normal mammary epithelia [255], suggesting that PVRL4 expression can be upregulated through mechanisms other than focal amplification. In addition, PVRL4-positive tumors were strongly associated with the poor five-year prognosis, and this striking correlation held true for patients with breast cancer ($p<0.0001$, [258]) and patients with non-small cell lung cancer ($p<0.0001$, [256]).

In summary, PVRL4 is strongly overexpressed and amplified in several types of cancers and is a predictor of poor outcome. The utility of PVRL4 as a potential tumor marker has been proposed; however, its functional involvement in transformation remains unclear. As discussed above, the investigation of the contribution of specific mediators of cell-cell adhesion in tumorigenesis is a conceptually intriguing problem. Thus, we set out to investigate whether PVRL4 promotes cell-to-cell attachment in TL-HMECs and if so, whether (and how) these contacts play a role in anchorage-independence phenotype.

Results

The cytoplasmic region of PVRL4 is dispensable for anchorage-independent growth

We focused our attention on the top scoring candidate identified in the screen, PVRL4 (poliovirus receptor-like 4). To identify regions of PVRL4 responsible for driving anchorage-independent colony formation, we created a series of PVRL4 deletion constructs, stably transduced them into TL-HMECs and verified their expression by Western blot (Figure 6a, b). Importantly, the endogenous expression of PVRL4 was not detectable in TL-HMECs (Figure 6b), which is consistent with the absence of its expression in normal mammary gland by immunohistochemical analysis [255].

Ectopic expression of a full-length PVRL4 strongly induced anchorage-independent colony formation. Remarkably, deletion of the afadin-binding region of PVRL4, or of entire cytoplasmic region of PVRL4 did not affect its ability to promote anchorage-independent colony formation in TL-HMECs (Figure 6c).

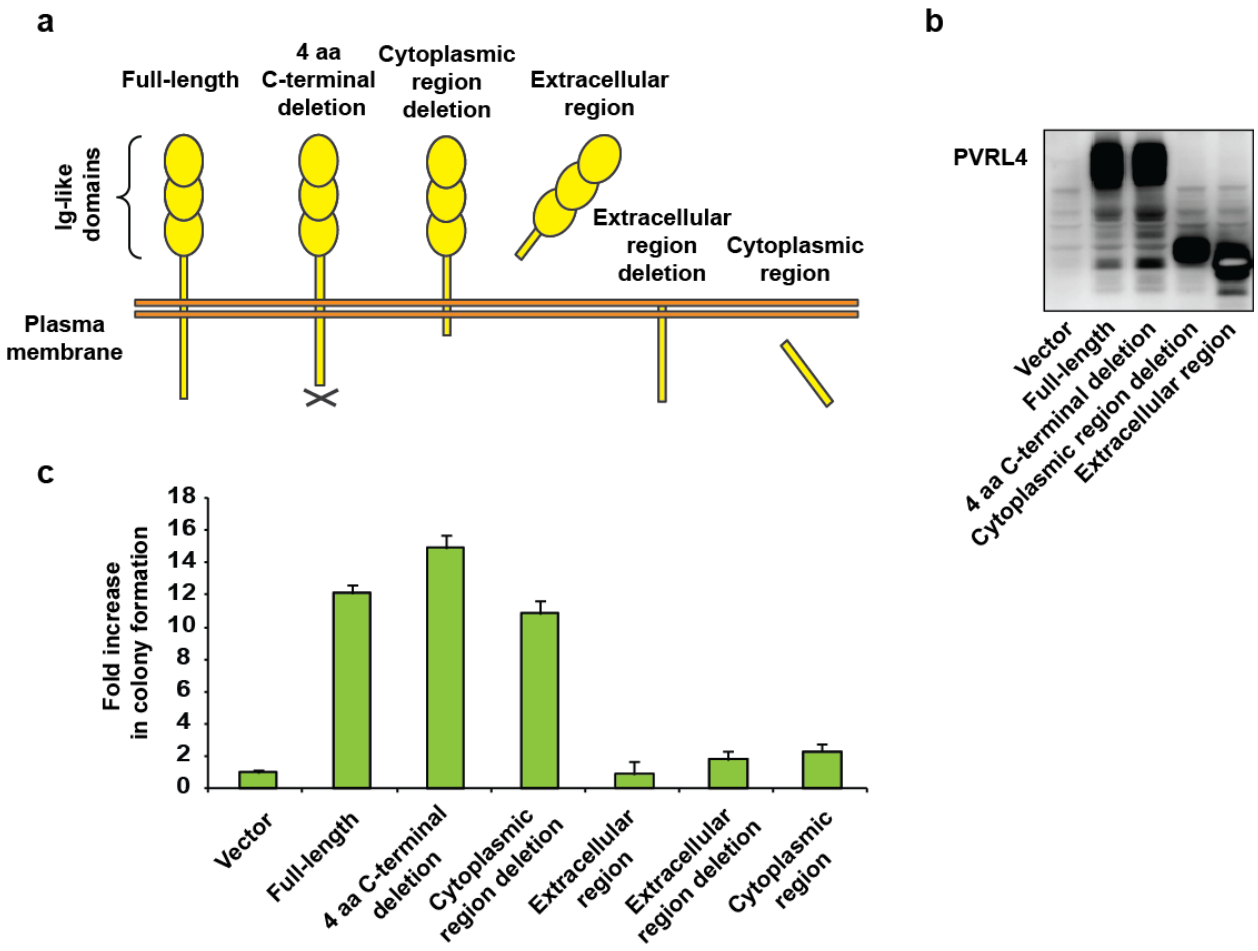


Figure 6. PVRL4-induced anchorage-independent colony formation is carried out through its extracellular region. a, b, A series of PVRL4 deletion constructs were designed and their expression confirmed by Western blot. **c,** PVRL4 mutants from **(a)** were tested for their ability to induce ECM-independent colony formation in triplicate (error bars ± SD).

The methylcellulose colony formation assay measures not only cell survival in the absence of attachment but also clonogenic potential of individual cells. We next asked whether cell viability in the absence of attachment will be enhanced by PVRL4 in the absence of the clonogenic growth requirement - thus, in conditions less stringent than those of the transformation assay - we cultured a population of TL-HMECs in suspension (as described in Methods section) and measured their ATP content by CellTiterGLO assay. Both the full-length

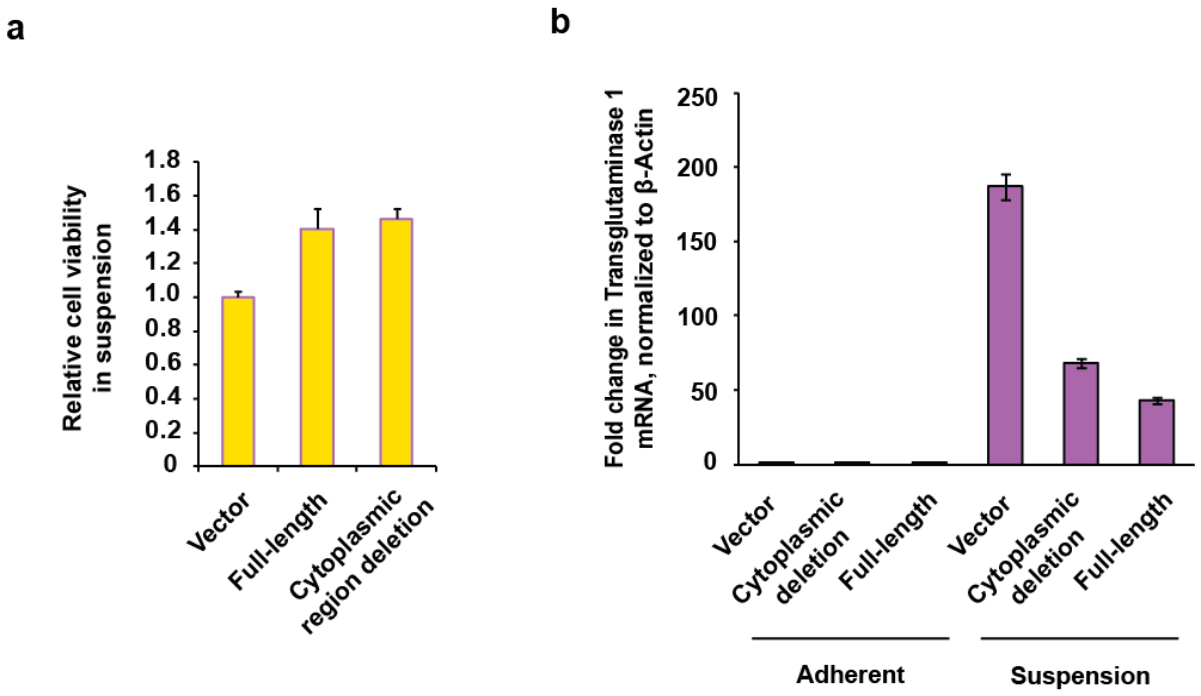


Figure 7. PVRL4 promotes cell viability and blocks the initiation of squamous differentiation of TL-HMECs is suspension. a, Cells with full-length PVRL4 or the cytoplasmic region deletion mutant were assayed for viability under conditions of ECM deprivation by measuring total ATP content in cells cultured on ultra-low attachment plates for 72 hours. Values were normalized to empty vector-transduced sample. Assays were performed in triplicate (error bars ± SD). **b,** TL-HMECs expressing empty vector, full-length PVRL4 or cytoplasmic region deletion mutant containing cells were cultured on tissue culture-treated (adherent) or ultra-low attachment (suspension) dishes for 72 hours. RNA was isolated and mRNA levels of Transglutaminase 1, a marker of squamous differentiation, were measured by RT-qPCR. Transcript levels were normalized to β-Actin. qPCR was performed in quadruplicate (error bars ± SD).

PVRL4 and its cytoplasmic deletion mutant promoted cell viability, further verifying that PVRL4 promotes cell survival in the absence of attachment (Figure 7a).

Interestingly, when cultured in attachment-deprived conditions, non-transformed mammary epithelial cells are known to enter an alternative mode of detachment-triggered cell death, characterized with a robust induction of terminal squamous differentiation markers. This was previously described in hTERT-immortalized HMECs and MCF10A cells [142], and

parallels the response of keratinocytes to ECM deprivation [259]; a phenomenon that is perhaps best explained by a common epidermal lineage that these two cell types belong to. In agreement with what was seen in other non-transformed mammary epithelial cell lines, suspension-cultured TL-HMECs did not undergo apoptosis in response to anchorage deprivation, yet they potently induce squamous differentiation markers, such as transglutaminase TGM1 (Figure 7b), keratin 6A and involucrin (not shown). Notably, expressing full-length PVRL4 or its cytoplasmic region deletion mutant attenuated TGM1 induction. These data demonstrate that PVRL4 protects TL-HMECs from the attachment deprivation-associated differentiation and allows proliferation in attachment-free conditions. Furthermore, this phenotype is facilitated by the extracellular part of PVRL4 rather than by its intracellular region.

PVRL4 facilitates cell-cell contact assembly which is required for anchorage-independent growth

To verify experimentally whether PVRL4 can promote cell-to-cell attachment in TL-HMECs, we performed aggregate formation assays with PVRL4-expressing and control cells. We observed that while TL-HMECs do not readily form aggregates in suspension by themselves, expression of PVRL4 drives rapid association of TL-HMECs into multicellular clusters within a short time span of 0.5-1 hour (Figure 8a, b).

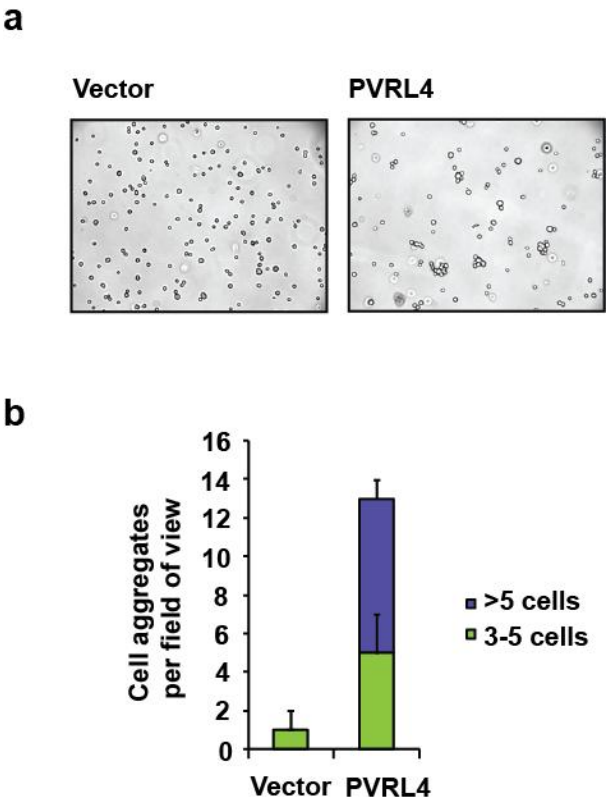
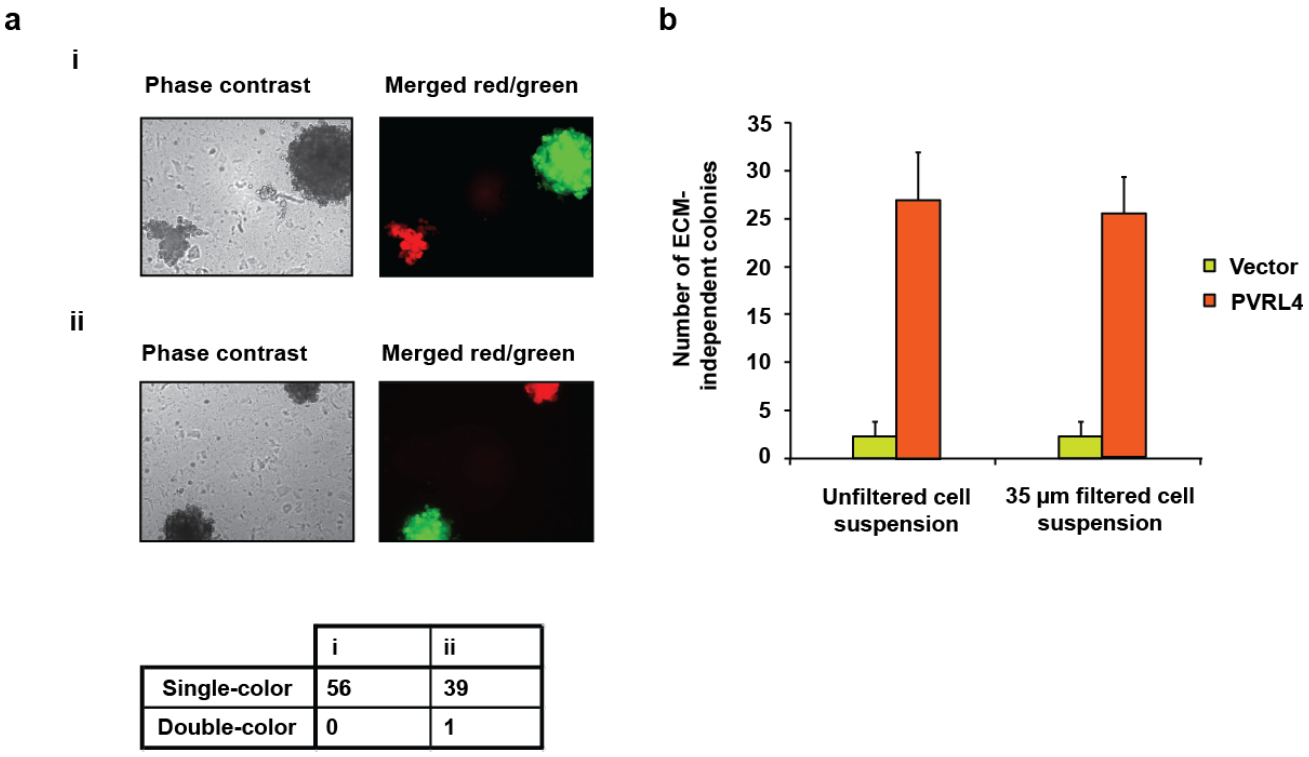


Figure 8. PVRL4 promotes cell clustering of TL-HMECs. a, Cells were dissociated off the tissue culture surface with trypsin-free cell dissociation buffer and kept in suspension for 1 h. **b,** small (3-5 cells) and large (>5 cells) cell clusters per field of view were counted, n = 3 (error bars ± SD).

We sought to rule out the possibility that anchorage-independent colony formation that we observed in PVRL4-expressing TL-HMECs was simply a result of a tissue culture artifact associated with either incomplete dissociation of cells prior to seeding into semi-solid medium or a potential *de novo* association of cells during growth in anchorage-independent conditions. To address this, we mixed equal numbers of dsRed- and GFP-labeled TL-HMECs transduced with PVRL4 and (i) seeded them into methylcellulose; or (ii) co-cultured the mixed population on adherent surface, subsequently seeding them into methylcellulose. Examining the colors of



	i	ii
Single-color	56	39
Double-color	0	1

Figure 9. PVRL4-induced ECM-independent colonies originate from single cells. a, PVRL4 expressing TL-HMECs were stably transduced with dsRed or GFP and mixed in equal proportions, followed by (i) immediate plating into semi-solid medium, or (ii) co-culturing on adherent surface for 2 days, followed by plating into semi-solid medium. Resulting colonies were visualized under a fluorescent microscope and each colony was assessed for presence of red and green fluorescence. Representative phase-contrast and fluorescent images (red and green channel) are shown. **b,** Colony formation efficiency of TL-HMECs transduced with empty vector or vector expressing PVRL4 was compared between cell suspensions that were filtered through a 35 µm nylon mesh strainer prior to plating into methylcellulose, or left unfiltered. ECM-independent colony formation assays were performed in triplicate (error bars ± SD).

resulting anchorage-independent colonies revealed that out of 56 colonies from the sample (i), all 56 were single-color colonies, whereas in the sample (ii), 39 out of 40 colonies were single-color, and only one colony contained both GFP and dsRed-positive cells (Figure 9a). The rarity of doubly-colored colonies demonstrates that PVRL4-induced colonies originate predominantly from individual cells. Consistent with this observation, passing cells through a 35 µm cell

strainer immediately prior to being plated in anchorage-independent conditions did not affect colony numbers (Figure 9b), further confirming that PVRL4-induced colonies are clonal and that the observed increase in anchorage-free growth is not a result of pre-existing multicellular clusters having a survival advantage over single cells when grown in conditions of matrix loss.

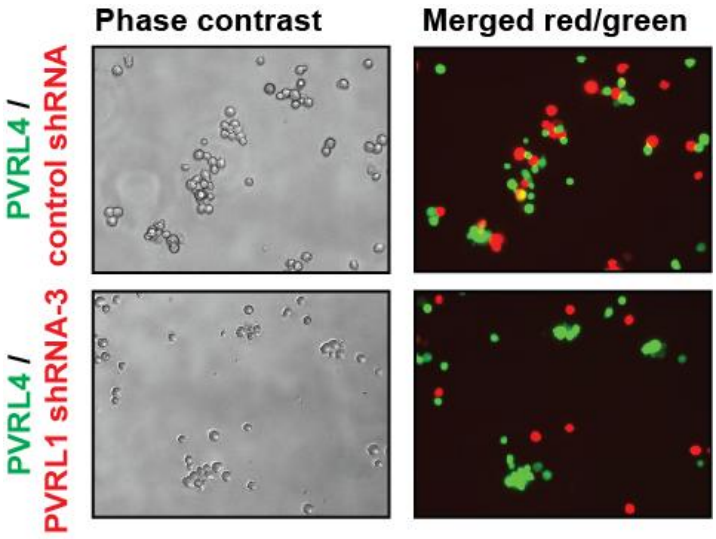


Figure 10. PVRL4-driven cell-cell clustering is dependent on its putative *trans*-interaction partner PVRL1. GFP-labeled PVRL4-expressing TL-HMECs were allowed to aggregate with dsRed-labeled cells expressing either a PVRL1-targeting shRNA or a control shRNA. Representative phase-contrast and fluorescent images (red and green channels superimposed) are shown.

The extracellular region of PVRL4 exhibits preferential affinity for *trans*-interacting with its counter-receptor PVRL1 [232] which is endogenously expressed in TL-HMECs. To test whether PVRL1 was necessary for PVRL4-driven cell-cell adhesion in TL-HMECs, we used an shRNA to stably deplete the PVRL1 transcript in a dsRed-labeled population of TL-HMECs and allowed cells to aggregate with PVRL4-expressing GFP-labeled TL-HMECs. PVRL1 depletion resulted in exclusion of dsRed-labeled cells from multicellular clusters, whereas control shRNA-expressing cells were readily incorporated (Figure 10), confirming that PVRL4-mediated cell-cell contacts are carried out through its interaction with a counter-receptor PVRL1.

We then asked whether disrupting PVRL4-driven cell-cell contact formation via stably depleting its *trans*-interacting partner PVRL1 affects the ability of PVRL4 to promote colony formation in the absence of anchorage. To address this, we stably depleted PVRL1 transcript with two independent shRNA constructs and verified the degree of depletion by qPCR (Figure 11a). Seeding PVRL1 shRNA- or control shRNA-transduced PVRL4-expressing TL-HMECs revealed that anchorage-independent colony formation was abolished by PVRL1 depletion (Figure 11b). This result paralleled the effect of PVRL1 depletion on PVRL4-driven cell-cell clustering and strongly implicated PVRL4/PVRL1-dependent cell-cell contacts in driving anchorage-independent growth.

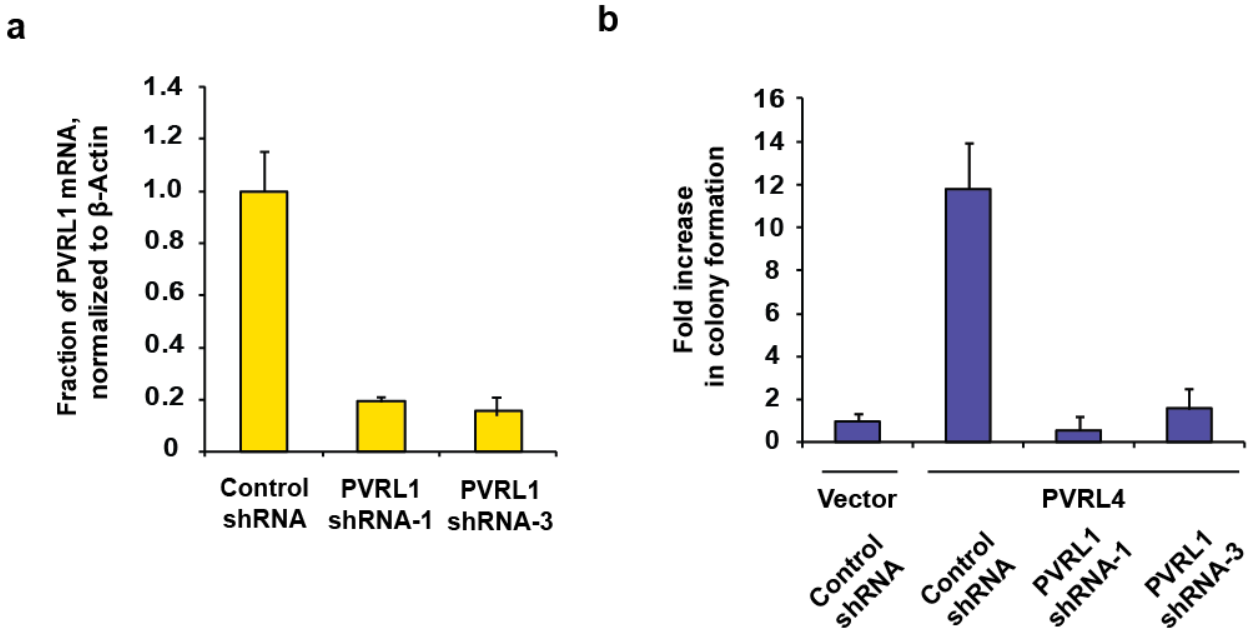


Figure 11. PVRL4-driven anchorage independence requires PVRL1. a, Efficiency of shRNA-mediated PVRL1 mRNA depletion was measured by RT-qPCR. PVRL1 transcript abundance was normalized to β-Actin. qPCR was performed in quadruplicate (error bars ± SD). **b,** PVRL4 was co-expressed with the indicated shRNAs and ECM-independent colony formation in TL-HMECs was assayed. Values were normalized to empty vector-transduced sample. Assays were performed in triplicate (error bars ± SD).

To further assess the involvement of PVRL4-PVRL1 *trans*-interaction on juxtaposed cells in driving anchorage-independent colony formation, we sought a physical means by which to disrupt PVRL4-induced cell-cell contacts to examine its effects on colony formation. Specifically, we asked whether antibodies directed towards the extracellular domain of PVRL4 could block PVRL4-induced cell clustering and anchorage-independent growth. Indeed, cell clustering was completely abrogated in presence of three independent PVRL4 antibodies, whereas control IgG or a blocking antibody against E-cadherin (DECMA-1) did not produce such an effect (Figure 12a, b). In addition, an antibody targeting the extracellular region of PVRL1 also inhibited PVRL4-induced cell clustering (Figure 12c). Next, we tested whether a monoclonal antibody against PVRL4 affects anchorage-independent growth induced by it. Indeed, PVRL4-driven colony formation was inhibited to almost a basal level in the presence of a monoclonal antibody targeting the extracellular region of PVRL4, but no such effect was observed on colonies formed by cells transduced with PTEN shRNA, another transforming construct (Figure 12d), demonstrating that the observed inhibition is due to a PVRL4-specific effect of an antibody and not due to nonspecific effects. Taken together, our data indicate that PVRL4 enables anchorage-independent cell growth in a manner which is dependent on the assembly of PVRL4-PVRL1 cell-cell contacts, suggesting that these contacts may be providing a survival benefit in the context of the absence of anchorage.

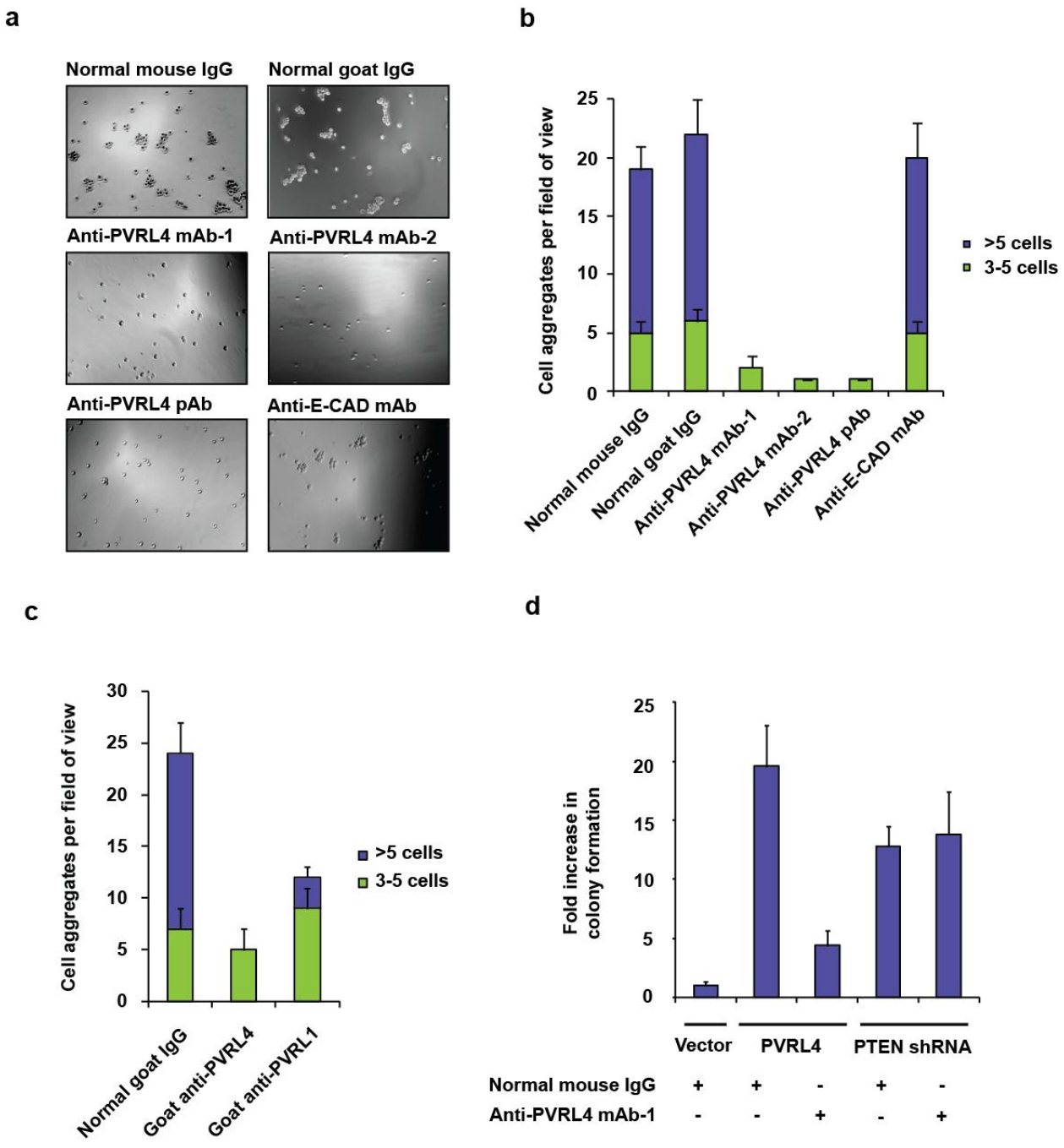


Figure 12. Antibodies directed against the extracellular region of PVRL4 disrupt PVRL4-driven cell-cell clustering and anchorage-independent growth. a-c, PVRL4-expressing TL-HMECs were allowed to aggregate in presence of the indicated antibodies or isotype controls. Representative images are shown. Cell clusters were quantified as before. **d,** Anchorage-independent growth induced by PVRL4 or an shRNA against PTEN was assayed in the presence of PVRL4-targeting antibody or control IgG. Colony numbers were normalized to control sample. Anchorage-independent colony formation assays were performed in triplicate (error bars ± SD).

The transmembrane and cytoplasmic regions of both PVRL4 and PVRL1 are dispensable for cell-to-cell attachment and anchorage-independent growth

To further probe the functional link between PVRL4-driven cell-cell contact assembly and anchorage-independent growth, we asked whether the PVRL4-PVRL1-mediated cell surface interaction alone is sufficient for driving this phenotype. To address this question, we created chimeric constructs in which extracellular regions of PVRL1 and PVRL4 were fused to transmembrane regions of an unrelated transmembrane molecule, CD8 (Figure 13a), while the cytoplasmic regions of each of the two molecules were deleted. We first introduced PVRL4-CD8tm into TL-HMECs, concomitantly depleting endogenous PVRL1 by RNAi (Figure 13d). Membrane localization of the chimeric construct was verified by immunofluorescence (Figure 13f). Expression of PVRL4-CD8tm induced both cell clustering and ECM-independent colony formation, and both phenotypes were suppressed by stable depletion of endogenous PVRL1 (Figure 13b, c). Importantly, clustering was restored when two populations of clustering-incompetent cells - PVRL4-CD8tm expressing, PVRL1-depleted cells ($PVRL4^{+}$; $PVRL1^{-}$) and control cells ($PVRL4^{-}$; $PVRL1^{+}$) - were mixed together, independently verifying that such cell contacts assemble through formation of a PVRL4-PVRL1 *trans*-interacting module (Figure 13e). Finally, both cell clustering and colony formation defects induced by PVRL1 shRNA were rescued by expression of the shRNA-resistant PVRL1-CD8tm construct (Figure 13b, c). These data indicate an on-target nature of PVRL1 depletion phenotype and demonstrate that ability to withstand altered ECM environment is driven by the PVRL4-PVRL1 cell-surface *trans*-interaction and not by intracellular or transmembrane regions of either molecule.

Figure 13. Expression of extracellular regions of PVRL4 and PVRL1 on the cell surface is sufficient for ECM-independent growth. a, Schematics representation of chimeric constructs consisting of extracellular domains of PVRL4 or an shRNA-resistant version of PVRL1 fused to the transmembrane domain of CD8 (blue). **b, c** TL-HMECs were stably transduced with the indicated combinations of expression constructs and assayed for anchorage-independent growth **(b)** and clustering **(c)**. Anchorage-independent colony numbers were normalized to control sample. Anchorage-independent colony formation assays were performed in triplicate (error bars ± SD). **d,** Expression levels of endogenous and chimeric proteins were verified by Western blot. **e,** Clustering assays were performed with TL-HMECs expressing the following transgenes: (i) empty vector/control shRNA; (ii) PVRL4-CD8tm/control shRNA; (iii) PVRL4-CD8tm/anti-PVRL1 shRNA; (iv) 1:1 mixture of (iii) and (i). **f,** TL-HMEC cells infected with PVRL4-CD8tm or empty vector were fixed with methanol and stained with goat polyclonal anti-PVRL4 antibody followed by anti-goat Alexa Fluor 488 secondary antibody.

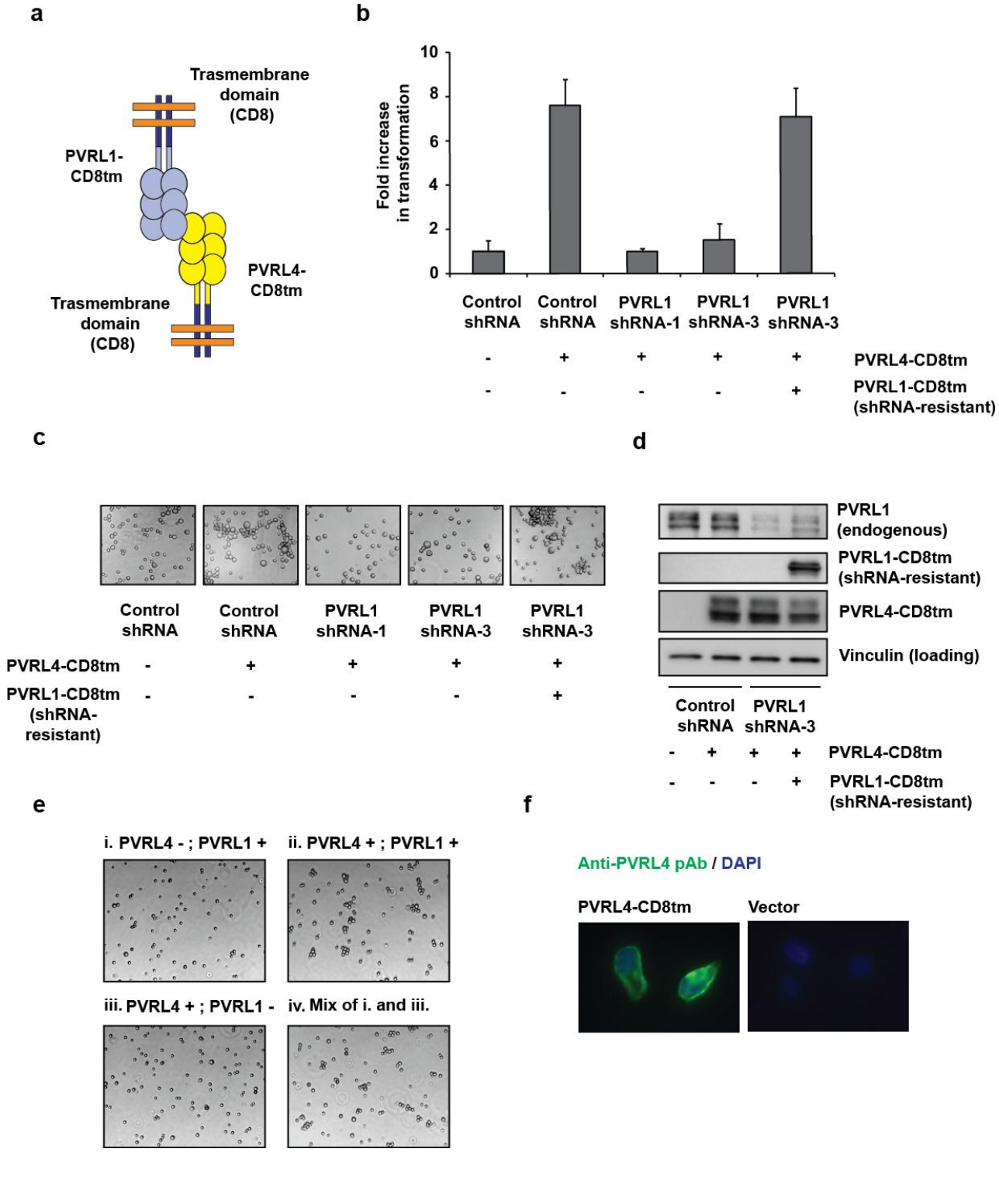


Figure 13 (Continued).

Multiple oncogenic perturbations converge upon cell-cell clustering phenotype

We have shown that PVRL4-driven anchorage-independence is dependent on its ability to induce cell-cell contacts, suggesting that in conditions of an altered extracellular matrix, certain types of intercellular interactions may act to enable matrix-independent survival. We hypothesized that other oncogenic perturbations may also promotes cell-cell contact formation in TL-HMECs (Figure 14a), and that such contacts may play a role in anchorage-free survival. Strikingly, mutant Ras^{V12}, myristoylated catalytic subunit of PI3K (Figure 14b), and shRNA-mediated depletion of PTEN (Figure 14c, d) all promoted cell-cell clustering in TL-HMECs. None of the perturbations induced endogenous PVRL4 expression, as measured by FACS (not shown), which suggests that cell-cell attachment was mediated by other adhesion mechanisms. Mixing PTEN shRNA-expressing GFP-labeled cells with control shRNA-expressing dsRed-labeled cells triggered formation of multicellular clusters that contained both GFP- and dsRed-labeled cells (Figure 14e), showing that, in a manner similar to PVRL4, PTEN-depleted cells can heterotypically attach to other cells. Our experiment reveals that increased cell-to-cell attachment is a phenotype that multiple oncogenic perturbations converge upon, and it would be of interest to investigate further, what drives the increased adhesiveness in each of these settings.

Taken together, our findings reveal a new mechanism which potentiates anchorage-independent survival that may be operational in tumors. PVRL4-driven anchorage-independent growth is carried out in a manner dependent on cell attaching to one another and is readily blocked by antibodies that disrupt this attachment, hinting at it being a node for a potential therapeutic intervention. In addition, exploring whether increased cell-to-cell attachment in oncogene-transformed cells contributes to the prosurvival effect can further shed light on how various types of cell-cell adhesions contribute to tumorigenesis.

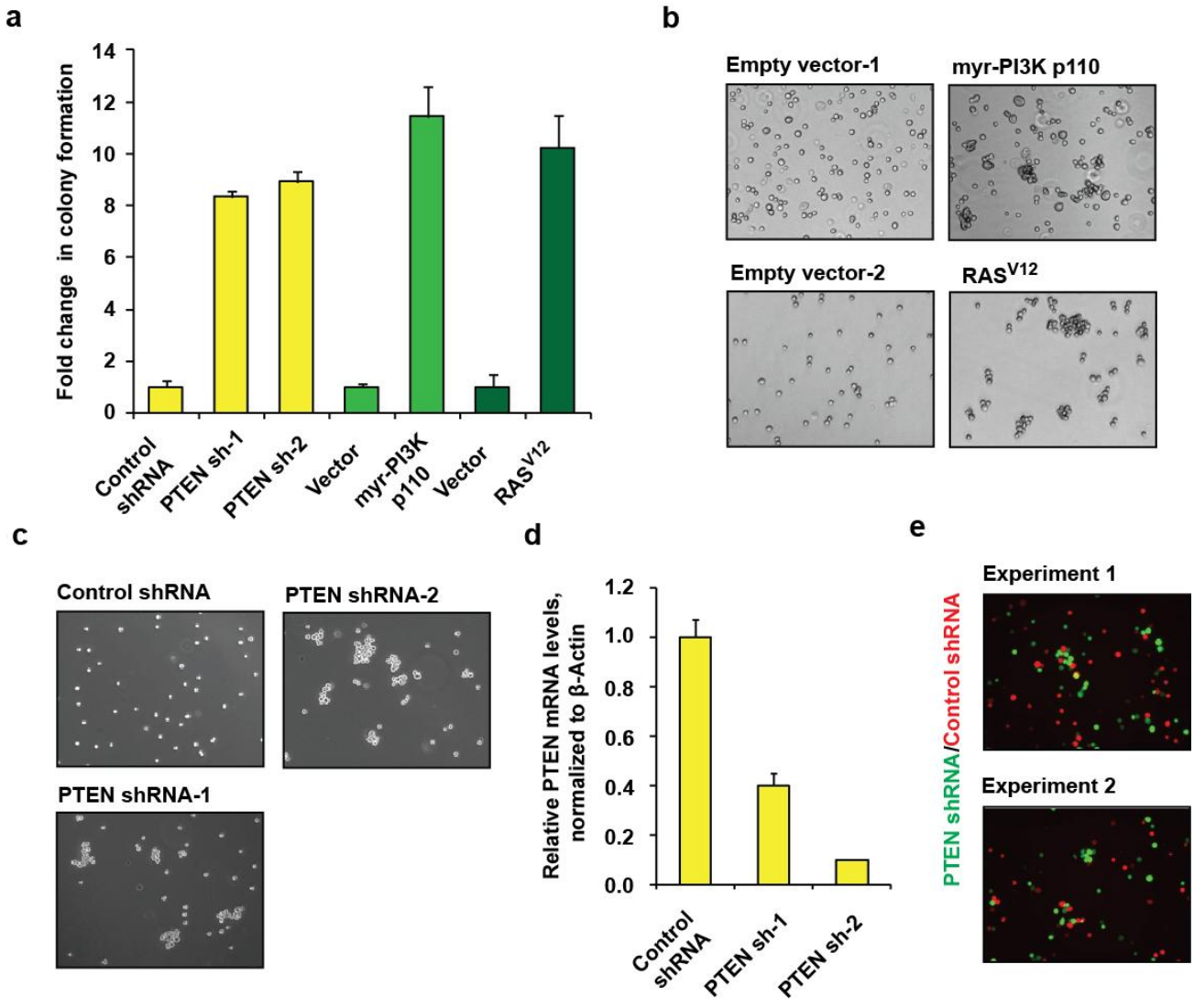


Figure 14. Clustering phenotypes are induced by various oncogenic perturbations. a, TL-HMECs transduced with the indicated constructs were assayed for ability to promote anchorage-independent colony formation in triplicate (error bars ± SD). **b,** TL-HMECs were stably transduced with indicated constructs and assayed for cell-cell clustering and colony formation in the absence of attachment. Representative clustering assay images are shown. **c,** TL-HMECs expressing two independent PTEN shRNAs or control shRNA were assayed for cell-cell clustering. Representative images are shown. **d,** Efficiency of PTEN mRNA depletion was measured by RT-qPCR. PTEN transcript abundance was normalized to β-Actin. qPCR was performed in quadruplicate (error bars ± SD). **e,** GFP-labeled PTEN-depleted TL-HMECs were allowed to aggregate with dsRed-labeled control shRNA-expressing cells. Images were taken and processed as before.

Materials and Methods

Constructs and virus production. For PVRL4 structure-function analysis, full-length ORF (accession number BC010423, aa 1-510) or indicated fragments (Supplementary Table 1) were generated by PCR and subcloned into pQCXIN retroviral vector (Clontech). PVRL4-CD8 and PVRL1-CD8 chimeric fusions were created the following way: extracellular region of PVRL4 (accession number BC010423, aa 1-342) or PVRL1 (accession number BC113471, aa 1-354) was amplified by PCR using a reverse primer containing a sequence for a 28-aa-long transmembrane domain of CD8A gene (accession number NM_001768, 543-626 bp), followed by a STOP codon. The resulting PCR product was subcloned into pQCXIN vector. To create an shRNA-resistant PVRL1-CD8 construct, silent mutations were introduced via site-directed mutagenesis with the following primers: sh3ResFW 5'-CCAGGCGTCCACAGTCAAGTTGTGCAAGTCAATGACTCCATGTATG-3' and sh3ResRV 5'-CATACATGGAGTCATTGACTTGCACAACTTGACTGTGGACGCCTGG-3' using QuikChange II Site-directed mutagenesis kit (Agilent). Stable RNAi-mediated depletion was performed with shRNAs expressed in either pMSCV-PM or pGIPZ vector in miR-30 context that were either picked from the Hannon-Elledge shRNA library (Open Biosystems) or designed *de novo* (design and cloning protocol described in [260]). 21-nt sense sequences of shRNAs used in this study are listed in Supplementary Table 2. For stable labeling with fluorescent markers, cells were transduced with pHAGE-dsRed and pMSCV-CMV-GFP viruses. For assaying cell clustering in presence of oncogenes, TL-HMECs were transduced with pWZL-myr-p110-PI3K-Neo or empty pWZL-neo, and pBABE-H-RAS^{V12}-puro or empty pBABE-puro. Retro- and lentiviral supernatants were generated by transient transfection of 293T cells following Mirus Bio's TransIT transfection protocol and harvested 48 hrs later.

Cell Culture. TL-HMECs expressing hTERT and SV40 Large T antigen [178] were cultured in MEGM (Lonza). Retroviral infections were performed in presence of 8 ug/mL of polybrene (Sigma). Successful viral integrants were selected with puromycin (2 ug/mL) or Geneticin (200 ug/mL).

ECM-independent colony formation and anoikis assays. ECM-independent colony formation assays were performed as previously described [98] with minor modifications. Briefly, cells were suspended in reduced growth factor MEGM (containing 50% of kit-supplied BPA, Insulin, EGF and hydrocortisone) with 2% methylcellulose (Sigma) and plated on tissue culture dishes precoated with 0.6% Noble Agar (Sigma) in MEM (Invitrogen). For assays performed in 6 cm dishes, 4.5×10^4 cells per dish were plated. For assays performed in 6-well plates, 1.2×10^4 cells per well were plated. Colonies were counted after three weeks of growth. For each assay, an average of three replicates +/- SD is shown. For ECM-independent colony formation assays in presence of antibodies, the following antibodies were used: normal mouse IgG (MAB004, R&D Systems), mouse anti-human PVRL4 IgG2B (MAB2659, R&D Systems) at 4 μg/mL. For anoikis assays, cells were seeded on ultra-low attachment dishes (Corning) in reduced growth factor MEGM with 1% methylcellulose. For assays performed in 10 cm dishes, 1.4×10^5 cells were plated; for assays performed in 6-well dishes, 2.0×10^4 cells per well were plated. Total ATP measurements were performed using CellTiterGLO reagent (Promega) according to the manufacturer's protocol after 72 hours of growth in suspension, and luminescence values were read with Victor X5 plate reader. For isolation of RNA or protein lysates, cell pellets were harvested after 72 hours of growth in suspension and washed with cold PBS prior to lysis.

Clustering Assays. Cells were gently detached off the tissue culture surface with enzyme-free cell dissociation buffer (Invitrogen) and washed once with complete medium. 1.0 x

10^5 cells were allowed to aggregate in 1 mL of complete medium in a 15-mL conical tube at room temperature. Tubes were gently flicked in the process to visually assess the progression of clustering. After 1-1.5 hr of incubation, cell suspension was poured into wells of 12-well dishes and allowed to attach to the bottom of the dish for 5-10 minutes. Cells were promptly visualized and photographed under phase contrast and fluorescent filters using AxioVert inverted microscope. When clustering assays were performed in the presence of antibodies, following antibodies were used: normal mouse IgG (MAB004, R&D Systems), normal goat IgG (AB-108-1C, R&D Systems), mouse anti-human PVRL4 IgG2A (MAB26591, R&D Systems), mouse anti-human PVRL4 IgG2B (MAB2659, R&D Systems), goat anti-human PVRL4 (AF2659, R&D Systems), goat anti-human PVRL1 (AF2880, R&D Systems), DECMA-1 (anti-human E-Cadherin, ab11512, Abcam). All antibodies were used at a concentration of 4 μg/mL. For short-term culture of TL-HMECs in suspension, 4.0 x 10^5 cells were allowed to aggregate in 1 mL of complete medium in a 15-mL conical tube at room temperature, mixed with 5 mL of 0.5% methylcellulose in reduced growth factor MEGM and incubated in wells of a 6-well ultra-low attachment dish (2 wells per sample) for indicated periods of time.

RT-qPCR. Total RNA was isolated from cells using RNAeasy Plus kit (QIAgen). cDNA was synthesized from 1 μg of total RNA, using Superscript III Reverse Transcriptase (Invitrogen) and Oligo-dT primer (Invitrogen), following the manufacturer's protocol. Quantitative PCR was performed with the Platinum SYBR Green qPCR Supermix-UDG kit (Invitrogen) on Applied Biosystems 7500 Fast Real Time PCR machine. Gene-specific primers were designed using Universal Probe Library (Roche Applied Science). PCR reactions were carried out in triplicates or quadruplicates. For each value, an average of at least three technical

replicates +/- SD is shown. Sequences of gene-specific primers used for qPCR are listed in Supplementary Table 3.

Immunofluorescence. TL-HMEC cells were cultured on chamber slides (BD Biosciences), fixed with cold methanol following by blocking in blocking buffer containing BSA and cold water fish gelatin (Sigma-Aldrich). Cells were incubated with primary goat anti-PVRL4 antibody (AF2659, R&D Systems), at 1:500 dilution at 4C overnight, followed by 1 hour incubation with chicken anti-goat Alexa-Fluor 488 secondary antibody (Invitrogen) at 1:2500 dilution. Cells were mounted in Vectashield mounting medium with DAPI (Vector Labs) and visualized under a fluorescent microscope.

Chapter IV: Genetic and pharmacological validation of PVRL4 as a drug target

Background and Rationale

Antibody-based cancer therapeutics

The proverbial "magic bullets" against cancer, monoclonal antibodies are arguably among the safest and most potent kinds of cancer therapeutics. Therapeutic antibodies are selected so that an antigen specifically overexpressed on the surface of cancer cells is targeted. This creates a wide therapeutic window which largely allows sparing healthy tissues from the antibody-elicited adverse effects. In addition, antibodies are highly efficacious as antitumor agents due to the multipronged nature of their attack on a tumor cell. Finally, a variety of chemical and genetic approaches can be used to modify antibodies and further enhance their tumor-killing capabilities.

There are two distinct ways by which antibodies act to suppress tumor growth. First, antibody binding can result in **direct inhibition of the function of an oncogene** to which tumor cells display "addiction" to. Antibody binding to its antigen may directly inhibit its function via, for example, blocking its dimerization, assumption of an active conformation or interactions with ligands. In addition, antibody-antigen binding can trigger internalization of the receptor and thus cessation of signaling associated with it. A second mechanism of anti-tumor action of therapeutic antibodies is carried out via coating or opsonization of tumor cells by antibodies, which triggers the **recruitment of a number of native immune system components**. Such recruitment is mediated through interactions of the Fc region of an antibody and Fc receptors which are

expressed by several types of immune effector cells. Importantly, for opsonization to trigger immune effector cell recruitment, the concentration of antigen molecules on the cell surface must be sufficiently high, which further widens the therapeutic window between tumor and healthy tissue.

The two major classes of immune cells that become recruited to tumor cells via their Fc receptors are (a) **natural killer (NK) cells**, which elicit a so-called antibody-dependent cellular cytotoxicity (ADCC) by releasing perforin which compromises cell membrane integrity and granzyme which initiates the apoptotic cascade, and (b) **macrophages**, which carry out the antibody-dependent cellular phagocytosis (ADCP). In addition to cell-based immune responses, antibodies attached to tumor cells trigger activation of **immune complement,** which also compromises cellular membrane integrity via assembly of a membrane attack complex (MAC). This mode of antibody-triggered cell killing is termed complement-dependent cytotoxicity (CDC).

The specificity of an antibody molecule is mediated by two relatively compact complementarity-determining regions (CDRs) which are situated on Fab regions of the molecule. This property makes antibodies highly modular as therapeutic agents and their safety and efficacy can be further enhanced in a number of distinct ways. Thus, the **humanization of mouse monoclonal antibodies**, a process in which all of the antibody sequences with the exception of those that are engaged in antigen recognition are substituted with human sequences using recombinant DNA technology, greatly reduces the non-specific immune response to antibody infusion and permits their safe use in patients [261]. In addition, the modular nature of antibody molecules fosters the creation of newer generations of antibody-based therapeutics. A prominent example of antibody engineering to enhance their therapeutic potential is the creation

of **bispecific antibodies** where one of the two Fab regions targets the tumor-specific antigen and another is directed towards a CD3 receptor. A molecular design of this type allows a simultaneous recruitment of both the native immune system components via Fc region, as well as CD3-expressing cytotoxic T lymphocytes through anti-CD3 Fab entity. As a successful example, anti-EpCAM/anti-CD3 bispecific antibody catumaxomab is efficacious in treating malignant ascites (tumor cells that invade into peritoneum) [262]. Yet another direction is creation of antibodies that are chemically conjugated to chemotherapeutics, bacterial toxins and radionuclides, which allows using antibodies as vehicles for **delivery of toxic "payloads"** directly to tumor cells. This short overview covers just some of the examples of the great versatility of antibody-based therapeutics.

As a next step of this study, we sought to verify, in a preclinical setting, whether PVRL4 can have a potential utility as a target for antibody-based therapy against cancer. Indeed, we have already shown that PVRL4-driven anchorage-independent growth is dependent on cell-cell contact formation, and can be directly inhibited by monoclonal antibodies targeted against its extracellular region. In addition, PVRL4 becomes overexpressed in at least three types of solid tumors whereas in corresponding normal tissues its expression is not detected. Taken together, this suggests a potential applicability of PVRL4 as a therapeutic target. As a next step, we sought to validate whether cancer cells that overexpress PVRL4 endogenously also require it for the maintenance of transformed phenotype *in vitro* and *in vivo*. Moreover, we directly tested the proposed therapy hypothesis by asking whether PVRL4-driven xenograft growth *in vivo* can be suppressed by antibodies targeted towards its extracellular region.

Results

PVRL4 is essential for the transformed phenotype of breast cancer cells *in vitro*

Having shown that PVRL4 enables the escape of epithelial cells from the growth restriction associated with the lack of proper ECM, we next wanted to test its functional relevance to breast tumor-derived lines. We have analyzed a publicly available dataset of copy number alterations from 541 breast tumors [263] and identified high-level amplifications of PVRL4 in 6.5% of samples (Table 2). Importantly, high-level amplifications of PVRL4 were statistically significantly overrepresented in breast tumors with basal-like expression profile compared to the rest of subtypes classified by PAM50 qPCR assay [264].

Table 2. The prevalence of PVRL4 amplifications across breast cancer subtypes. The fourth column represents how many tumors in each subtype are expected to have PVRL4 amplification if there was no correlation between tumor subtype and PVRL4 amplification.

Expression subtype	Total samples in database	Samples with PVRL4 amplification	Expected by random chance	P value (Z-score statistic)
Luminal A	235	6	16.5	**0.004**
Luminal B	133	8	9.3	0.33
HER2-enriched	58	5	4	0.32
Basal	99	16	7	**0.0002**
Claudin-low	8	0	0.6	0.22
Normal-like	8	0	0.6	0.22

In particular, a high-level copy number amplification was detected in 16% of all basal-like tumors (Figure 15a), thus strongly implicating PVRL4 in the pathogenesis of a particularly aggressive breast cancer subtype. A particularly strong focal amplification (~50 kb) of a genomic region which included PVRL4 was harbored by an inflammatory breast cancer cell line SUM190 (Figure 15b) [34]. This region of focal amplification contained a total of just five genes - none of them with known roles in tumorigenesis, which raised a possibility that the amplification of this locus was due to a potential survival advantage that PVRL4 provides to cells.

SUM190 cells exhibited a strong propensity for cluster formation when incubated in suspension for 0.5-1 hours. Importantly - and in parallel with the effect seen in TL-HMECs - anti-PVRL4 antibodies (Figure 16a), as well as an shRNA against PVRL4 (Figure 16b) completely abrogated clustering of SUM190 cells. Because PVRL4-driven cell contacts are

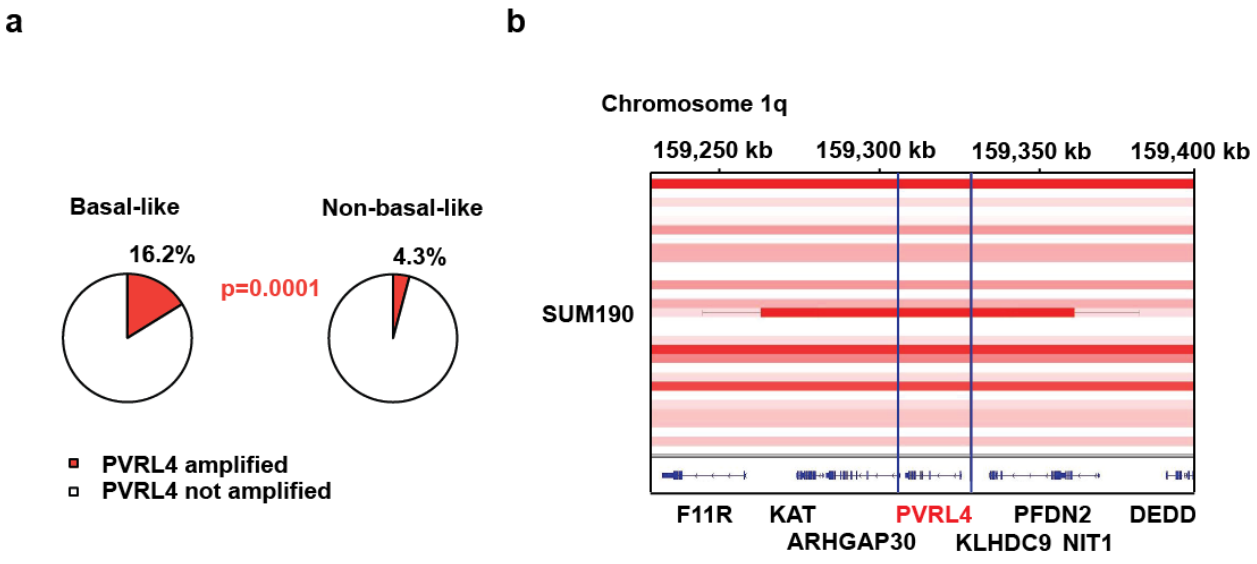


Figure 15. Amplification of PVRL4 in breast cancer. a, PVRL4-amplified samples are overrepresented in breast tumors with basal-like expression signatures. **b,** A view from the Integrated Genome Viewer program showing focal amplification of PVRL4 locus in SUM190 (HER2-amplified) cell line. The degree of amplification is denoted by the intensity of the color.

assembled via PVRL4 interaction with PVRL1, PVRL4 can potentially facilitate cell adhesion to any PVRL1-expressing cell type. Clustering phenotype is prominent in circulating tumor cells and is associated with greater metastatic capacity in mouse models. In addition, adhesion of CTCs to the endothelium can facilitate extravasation and metastatic seeding. To test this *in vitro*, we asked whether SUM190 can adhere to HMVEC-Ls, a primary microvascular endothelial cell line from human lung, which is a target organ for breast cancer metastasis. Indeed, we have observed heterotypic clustering of SUM190 and HMVEC-L cells, and just like self-self SUM190 clustering, SUM190-to-HMVEC-L clustering was abrogated by anti-PVRL4 antibody (Figure 16c) as well as by an shRNA against PVRL4 (Figure 16d).

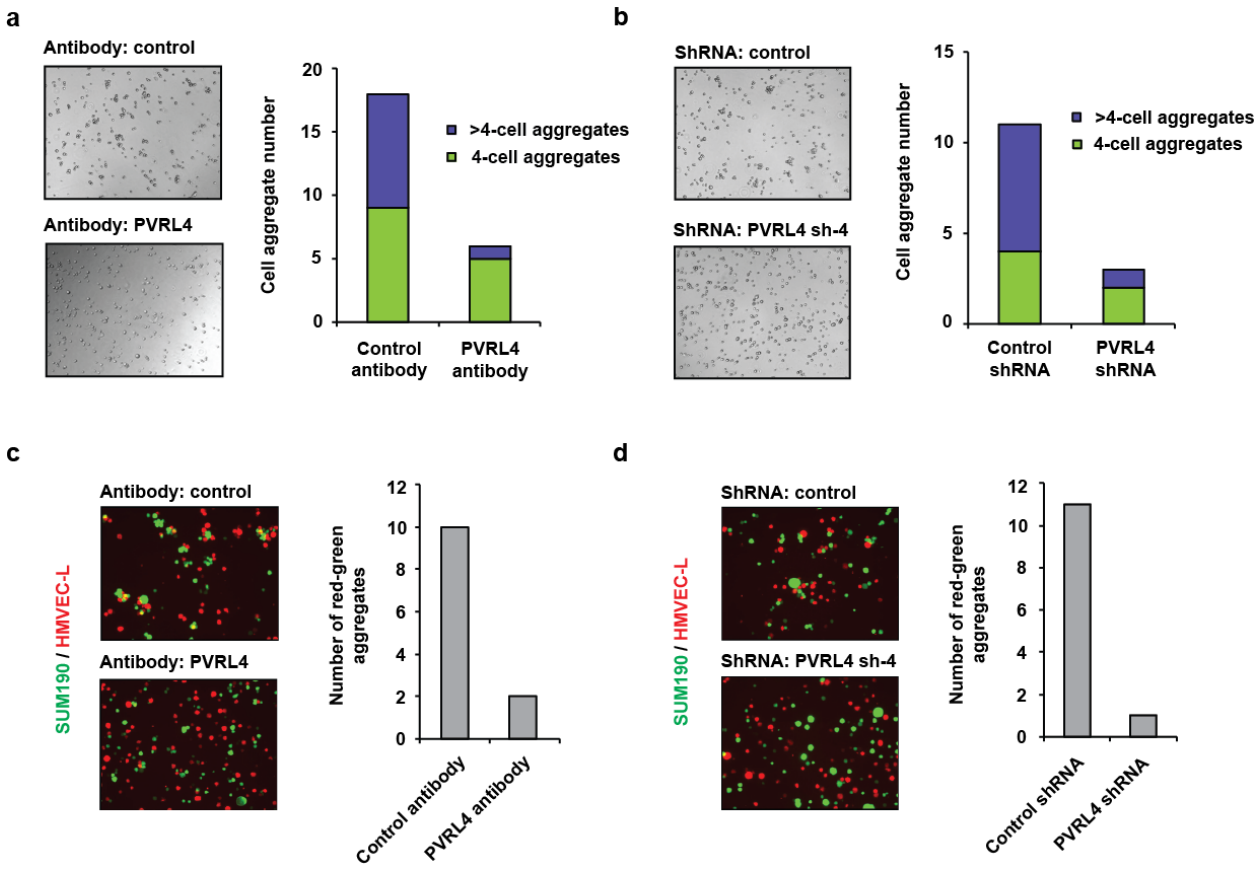


Figure 16. SUM190 cell clustering is PVRL4-driven. a, b, SUM190 cells were assayed for cell clustering in presence of the indicated antibodies (**a**) or shRNAs (**b**). **c, d,** GFP-labeled SUM190 cells were assayed for heterotypic clustering with dsRed-labeled HMVEC-L cells in presence of antibodies (**c**) or shRNAs (**d**).

We next asked whether PVRL4 is functionally involved in driving transformation of SUM190 cells. To test this, we stably expressed four independent shRNAs against PVRL4 in SUM190 cells, confirming the extent of the transcript depletion by RT-qPCR. In agreement with the phenotype seen with PVRL4 overproduction in TL-HMEC line, RNAi against PVRL4 potently reduced anchorage-independent colony formation as well as clonogenic survival of adherent SUM190 cells, indicating that PVRL4 plays an important prosurvival role in breast cancer cells. Importantly, observed effects correlated with the degree of RNAi-mediated mRNA depletion (Figure 17).

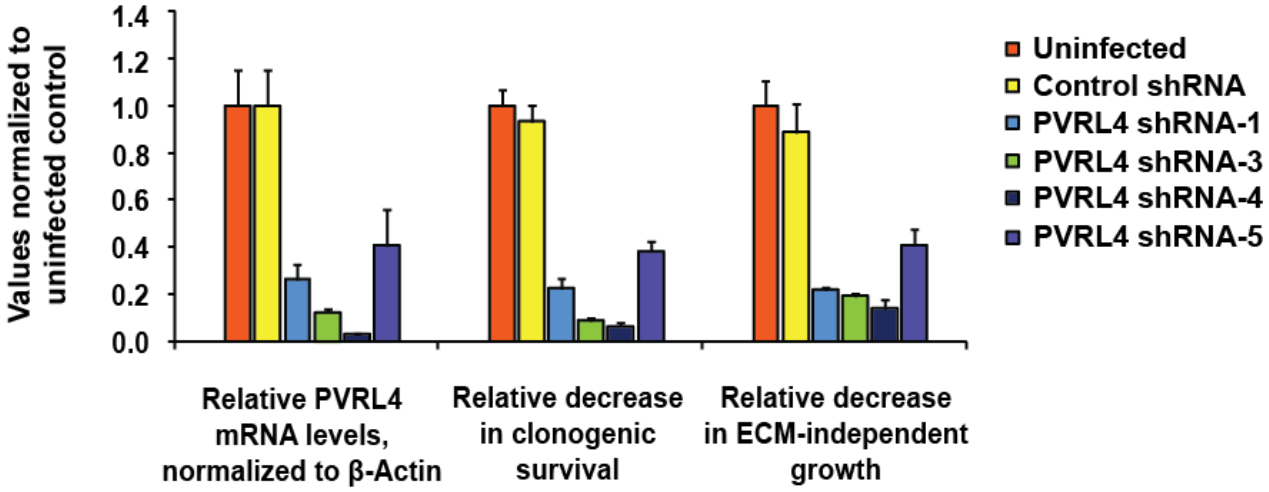


Figure 17. PVRL4 is essential for the transformation of SUM190 breast cancer cells. PVRL4 mRNA was stably depleted from SUM190 cells by four independent shRNAs. Transcript levels were measured by RT-qPCR and normalized to β-actin. qPCR was performed in quadruplicate (error bars ± SD). PVRL4-depleted and control cells were assayed for clonogenic survival and anchorage-independent colony formation. Assays were performed in triplicate (error bars ± SD). All values were normalized to uninfected control sample.

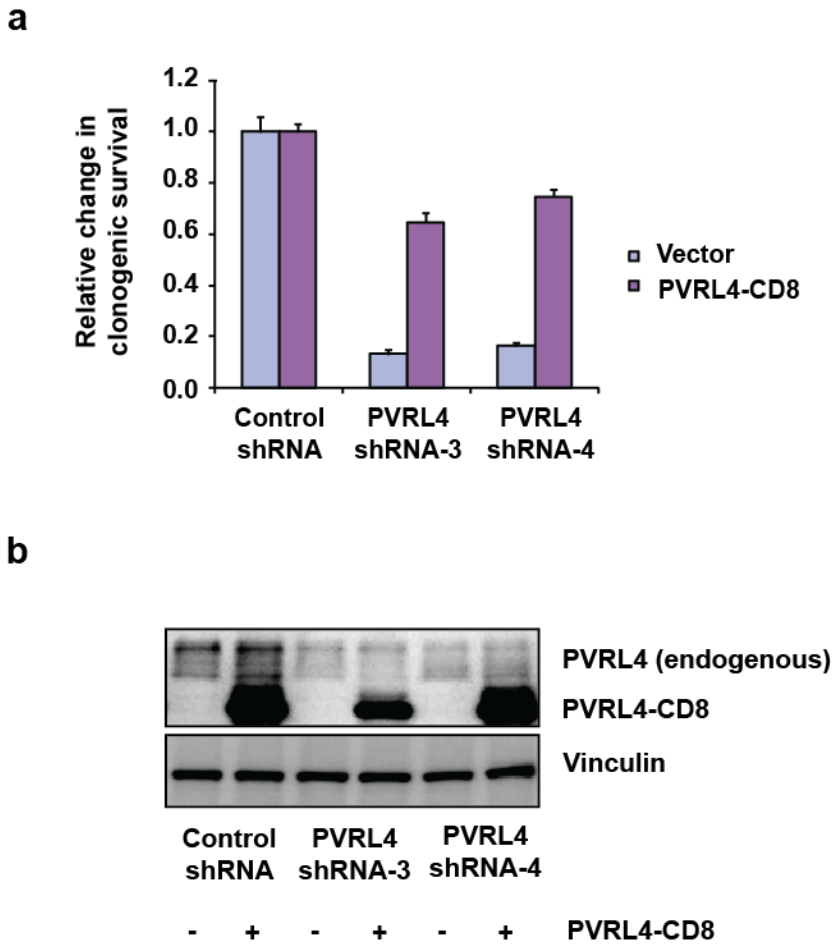


Figure 18. Transforming phenotype of PVRL4 in SUM190 cells is carried out through its extracellular region. a, The PVRL4-CD8 chimeric construct was used to rescue the defect in clonogenic survival observed with RNAi-mediated PVRL4 depletion. Assays were performed in triplicate (error bars ± SD). Colony numbers were normalized to the control shRNA sample. **b,** Expression levels of endogenous and chimeric proteins were verified by Western blot.

To confirm that the observed phenotype was specific for PVRL4 depletion, we used a PVRL4-CD8tm construct to rescue the colony formation defect. Coexpression of PVRL4-CD8tm in presence of PVRL4-targeting shRNAs alleviated the clonogenic survival defect, confirming its on-target nature (Figure 18a, b). In addition, this result demonstrates that the prosurvival function of PVRL4 is carried out via its extracellular region in breast cancer cells, which is consistent with the ability of PVRL4-CD8tm construct to enable colony formation in conditions of anchorage independence in TL-HMECs.

Targeting PVRL4 inhibits tumor growth *in vivo*

To determine whether growth inhibition observed with PVRL4 depletion *in vitro* was also relevant for growth of breast cancer cell lines *in vivo*, we stably transduced SUM190 cells with a control shRNA or shRNA targeting PVRL4, and orthotopically implanted them into mammary fat pads of nude mice. Depletion of PVRL4 by RNAi from SUM190 resulted in a significantly reduced xenograft growth as compared to control shRNA (Figure 19a, b), demonstrating that PVRL4 is an important determinant of cancer cell growth *in vivo* as well as *in vitro*. A similar effect was achieved with a triple-negative breast cancer line SUM185, transduced with an anti-PVRL4 shRNA (Figure 19c), demonstrating that the observed effect is not limited to an isolated cell line.

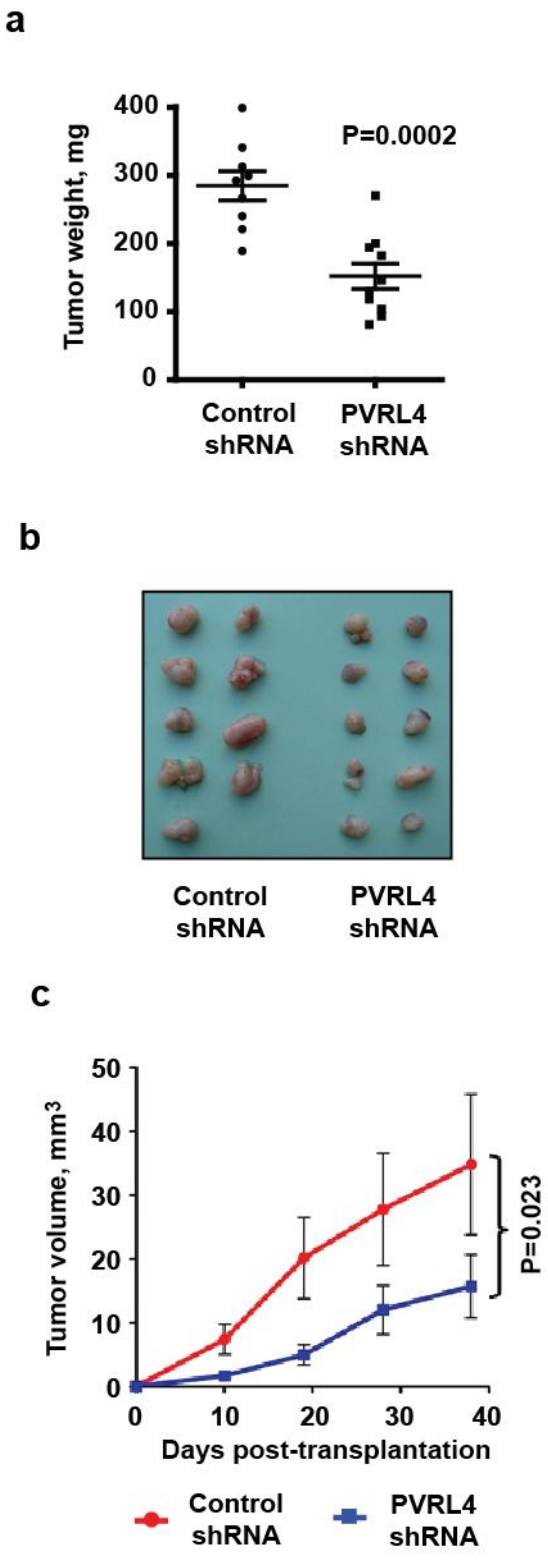


Figure 19. RNAi-mediated suppression of PVRL4 inhibits xenograft growth. a, b, Female nude mice were injected into mammary fat pads with SUM190 cells expressing indicated shRNAs (n=10 per group, error bars ± SD). Xenografts were excised, scaled **(a)** and photographed **(b). c,** SUM185 cells expressing indicated shRNA were injected into mammary fat pads of female nude mice (n=10 per group, error bars ± SD). Tumor volume was measured with calipers at indicated time points.

Having previously shown that a monoclonal antibody which blocks PVRL4-driven cell clustering and anchorage-independent growth of TL-HMECs *in vitro*, we sought to determine whether this antibody would similarly alleviate SUM190 xenograft growth in an *in vivo* setting. To address this, we treated mice bearing ~50 mm^3 SUM190-eGFP xenografts with four consecutive intravenous injections of either anti-PVRL4 monoclonal antibody or isotype control IgG at 15 mg/kg each. After the last treatment, we continued to monitor tumor growth for seven more days. Whereas tumor volumes in the control group steadily increased over time, the PVRL4 antibody-treated group displayed a remarkable stalling of tumor growth throughout the course of injection regimen (Figure 20a). Immunoblotting of tumor lysates revealed that PVRL4 antibody treatment resulted in a precipitous decline of PVRL4 protein levels even after 7 days after the last antibody infusion, suggesting that high PVRL4-expressing cells were preferentially eliminated over the course of the treatment (Figure 20b). Dissection of excised xenografts revealed a softer, paste-like composition of PVRL4 antibody-treated tumors compared to the more solid consistency of control-treated samples. This latter observation was corroborated by histological analysis (Figure 20c), which revealed areas of widespread necrosis and, importantly, loss of cell contacts, in tumors with the strongest response to treatment (D-F) when compared to control IgG-treated tumors (A-C). Finally, examination of a three-dimensional tumor architecture in control- and anti-PVRL4 antibody-treated tumors by two-photon confocal microscopy revealed reduced cell-cell contacts in anti-PVRL4 antibody treated samples as compared to control (Figure 20d). Loss of cell contacts and necrosis observed in explanted tumors provides *in vivo* support to our model that PVRL4-driven ability to tolerate loss of anchorage by virtue of direct interaction between neighboring cells.

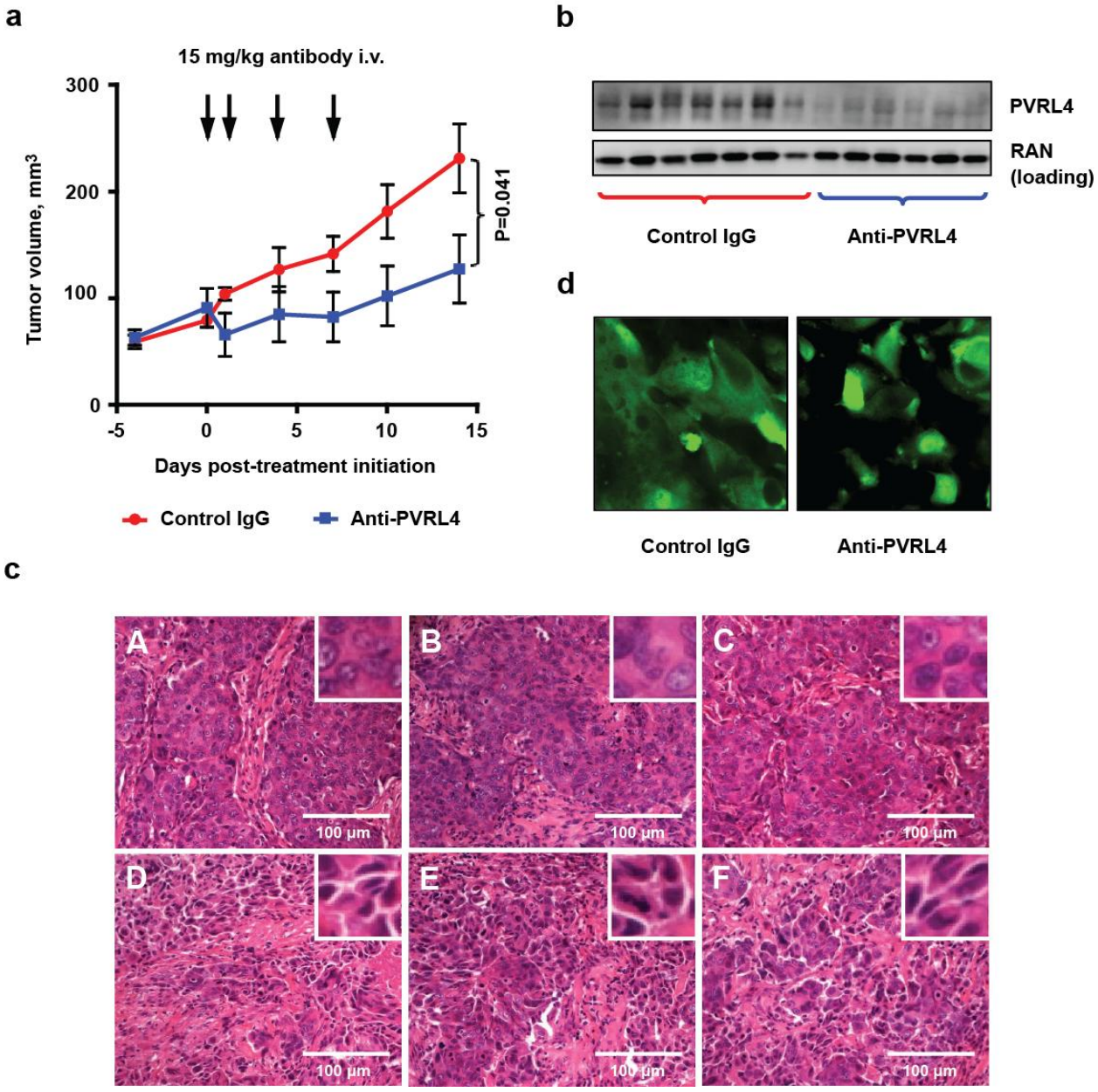


Figure 20. Antibodies against PVRL4 inhibit xenograft growth and reduce cell-to-cell adhesion within tumors. a, Female nude mice with ~50 mm^3 SUM190-eGFP xenografts were randomized into two cohorts (N=7 per group) and injected with anti-PVRL4 monoclonal antibodies or control IgG at indicated days. Tumor volume was measured with calipers (error bars ± s.e.m.). **b,** Levels of PVRL4 protein were measured in tumor lysates from anti-PVRL4 antibody or control-treated mice, seven days after the last treatment. **c,** Tumor sections from control IgG (A-C) or anti-PVRL4 antibody-treated (D-F) mice were stained with hematoxylin/eosin and photographed. Representative images are shown. **d,** Freshly explanted tumors from control IgG or anti-PVRL4 antibody-treated mice (N=3 per group) were visualized using multiphoton confocal microscope. Representative images are shown.

One possible explanation for the *in vivo* tumor inhibitory effect could be that the Fc-region drove recruitment of components of innate immunity by the anti-PVRL4 antibody. To test this possibility, we asked whether the anti-PVRL4 antibody which we used, was capable of inducing ADCC (antibody-dependent cytotoxicity) *in vitro*. Specifically, we mixed SUM190 cells with fresh human NK cells and measured the relative degree of cell lysis induced by either anti-PVRL4 antibody or control IgG. No increase in cell lysis was observed with anti-PVRL4 antibody over isotype control-incubated cells, demonstrating that the Fc region of this antibody was inefficient at recruiting Fc receptor-containing cells. These data suggest that the observed tumor inhibitory effect is likely a consequence of a direct blockade of PVRL4 function (Figure 21).

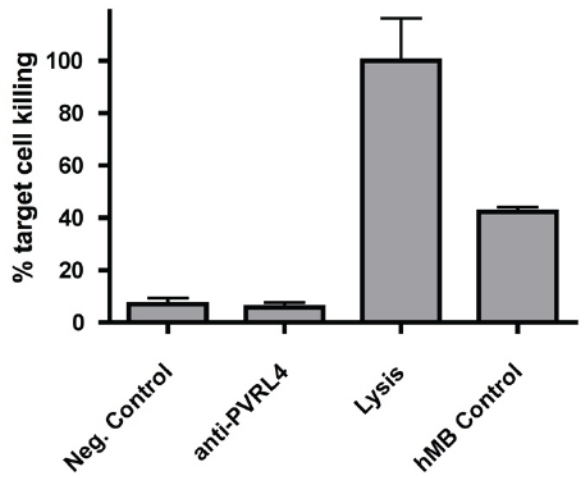


Figure 21. Anti-PVRL4 antibodies do not trigger *in vitro* ADCC of SUM190 cells. Europium-labeled SUM190 cells were incubated with fresh human NK cells in presence of an isotype control or anti-PVRL4 antibody, and the degree of lysis was measured by the DELFIA Europium assay. The maximum signal was determined by a complete lysis of labeled SUM190 cells in DELFIA lysis buffer. As a positive control, hMB humanized mouse lymphoma cells were mixed with effector cells in presence of ADCC-competent anti-CD52 antibody.

Taken together, these data suggest that targeting PVRL4-driven tumors with a monoclonal antibody directed towards its extracellular region results in the dramatic inhibition of growth and disruption of cell-cell contacts, demonstrating a therapeutic efficacy of such approach. One caveat associated with targeting junctional proteins as an anticancer therapy strategy is that it may inadvertently

induce an EMT phenotype and, while suppressing the primary tumor growth, concomitantly instigate metastasis. To test this, we measured levels of E-cadherin (an epithelial marker) and vimentin (a mesenchymal marker) in SUM190 xenografts treated with anti-PVRL4 antibody *in vivo* (Figure 22a), as well as in cultured SUM190 cells in which PVRL4 expression was inhibited by RNAi (Figure 22b). In each case, E-cadherin and vimentin protein levels remained unchanged, demonstrating that inhibiting PVRL4 in SUM190 cells does not cause EMT; neither does it select for cells with EMT-like characteristics *in vivo*. However, this analysis cannot rule out the possibility in which a small subset of cells undergo EMT as a result of treatment.

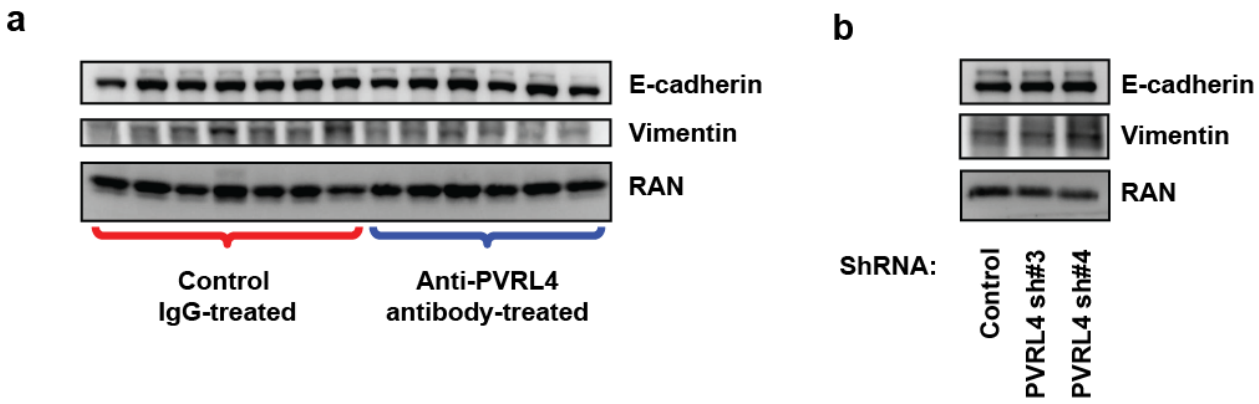


Figure 22. PVRL4 inhibition does not trigger EMT in SUM190 cells. a, Tumor lysates from control antibody- or anti-PVRL4 antibody-treated mice were blotted for E-cadherin and vimentin. **b,** PVRL4 was stably depleted by two independent shRNAs in SUM190 cells and lysates were blotted for E-cadherin and vimentin.

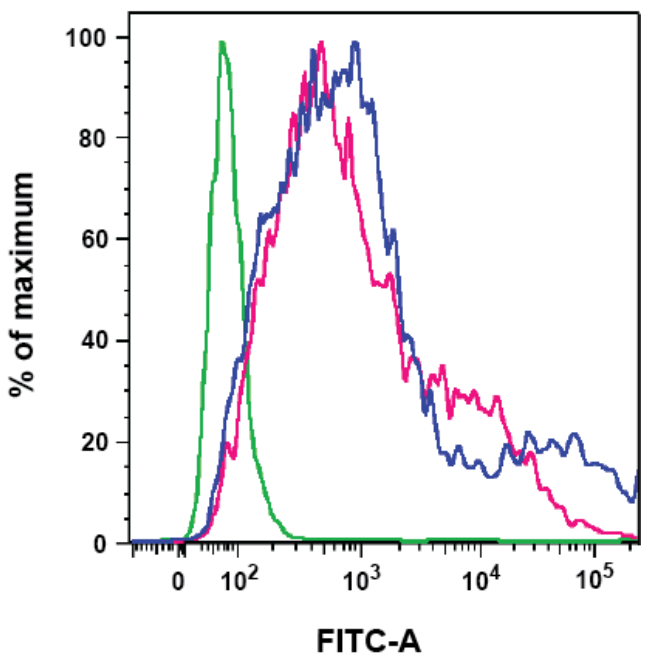


Figure 23. Both mouse and human PVRL4 are recognized by the anti-PVRL4 antibody. 293T cells were transfected with empty pQCXIN (green line), pQCXIN-human PVRL4 (blue line) or pCMV-SPORT6-mouse PVRL4 (magenta line). Live-cell FACS was performed with mouse anti-human PVRL4 antibody followed by anti-mouse secondary antibody conjugated to Alexa Fluor 488 fluorophore. FITC-A fluorescent signal for three labeled cell populations is shown.

Another safety concern associated with targeting PVRL4 with a monoclonal antibody is that such treatment has a potential for inducing damaging effects in tissues that normally expressing the antigen. Both human and mouse PVRL4 were strongly recognized by anti-PVRL4 antibody when expressed on the cell surface (Figure 23). Among normal mouse tissues, PVRL4 expression is strongest in cornea and skin epidermis [265]. Skin surface of mice treated with anti-PVRL4 antibodies was not visibly affected by treatment, and mice appeared healthy overall, demonstrating that targeted therapy against PVRL4 does not induce acute side effects in this organism, not ruling out, however, a potential side effects with the chronic exposure. Taken together with our findings that functionally link PVRL4 to tumorigenesis, as well as with its widespread expression in multiple tumor types, these data validate, in a preclinical setting that PVRL4-driven cell-cell contacts can be targeted with antibodies as a potential therapeutic strategy directed against a broad spectrum of tumor types.

Materials and Methods

Cell Culture. SUM190 and SUM185 cells (provided by K. Polyak) were cultured in SUM medium, which is a 1:1 mix of MEGM and F12:DMEM (Invitrogen), supplemented with 5% FBS (Invitrogen). 293T cells were cultured in DMEM (Invitrogen), supplemented with 10% FBS. HMVEC-L cells (Lonza) were cultured in EGM-2 medium (Lonza). Retroviral infections were performed in presence of 8 ug/mL of polybrene (Sigma). For SUM190 and SUM185 infections, cells were plated in 6-well dishes and centrifuged in presence of viral supernatant and polybrene for 1 hr at 2,000 r.p.m. Successful viral integrants were selected with puromycin (2 ug/mL) or Geneticin (200 ug/mL for TL-HMECs, 750 ug/mL for SUM190). Mouse pSPORT6-PVRL4 was purchased from Open Biosystems and transiently transfected into 293T cells following Mirus Bio's TransIT transfection protocol.

Anchorage-independent colony formation assays. ECM-independent colony formation assays were performed as previously described [98] with minor modifications. Briefly, cells were suspended in SUM medium with 2% methylcellulose (Sigma) and plated on tissue culture dishes precoated with 0.6% Noble Agar (Sigma) in MEM (Invitrogen). For assays performed in 6 cm dishes, 4.5×10^4 cells per dish were plated. For assays performed in 6-well plates, 1.2×10^4 cells per well were plated. Colonies were counted after three weeks of growth. For each assay, an average of three replicates +/- SD is shown.

Clonogenic Assays. For assaying clonogenic potential, 1.0×10^3 SUM190 cells were seeded in 6 cm tissue culture-treated dishes. After 3 weeks of growth, resulting colonies were stained with 1% Methylene Blue and counted. For each assay, an average of three replicates +/- SD is shown.

Clustering Assays. Cells were gently detached off the tissue culture surface with enzyme-free cell dissociation buffer (Invitrogen) and washed once with complete medium. 1.0 x 10^5 cells were allowed to aggregate in 1 mL of complete medium in a 15-mL conical tube at room temperature. Tubes were gently flicked in the process to visually assess the progression of clustering. After 1-1.5 hr of incubation, cell suspension was poured into wells of 12-well dishes and allowed to attach to the bottom of the dish for 5-10 minutes. Cells were promptly visualized and photographed under phase contrast and fluorescent filters using AxioVert inverted microscope. When clustering assays were performed in the presence of antibodies, following antibodies were used: normal mouse IgG (MAB004, R&D Systems), mouse anti-human PVRL4 IgG2B (MAB2659, R&D Systems). All antibodies were used at a concentration of 4 μg/mL.

Western Blotting. Cells were lysed in NP-40 buffer (1% NP-40, 25 mM Tris-HCl pH=7.4, 150 mM NaCl, 1mM EDTA, 10% Glycerol) in the presence of protease and phosphatase inhibitor tablets (Roche). Adherent cells were lysed for 15 minutes on ice, followed by scraping into Eppendorf tubes and centrifugation at 14,000 rpm for 15 min at 4C. Protein concentration in supernatants was measured using BCA assay (Pierce) and lysates were brought to identical concentrations with lysis buffer. Samples were mixed 1:1 with 2X Laemmli buffer (125 mM Tris-HCl, pH = 6.8, 4% SDS, 20% Glycerol, 0.004% Bromophenol Blue) and DTT was added to final concentration of 25 mM. Samples were boiled for 8 minutes and loaded on Tris-Glycine 4-20% or 4-12% gradient gels (Invitrogen). Transfer/blotting were performed as described elsewhere. Western blotting was performed with the following antibodies: goat anti-PVRL4 (AF2659, R&D Systems), goat anti-PVRL1 (AF2880, R&D Systems), mouse anti-Vinculin (V9131, Sigma), mouse anti-RAN (610340, BD Biosciences).

Live–cell FACS analysis. 293T cells were gently detached off the adherent surface with enzyme-free cell dissociation buffer (Invitrogen) and washed once with serum-free DMEM. Cells were incubated with 10% normal goat serum (Invitrogen) for 10 min at room temperature, then with a primary antibody at 1:100 dilution in 5% goat serum for 30 min on ice, followed by a goat-anti-mouse secondary antibody coupled to Alexa Fluor 488 (Invitrogen) at 1:1000 dilution in PBS for 30 min on ice. Fluorescent signal was measured on LSR II FACS Analyzer and analyzed with FlowJo software.

***In vivo* experiments.** For subcutaneous xenograft assays with shRNA-transduced cells, nude mice (Taconic) were injected orthotopically into the mammary fat pad with 1.0×10^6 SUM190 or SUM185 cells in serum-free medium with 50% Matrigel (BD Biosciences), one injection site per mouse. Tumors were excised after four weeks of growth, scaled and photographed. For antibody treatment experiment, 15 mg/kg of anti-PVRL4 antibody or normal IgG control (R&D Systems) was injected 4 times at days 0, 1, 4 and 7. Treatment response was assessed by caliper measurements of the tumors in anesthetized mice. Mice were euthanized 15 days post treatment initiation and tumors were harvested and representative parts subjected for paraffin embedding, western blot analysis and direct microscopy on an inverted Olympus Multiphoton Laser Scanning Confocal Microscope using a 25x objective. Images were analyzed by IMARIS software package.

Antibody dependent cell-mediated cytotoxicity (ADCC) assay. PVRL4 antibody-dependent cell-mediated cytotoxicity was assessed by using europium-labeled (DELFIA cell cytotoxicity assay, Perkin Elmer) SUM190 cells as target cells in a 96-well format. Primary human NK cells were isolated from a fresh buffy coat using a magnetic purification (EasySep Human CD56 positive selection Kit, StemCell Technologies). Cells were co-incubated at 1:1,

1:10 and 10:1 effector-to-target cell ratio. 25 µg/ml anti-PVRL4 antibody was added to respective wells. Cells were co-incubated for 4 hours and cell-free supernatants further subjected to time-lapsed fluorescence quantification using a TECAN Infinite 200 PRO plate reader. To determine maximal range of europium release, target cells were lysed in DELFIA lysis buffer. Levels of released europium are displayed as percentage values of maximum release determined by lysis. To control for NK cell effector functionality, ADCC assay was performed using the hMB lymphoma model [266] as a target cell line in presence of 25 µg/ml of anti-CD52 antibody (alemtuzumab).

Chapter V: The signaling mechanism of PVRL4

Background and Rationale

Interactions of cell adhesion molecules with signaling proteins

Far from being passive intercellular "glue", cell adhesion molecules (CAMs) are known to affect cell signaling in a number of ways. Even though CAMs, as a rule, lack catalytic domains themselves, they make use of their extracellular and cytoplasmic regions to recruit signaling-competent proteins. The surface of the cell and the subcellular region is densely populated with a variety of signaling-competent molecules. Among these molecules are receptors of intercellular signals – first and foremost, integrins and receptor tyrosine kinases, as well as non-receptor tyrosine kinases, signaling adaptor proteins and phosphatases. Various signaling proteins are known to physically associate with CAMs, which influences their downstream signaling. The great variety of the types of interaction between CAMs and other proteins can be divided into distinct categories with respect to directionality and the part of the molecule involved in interaction: (1) interactions of **extracellular regions of CAMs** with signaling receptors on neighboring cells; (2) interactions with intracellular proteins through **cytoplasmic regions of CAMs** and (3) **lateral *cis*-interactions** with transmembrane receptors (Figure 24).

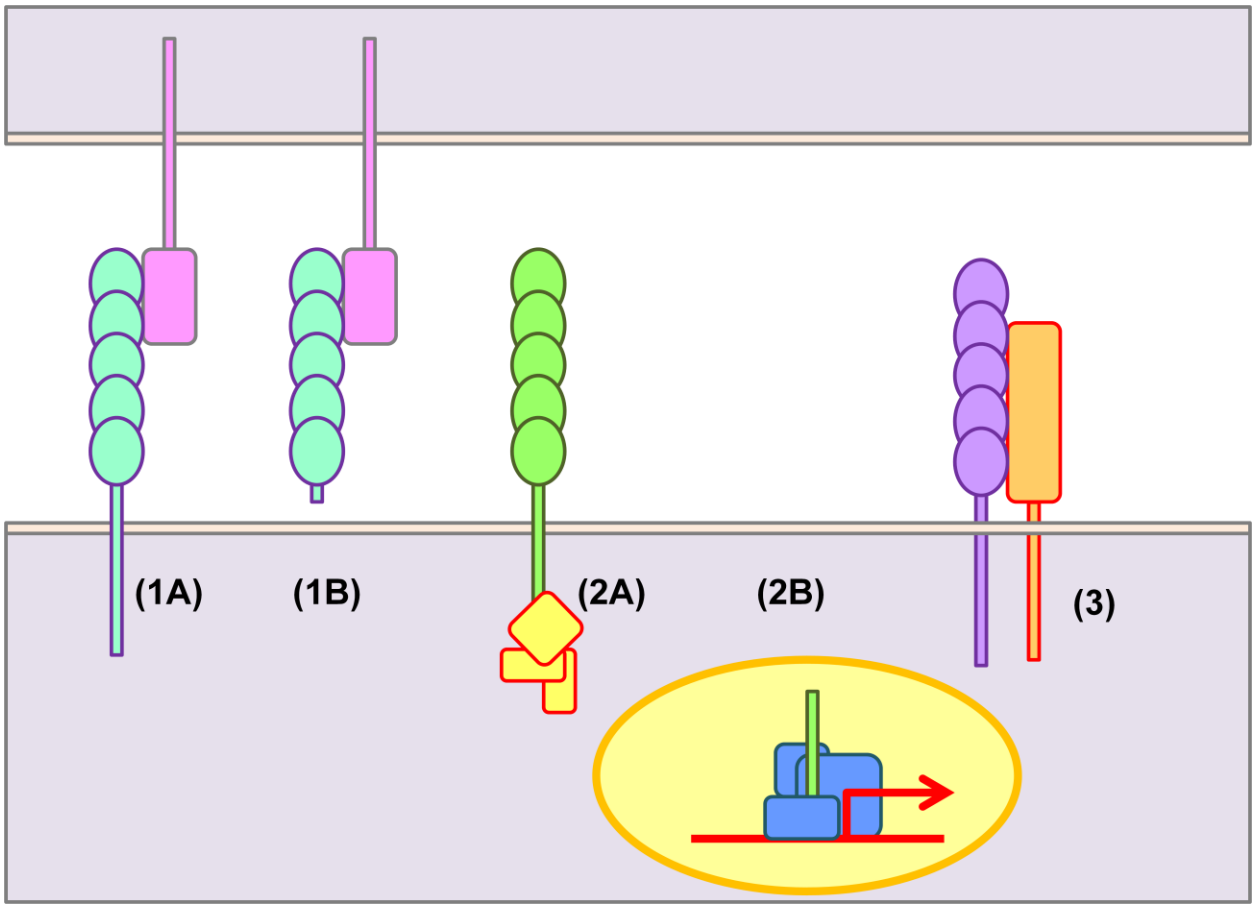


Figure 24. Cell adhesion molecules interact with other signaling proteins in a variety of ways. 1A, *Trans*-interaction between a CAM and a signaling receptor, such as integrin. **1B,** *Trans*-interaction of a cleaved extracellular region of a CAM with a signaling receptor. **2A,** Recruitment of signaling adaptors to the cytoplasmic region of a CAM. **2B,** Nuclear translocation of cleaved cytoplasmic regions of CAMs and their participation in transcriptional regulation. **3,** Lateral *cis*-interaction of CAMs with integrins and receptor tyrosine kinases.

A prototypical example of a *trans*-interaction of a CAM with another cell-surface signaling molecule (Figure 24-1A) is at the core of an immune synapse formation mechanism which is mediated via juxtacrine interactions of leukocyte-specific IgCAMs and integrins. Thus, a *trans*-interaction between ICAM-1 on the surface of antigen-presenting cells and αLβ2 and αMβ2 integrins on T cells promotes the immune synapse formation between these two cell types, followed by T-cell activation [267]. An additional layer of complexity to this mode of CAM action is added by the susceptibility of extracellular regions of some CAMs for becoming

cleaved by a class of metalloproteinases called sheddases. The release of an extracellular region of a CAM into the intercellular space allows it to engage with its respective receptor on cells at a distance (Figure 24-1B). For example, the interaction of cleaved extracellular region of L1CAM with αvβ5 integrin was shown to promote the migratory behavior in CHO cells, and this phenotype can be blocked by integrin-specific antibodies [203]. Importantly, various sheddases, such as ADAM17, become upregulated in cancer, raising an important possibility that such mode of CAM-mediated signaling can be selected for during the process of tumorigenesis [268].

Cytoplasmic regions of CAMs can bind a variety of signaling adaptors which may result in stimulation or, on the contrary, suppression of intracellular signaling (Figure 24-2A). As described previously, adherens junction-localized E-cadherin molecules sequester critical signaling adaptors β-catenin and p120-catenin, thus inhibiting Wnt- and small GTPase-associated signaling. In other instances, cytoplasmic regions of CAMs can promote pro-survival signaling events. As an example, homotypic *trans*-interactions of NCAMs on juxtaposed neuronal cells trigger the recruitment of RPTPα phosphatases to C-termini of NCAM. This, in turn, activates a non-receptor tyrosine kinase Fyn, a member of the Src family of kinases, which triggers neurite outgrowth [269].

Not only extracellular, but cytoplasmic regions of CAMs as well are susceptible for cleavage by proteases, and resulting fragments can carry out autonomous functions (Figure 24-2B). As an example, γ-secretase-cleaved C-terminal region of EpCAM, the cell adhesion molecule upregulated in a variety of tumors, translocates to the nucleus where it directly influences Wnt-regulated gene expression through forming a complex with FHL2, β-catenin and LEF [270].

A third mode of cell adhesion molecule-influenced signaling is the lateral recruitment of transmembrane signaling molecules, such as integrins and receptor tyrosine kinases (Figure 24-3). A well-studied example is a *cis*-interaction of NCAM and FGFR in neuronal cells, which facilitates FGFR activation independently of its ligand [271, 272]. Furthermore, as was described in chapter II, nectin-3, through its extracellular region, was shown to engage in lateral interactions with αvβ3 integrin, inhibiting its activation [253]. In addition, nectin-3 was shown to bind and activate PDGF receptor at sites of cell-cell junctions in MDCK cells in a manner that is dependent on its cytoplasmic binding partner, afadin [254].

In conclusion, cell adhesion molecules employ a variety of interaction modes, thereby influencing a wide range of signaling events. Some of the signaling events associated with CAMs are cell-cell adhesion-dependent and are thought to be further enhanced via formation of supramolecular chains of *cis*- and *trans*-interacting CAM dimers. As an example, formation of such supramolecular structures has been proposed for both homotypically *trans*-interacting CAMs, such as NCAM [273], as well as for heterotypically interacting SynCAM1 and SynCAM2 [274]. Yet other signaling events modulated by CAMs are independent from their participation in cell-cell contacts, being carried out by autonomously acting proteolytic fragments.

Through structure-function characterization of PVRL4, we have demonstrated that, in normal mammary epithelial cells and in cancer cells alike, PVRL4 carries out its prosurvival action through its extracellular region. Importantly, a chimeric fusion of PVRL4 extracellular region and the transmembrane domain of CD8 protein is sufficient for driving cell-cell clustering and colony formation, whereas the extracellular region of PVRL4 that is not anchored to the

membrane fails to induce anchorage-independence in TL-HMECs. Thus, the extracellular region of PVRL4 must be tethered to the cell surface in order to exert its function.

Integrin β4 - an atypical member of the integrin family

In mammalian cells, the integrin family of adhesion molecules consists of 8 β and 18 α subunits, which associate into 24 distinct heterodimers. Both α and β subunits interact with the extracellular matrix, whereas intracellular interactions with actin filaments and signaling adaptors are carried out by β subunits [111].

Integrin β4 is a unique member of the integrin family. Whereas all of the other integrins have very short cytoplasmic regions (~50 amino acids), the cytoplasmic region of a β4 integrin is atypically long, consisting of 1017 amino acids. Integrin β4 forms heterodimers exclusively with α6 subunit, which determines its preference for laminin for a ligand. In further distinction from the rest of the integrin family, integrin β4 does not take part in forming canonical focal adhesions, but instead, creates attachment points for keratin-containing intermediate filaments in adherent cells, forming structures known as hemidesmosomes [275]. As opposed to other integrins, the C-terminus of integrin β4 does not bind FAK; however, it can activate Src family kinases (SFKs) in a FAK-independent manner via recruitment of SHP-2 phosphatase [276]. Activated SFKs, in turn, phosphorylate several key tyrosine residues in the C-terminus of β4 integrin, creating docking sites for Shc adaptor, which drives the activation of Ras and PI-3 kinase [277].

Originally identified as a tumor cell surface specific protein TSP180 [278], integrin β4 is upregulated in a variety of cancers and is functionally involved in tumorigenesis [275]. An extensive array of evidence functionally links integrin β4 to breast cancer. Similarly to PVRL4, integrin β4 expression is associated with poor prognosis and basal-like expression profile [279,

280]. Integrin β4-specific antibodies were shown to suppress anchorage-independent growth in a panel of breast cancer cell lines [281]. Integrin β4 interacts in *cis*- with a number of receptor tyrosine kinases, such as EGFR [282], c-Met [283] and HER2 [284], and C-terminal deletion mutant integrin β4 transgene causes delay of tumor onset and reduced invasiveness in the MMTV-HER2 mouse model of mammary tumorigenesis [284].

Due to its atypically long C-terminus which serves as an organizing scaffold for a number of downstream signaling effectors, integrin β4 is thought to exhibit a significant degree of ECM-autonomous signaling. This signaling autonomy was convincingly demonstrated in a study utilizing chimeric constructs where extracellular and transmembrane regions of integrin β4 were substituted with those on the TrkB receptor tyrosine kinase. Dimerization of two such chimeric molecules on the cell surface by adding TrkB ligand, BDNF, was sufficient to trigger SFK activation in a manner which was dependent on SHP-2 phosphatase [285].

In this chapter we use mass spectrometry analysis to identify novel binding partners of PVRL4 and identify integrin β4 as its *cis*-binding interactor. Next, we evaluate the relevance of this interaction as well as integrin β4-associated downstream signaling to PVRL4-driven transformation in TL-HMECs and SUM190 cells.

Results

Identification of integrin β4 as a novel interacting partner of PVRL4 via mass spectrometry

We have demonstrated that PVRL4-PVRL1 cell surface *trans*-interaction provides a survival advantage in anchorage-free conditions. As a next step, we sought to gain insight into the mechanism by which this interaction becomes sensed by a cell and interpreted as a prosurvival signal. Neither of two molecules possesses catalytic activity; in addition, our data show that transmembrane as well as cytoplasmic regions are dispensable for anchorage-independence phenotype. Therefore, we considered a possibility that the assembly of a PVRL4-PVRL1 interacting module on the interface of two neighboring cells triggers lateral recruitment and/or activation of cell surface-localized proteins, which, in turn, conveys a prosurvival signal.

To identify cell surface-localized binding partners of PVRL4, we created a C-terminally HA/FLAG-tagged PVRL4 construct and verified that cell-cell clustering as well as anchorage-independence phenotypes were not affected by the addition of a C-terminal tag (not shown). We then performed immunoprecipitations with anti-HA agarose beads from lysates of TL-HMECs induced to express either HA/FLAG-tagged PVRL4 or HA/FLAG-tagged GFP and subjected the eluates to tandem mass spectrometry analysis. Since only the extracellular regions of PVRL4 and PVRL1 are required for anchorage-independent colony formation, we searched the list of PVRL4 IP-specific peptides for those with at least three unique peptides that corresponded to cell surface-localized proteins, as classified by Gene Ontology.

In particular, we have identified the transmembrane protein integrin β4 as specifically interacting with HA/FLAG-PVRL4 (Figure 25a). To validate the putative PVRL4-integrin β4 interaction directly in cells under conditions of anchorage deprivation, we performed

immunoprecipitations with anti-HA beads from lysates of suspension-incubated TL-HMECs that expressed either HA/FLAG-tagged PVRL4 or HA/FLAG-tagged GFP. Immunoblotting for integrin β4 verified a specific association of integrin β4 with PVRL4 (Figure 25b). Reciprocally, PVRL4 was found to coprecipitate from suspension-incubated TL-HMECs subjected to immunoprecipitation with integrin β4-specific antibody but not with control IgG (Figure 25c). Similarly, we were able to coprecipitate endogenous PVRL4 from SUM190 cell lysates, when immunoprecipitations with integrin β4-specific antibody were performed (Figure 25d).

In addition, we tested whether integrin β4-PVRL4 association was triggered by PVRL4-PVRL1 cell-cell contact assembly. The amount of β4 integrin coprecipitating with HA/FLAG-tagged PVRL4 was not affected by whether cells were incubated to allow assembly of clusters or lysed immediately; neither was it affected by RNAi-mediated depletion of PVRL1 (Figure 25e). These data point to a *cis* mode of association between PVRL4 and β4 integrin on cell membrane and that cell-cell clustering is unlikely to be influencing this association.

a

Unique Peptides	Gene Symbol	Gene Description
29	PVRL4	POLIOVIRUS RECEPTOR-RELATED PROTEIN 4.
5	ITGB4	ISOFORM BETA-4C OF INTEGRIN BETA-4 PRECURSOR.
3	MMP14	MATRIX METALLOPROTEINASE-14 PRECURSOR.

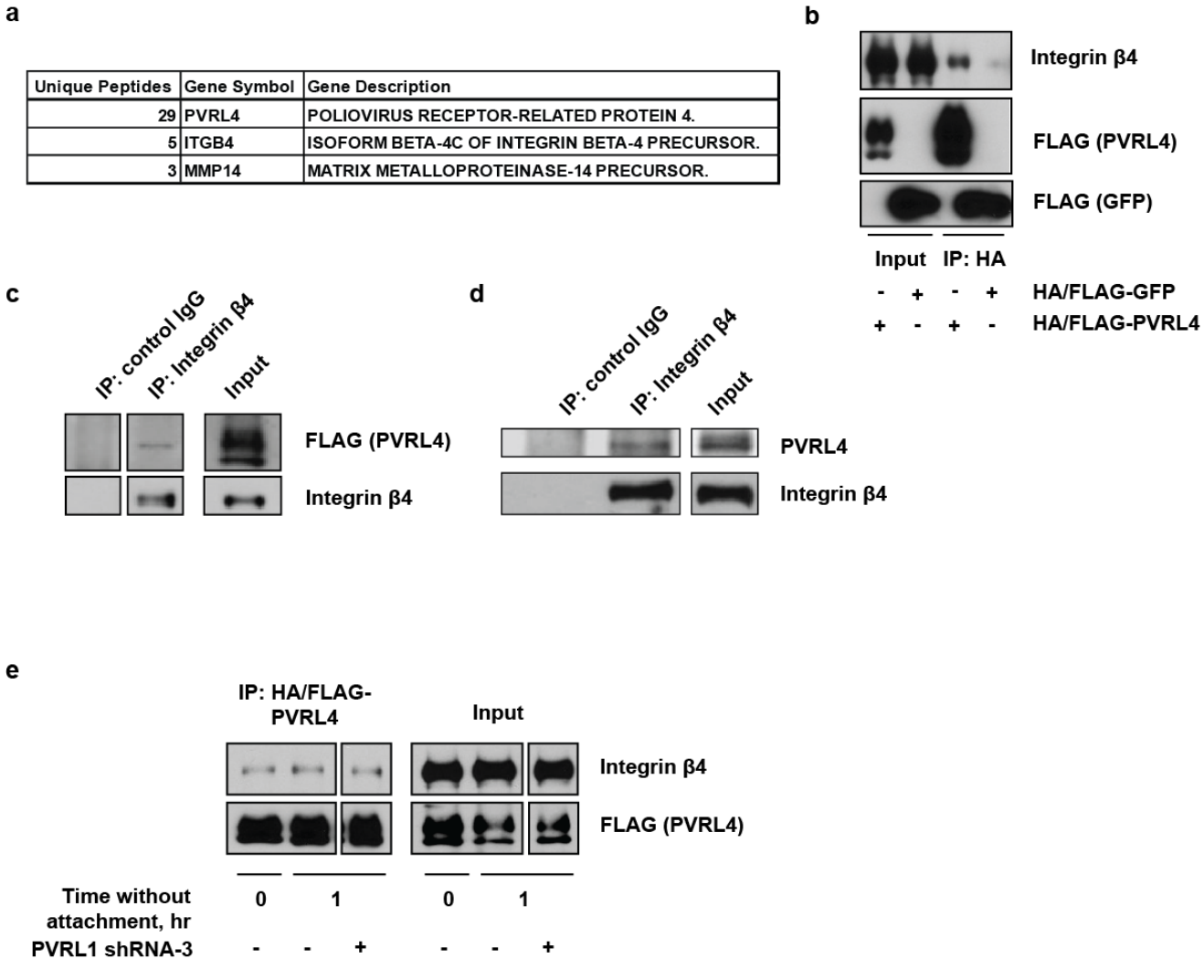


Figure 25. Interaction of PVRL4 with integrin β4. a, Cell surface-localized proteins interacting with HA/FLAG-tagged PVRL4, but not with HA/FLAG-tagged GFP, as determined by mass spectrometry. **b,** TL-HMECs expressing HA/FLAG tagged PVRL4 or HA/FLAG-tagged GFP were detached from adherent surface with the enzyme-free cell dissociation buffer and incubated in suspension for 1 hour. Immunopreciptiations were performed with HA beads, followed by Western blot with FLAG and integrin β4 antibody. **c,** Immunopreciptiations with anti-β4 integrin or control IgG were performed on lysates from suspension-incubated TL-HMECs stably expressing HA/FLAG-tagged PVRL4. Immunoprecipitates and input lysates were blotted with anti-integrin β4 and anti-FLAG antibodies. **d,** Immunopreciptiations with anti-β4 integrin or control IgG were performed on lysates from suspension-incubated SUM190 cells and blotted with anti-integrin β4 and anti-PVRL4 antibodies. **e,** Anti-HA beads were used to immunoprecipitate HA/FLAG-PVRL4 from indicated lysates. Immunoprecipitates were blotted with anti-integrin β4 and anti-FLAG antibodies.

Integrin β4 is necessary for the PVRL4-driven anchorage-independence

To ask whether PVRL4-β4 integrin association contributes to anchorage-independent colony formation phenotype, we stably depleted β4 integrin from TL-HMECs using two independent shRNA constructs (Figure 26a). Depletion of β4 integrin had no effect on TL-HMEC attachment to tissue culture vessel or on proliferation in adherent conditions; neither did it affect cell cluster formation induced by PVRL4 (not shown). However, PVRL4-mediated anchorage-independent colony formation was markedly reduced in the presence of β4 integrin-specific shRNAs (Figure 26b). Importantly, reliance on β4 integrin was specific to anchorage-independent colony growth promoted by PVRL4, whereas PI3K-driven colony numbers were not affected by its depletion (Figure 26c).

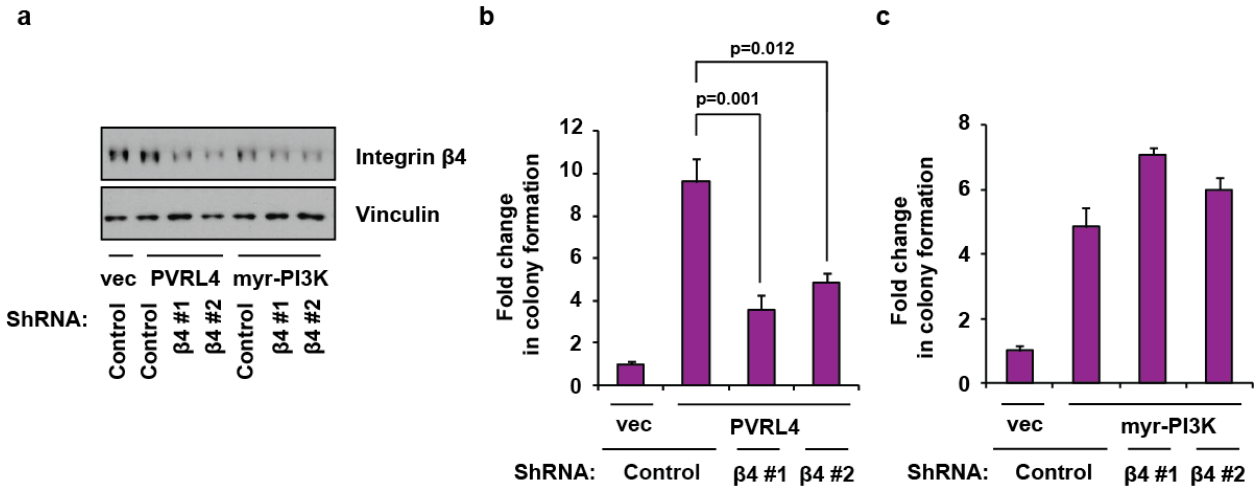


Figure 26. Integrin β4 is specifically required for PVRL4-driven anchorage-independent growth. a, TL-HMECs expressing vector control, PVRL4 or myr-PI3K were stably transduced with the indicated shRNA constructs and integrin β4 levels were assayed by Western blot. **b, c,** TL-HMECs from **(a)** were assayed for anchorage-independent colony formation induced by PVRL4 **(b)** and by myr-PI3K **(c)**. Colony numbers were normalized to vector control sample. Assays were performed in triplicate (error bars ± SD).

PVRL4-driven cell-cell contact assembly enables sustained Src family kinase activation in anchorage-deprived cells

In matrix-attached cells, ligation of integrins to ECM maintains a constitutive level of active Src family kinases (SFKs), whereas in cells that are deprived of anchorage, SFKs become inactivated. We hypothesized that PVRL4-induced cell clusters are able to maintain SFK activity even in the absence of anchorage. To test this, we measured levels of autophosphorylated SFKs in TL-HMECs after 6-hour incubation in suspension (Figure 27a). Levels of activated SFK dropped precipitously in control cells but were markedly higher in PVRL4-expressing cells, but not in PVRL4-expressing, PVRL1-depleted cells. Also, addition of anti-PVRL4 antibody sufficient to partially block cell clustering also reduced the level of SFK autophosphorylation in suspension. A similar effect was observed after a 1 hour-long incubation of PVRL4-expressing TL-HMECs in suspension (Figure 27b). Similarly, activated state of SFKs was

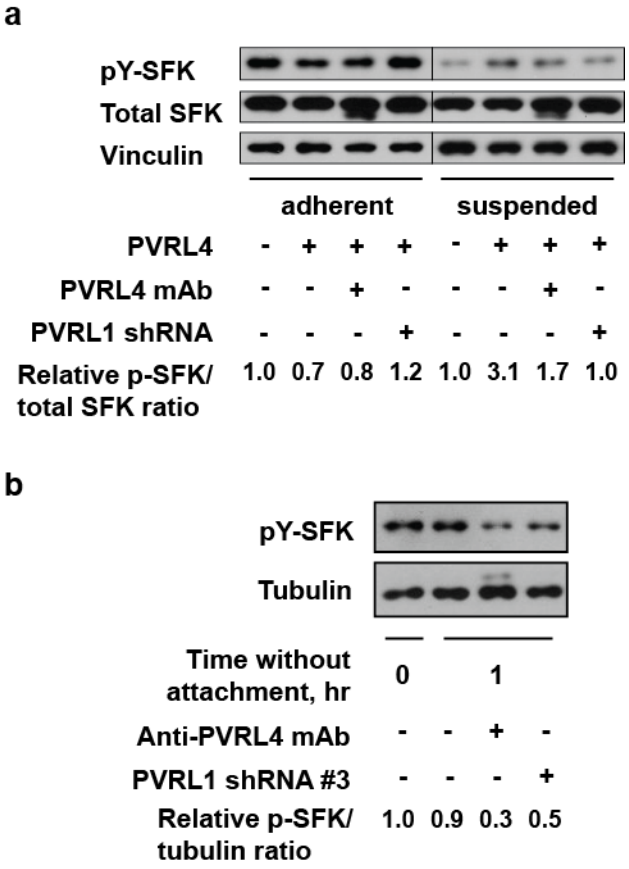


Figure 27. Src kinases become activated in PVRL4-expressing cells in a clustering-dependent manner. a, TL-HMECs were detached from the adherent surface and incubated in methylcellulose in suspension for 6 hours, or cultured on an adherent surface for 48 hours. **b,** PVRL4-expressing TL-HMEC cells transduced with control or anti-PVRL1 shRNA were incubated in suspension in conditions indicated.

maintained in cells that were allowed to form clusters but not in samples where clustering was blocked by either anti-PVRL4 antibody or by PVRL1 depletion.

SFK activity is necessary for colony growth in TL-HMECs and SUM190 cells

Having shown that PVRL4-driven cell-cell contact assembly could preserve SFK activation even in the absence of attachment, we next hypothesized that sustained activation of SFKs underlies the observed ability of PVRL4-expressing cells to survive and proliferate in the absence of attachment. In support of this, PVRL4-driven, but not PI3K-driven anchorage-independent colony formation was abrogated in presence of PP2, a chemical inhibitor of SFK activity (Figure 28a, b). Similarly, clonogenic survival of SUM190 cells was strongly inhibited by PP2 (Figure 28c).

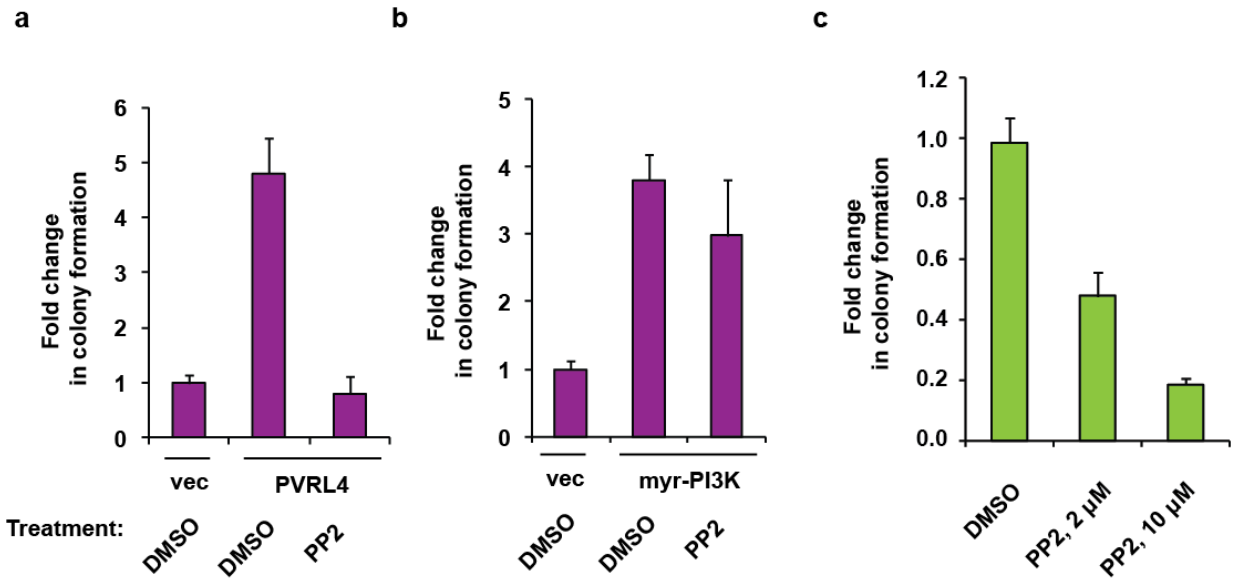


Figure 28. Chemical inhibition of Src suppresses anchorage-independence in TL-HMECs and clonogenic survival in SUM190 cells. a, b, TL-HMECs stably expressing PVRL4 **(a)** or myr-PI3K **(b)** were assayed for anchorage-independent colony formation in presence of PP2 or vehicle control. Colony numbers were normalized to vector sample. Assays were performed in triplicate (error bars ± SD). **c,** Clonogenic growth of SUM190 cells was assayed in presence of varying concentrations of a Src inhibitor PP2.

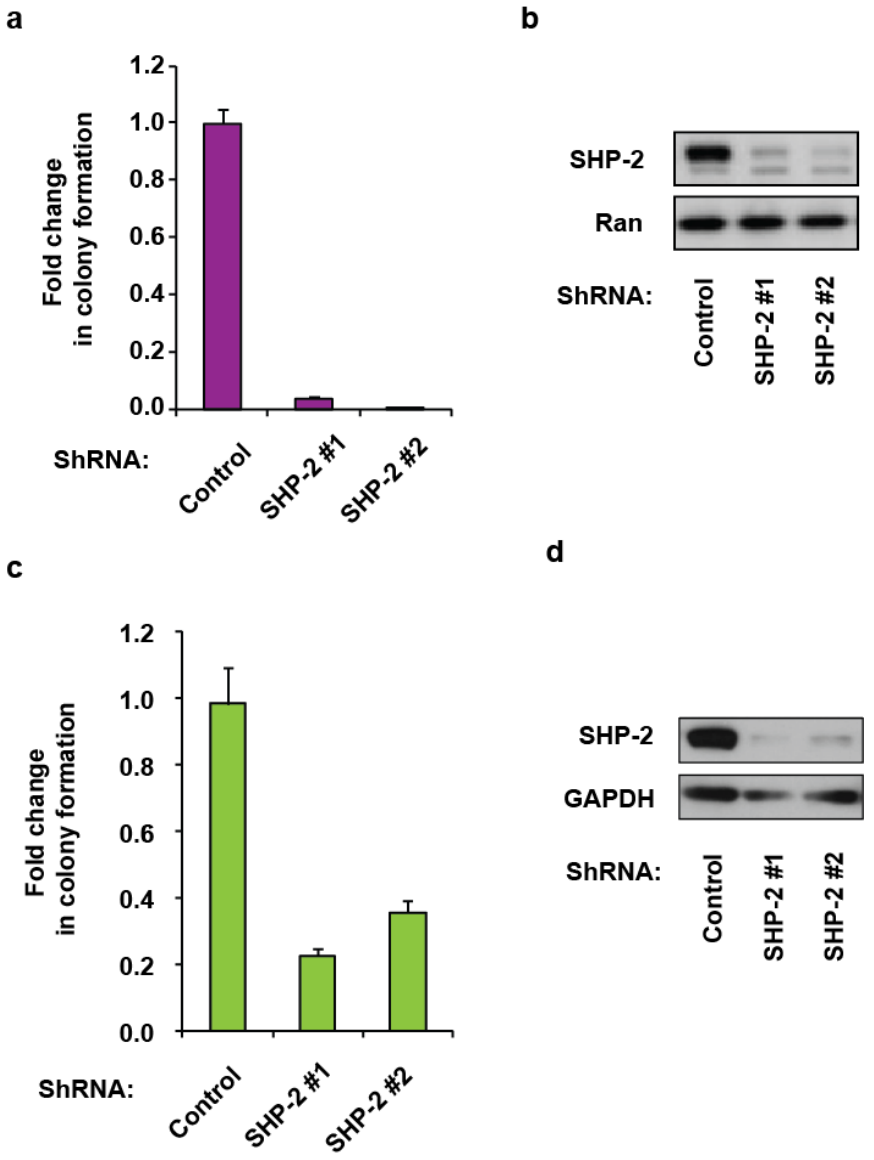


Figure 29. RNAi-mediated depletion of SHP-2 suppresses anchorage-independence in TL-HMECs and clonogenic survival in SUM190 cells. a, TL-HMECs expressing PVRL4 were stably transduced with indicated shRNA constructs and assayed for anchorage-independence. Colony numbers were normalized to vector control sample. Assays were performed in triplicate (error bars ± SD). **b,** SHP-2 levels were assayed by Western blot in TL-HMEC lysates from **(a)**. **c,** SUM190 cells were stably transduced with indicated shRNA constructs and assayed for clonogenic growth. **d,** SHP-2 levels were assayed by Western blot in SUM190 lysates from **(c)**.

Matrix-independent activation of SFKs by integrin β4 was previously shown to be carried out through SHP-2 phosphatase. Since PVRL4-driven anchorage-independent growth is inhibited by both integrin β4 depletion and by chemical inhibition of SFK activity, we reasoned that depletion of SHP-2 will have a similar effect. Indeed, two independent shRNAs against SHP-2 completely abrogated PVRL4-driven colony formation in TL-HMECs (Figure 29a, b) and in SUM190 cells (Figure 29c, d).

Taken together, these data suggest that PVRL4- potentiated cell survival relies upon activation of integrin β4/SHP-2/Src signaling pathway in a manner that is dependent on physical cell-cell interaction facilitated by PVRL4. This raises an interesting possibility that PVRL4 potentiates colony formation through assembling cell-cell contacts which allows cells to maintain some critical aspects of matrix-dependent signaling even in the absence of matrix anchorage.

Materials and Methods

Constructs and virus production. Stable RNAi-mediated depletion was performed with shRNAs expressed in either pMSCV-PM or pGIPZ vector in miR-30 context that were either picked from the Hannon-Elledge shRNA library (Open Biosystems) or designed *de novo* (design and cloning protocol described in [260]). 21-nt sense sequences of shRNAs used in this study are listed in Supplementary Table 2. Tandem HA/FLAG-tagged PVRL4 construct was obtained by Gateway recombination of PVRL4 entry clone into C-terminal iTAP vector (provided by W. Harper). Retro- and lentiviral supernatants were generated by transient transfection of 293T cells following Mirus Bio's TransIT transfection protocol and harvested 48 hrs later.

Cell Culture. TL-HMECs expressing hTERT and SV40 Large T antigen [178] were cultured in MEGM (Lonza). SUM190 (provided by K. Polyak) were cultured in a 1:1 mix of MEGM and F12:DMEM (Invitrogen), supplemented with 5% FBS (Invitrogen). 293T cells were cultured in DMEM (Invitrogen), supplemented with 10% FBS. Retroviral infections were performed in presence of 8 ug/mL of polybrene (Sigma). For SUM190 and SUM185 infections, cells were plated in 6-well dishes and centrifuged in presence of viral supernatant and polybrene for 1 hr at 2,000 r.p.m. Successful viral integrants were selected with puromycin (2 ug/mL) or Geneticin (200 ug/mL for TL-HMECs, 750 ug/mL for SUM190).

Anchorage-independent colony formation and anoikis assays. ECM-independent colony formation assays were performed as previously described [98] with minor modifications. Briefly, cells were suspended in reduced growth factor MEGM (containing 50% of kit-supplied BPA, Insulin, EGF and hydrocortisone) with 2% methylcellulose (Sigma) and plated on tissue culture dishes precoated with 0.6% Noble Agar (Sigma) in MEM (Invitrogen). For assays performed in 6 cm dishes, 4.5×10^4 cells per dish were plated. For assays performed in 6-well

plates, 1.2 x 10^4 cells per well were plated. Colonies were counted after three weeks of growth. For each assay, an average of three replicates +/- SD is shown. For ECM-independent colony formation assays in presence of PP2 inhibitor, PP2 (EMD/Millipore) was used at 10 μM final concentration.

Clonogenic Assays. For assaying clonogenic potential, 1.0 x 10^3 SUM190 cells were seeded in 6 cm tissue culture-treated dishes. After 3 weeks of growth, resulting colonies were stained with 1% Methylene Blue and counted. For each assay, an average of three replicates +/- SD is shown.

Clustering Assays. For short-term culture of TL-HMECs in suspension, 4.0 x 10^5 cells were allowed to aggregate in 1 mL of complete medium in a 15-mL conical tube at room temperature, mixed with 5 mL of 0.5% methylcellulose in reduced growth factor MEGM and incubated in wells of a 6-well ultra-low attachment dish (2 wells per sample) for indicated periods of time.

Western Blotting. Cells were lysed in NP-40 buffer (1% NP-40, 25 mM Tris-HCl pH=7.4, 150 mM NaCl, 1mM EDTA, 10% Glycerol) in the presence of protease and phosphatase inhibitor tablets (Roche). Adherent cells were lysed for 15 minutes on ice, followed by scraping into Eppendorf tubes and centrifugation at 14,000 rpm for 15 min at 4C. Suspension-cultured cells were washed once with cold PBS, followed by lysis and centrifugation. Protein concentration in supernatants was measured using BCA assay (Pierce) and lysates were brought to identical concentrations with lysis buffer. Samples were mixed 1:1 with 2X Laemmli buffer (125 mM Tris-HCl, pH = 6.8, 4% SDS, 20% Glycerol, 0.004% Bromophenol Blue) and DTT was added to final concentration of 25 mM. Samples were boiled for 8 minutes and loaded on Tris-Glycine 4-20% or 4-12% gradient gels (Invitrogen). Transfer/blotting were performed as

described elsewhere. Western blotting was performed with the following antibodies: goat anti-PVRL4 (AF2659, R&D Systems), mouse anti-Vinculin (V9131, Sigma), mouse anti-RAN (610340, BD Biosciences), mouse anti-ITGB4 (611232, BD Biosciences), mouse anti-FLAG-HRP (A8592, Sigma-Aldrich), rabbit anti-pY416 SFK (2101, Cell Signaling), mouse anti-SFK (2110, Cell Signaling), mouse anti-SHP-2 (610621, BD Biosciences) and mouse anti-α Tubulin (sc-8035, Santa Cruz).

HA-pulldown. TL-HMECs expressing HA/FLAG-PVRL4 or HA/FLAG-GFP were gently detached off the tissue culture surface with enzyme-free cell dissociation buffer, washed and lysed for 30 min in MCLB lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40) in presence of protease and phosphatase inhibitors (Roche), followed by centrifugation at 14,000 rpm at 4C. Lysates were precleared with protein A/G beads (sc-2003, Santa Cruz). Pulldowns were performed with anti-HA beads (A2095, Sigma Aldrich) overnight at 4C. Beads were washed 5 times with MCLB buffer, followed by 2 washes with elution buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol). Elutions were performed with 500 μg/mL of HA peptide (I2149, Sigma Aldrich) in elution buffer. Proteins were precipitated from this mixture with 20% trichloroacetic acid (TCA), and the resulting pellet was washed once with 10% TCA and four times with cold acetone.

Mass Spectrometry. TCA-precipitated proteins were dissolved in 100mM ammonium bicarbonate (pH 8.0) with 10% acetonitrile and 10 ng/μL trypsin (Promega) and incubated at 37°C for 5 hours. They were subsequently desalted, dissolved in 5% formic acid / 5% acetonitrile, and loaded onto a reversed phase microcapillary column (100 mm I.D.) packed with 18 cm of Maccel C18AQ resin (3 mm, 200 Å, The Nest Group, Inc). Peptides were eluted using a gradient of 4%–26% acetonitrile in 0.125% formic acid over 95 minutes and detected in a

hybrid linear ion trap-orbitrap mass spectrometer (LTQ-Orbitrap Discovery, ThermoFisher). Precursors selected for MS/MS fragmentation were corrected for errors in monoisotopic peak assignment, and tandem MS spectra were searched using the Sequest algorithm, with mass tolerance set to 25 ppm and two missed cleavages allowed. False discovery rates were estimated with the target-decoy method [286], and linear discriminant analysis (LDA) was utilized to filter peptides to an initial 1% peptide-level FDR. Peptides were then assembled into proteins and further filtered to a protein-level FDR of 0.84% [287], resulting in a final peptide-level FDR of 0.35%.

Immunoprecipitations. Cells were lysed in IP lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, 1% NP-40) in presence of protease and phosphatase inhibitors and centrifuged at 14,000 rpm at 4C. Immunoprecipitations were performed with anti-HA beads (A2095, Sigma Aldrich) for 2 hours or with anti-ITGB4 antibody (611232, BD Biosciences) for 2 hours, followed by protein A/G beads (sc-2003, Santa Cruz) for 1 hour. Beads were washed 4 times with lysis buffer and boiled in Laemmli buffer with 25 mM DTT.

Chapter VI: Conclusions and Future Directions

The utility of anchorage-independence screens for cancer gene discovery

Genetic screening is a versatile tool for a high-throughput testing of thousands of genes for functional contribution, or lack thereof, to the biological phenotype of interest. Genetic screening had firmly established itself as an approach that is central to the discovery of genetic determinants of tumorigenesis. Thus, gain- and loss-of-function screens in normal and transformed mammalian cell lines had produced datasets of candidate genes functionally involved in a wide variety of phenotypes relevant to cancer cell biology. Among these phenotypes are increased rates of cell proliferation, sensitivities to select drugs and stressors, anchorage-independent growth, replicative and oncogene-induced senescence, a balance between stemness and differentiation as well as cellular motility and invasiveness.

By staying anchored on the extracellular matrix, epithelial cells receive a sustained antiapoptotic message. Conversely, the cessation of this signal has to be compensated through acquisition of genetic changes associated with matrix-autonomous activation of various aspects of matrix adhesion-associated signaling. Anchorage-independent growth is a critical hallmark of epithelial cancers. To initiate and sustain tumor growth, cells must acquire some resistance to loss of matrix anchorage as early as at a stage of localized dysplasia, for example, at the ADH (atypical ductal hyperplasia), a precursor state to breast carcinoma *in situ*. Importantly, cancer cells remain addicted to anchorage-independence throughout the process of multistep tumorigenesis.

Cell-matrix attachment does not simply elicit a singular flux through an isolated pathway; on the contrary, it profoundly affects diverse aspects of an epithelial cell physiology – among

which are cell cycle, apoptosis, cytoskeletal tension, various aspects of metabolism and cellular differentiation. Thus, it is conceivable that not a single oncogene, but large networks of interconnecting oncogenic changes contribute to a cancer cell's resistance to anchorage loss.

To decipher genetic changes that enable cell survival in the absence of matrix anchorage, sensitive *in vitro* models of anchorage-independent growth have been developed. TL-HMEC system in particular is a useful tool for testing genes that either enable or suppress anchorage-independent growth in the context of a normal epithelial cell line. An important advantage of TL-HMEC system is that it can be made anchorage-independent through ectopic introduction of a singular genetic change, such as a constitutively active catalytic subunit of PI-3 kinase or depletion of a lipid phosphatase PTEN. In other words, an upregulation or a downregulation of a single gene may be sufficient to induce a switch from anchorage-dependent to anchorage-independent proliferation. This sensitivity makes TL-HMEC system a highly useful tool for discovery of novel genetic determinants of this phenotype.

At the same time, one might argue that along with being an advantage, an obvious sensitivity of TL-HMECs for becoming anchorage-independent via potentiating PI-3 kinase signaling can also be perceived as a drawback. Thus, one might suggest that, instead of uncovering broad spectra of anchorage-independence drivers, screens performed in TL-HMECs will repeatedly yield sets of negative and positive regulators of PI-3 kinase pathway only. If this is indeed the case, the utility of this system as a tool for the discovery of broadly-acting anchorage-independence genes will be limited.

However, if one is to examine the "track record" of validated ectopic genetic changes which, as our group has shown, can induce anchorage independence in TL-HMECs, they encompass a variety of genes that fall outside of spectrum of canonical PI-3 kinase pathway

signaling. Thus, among some of the validated genes that were discovered by our group as affecting anchorage-independence in TL-HMECs are (i) depletion of transcriptional repressor REST, which profoundly affects expression of a variety of protein-coding genes and non-coding RNAs [98], (ii) depletion of PTPN12 phosphatase, which affects activation states of a number of receptor tyrosine kinases [99], and (iii) depletion of TGF-β receptor II, a master regulator of a pathway known to be tumor-suppressive in epithelial cells [98]. Finally, as demonstrated in this work, expressing PVRL4 in TL-HMECs can sustain Src family kinase activity in the absence of matrix anchorage; and PVRL4-driven, but not PI-3 kinase-driven anchorage-independence, is sensitive to chemical inhibition of Src. Altogether, the broad spectra of genes discovered in anchorage-independence screens performed in TL-HMECs validate it as a sensitive tool for oncogene and tumor suppressor discovery, applicable for capturing the multifaceted nature of genetic determinants that converge upon the phenotype of anchorage-independent growth.

We describe some of the additional steps we had taken in order to improve the sensitivity of TL-HMEC system as a tool for oncogene and tumor suppressor discovery. These steps include (1) isolating transformation-sensitive clones from a polyclonal population, and (2) decreasing non-autonomous drivers of growth factor signaling by reducing concentrations of growth factors in growth medium.

Our observations provide some insight into additional aspects of TL-HMEC model which can be taken advantage of in order to facilitate cancer gene discovery. First, we demonstrate that distinct clones within the polyclonal TL-HMEC line exhibit profound differences in their susceptibility to transformation by PTEN shRNA. One intriguing hypothesis is that distinct clones of TL-HMECs may display distinct preferences with regard to what signaling pathways convey anchorage-independence phenotype upon them. For example, while some clones are

responsive to PI-3 kinase pathway stimulation, others might display sensitivity to activation of c-Src, and yet others become anchorage-independent upon TGF-β pathway suppression. If this is indeed the case, anchorage-independence screens could be performed with an oligoclonal mix of TL-HMEC clones in which each clone with a distinct pathway tropism is uniquely barcoded for identification. An approach like this can uncover unexpected functional interactions and interchangeabilities between distinct functional modalities within cellular signaling networks.

In addition, we observe that the number of spontaneously arising anchorage-independent colonies can be readily modulated by modifying growth medium composition. The sensitivity to paracrine environment can further be utilized for assessing interactions between various genetic and non-genetic factors and their combined contribution to the phenotype - for example, assaying anchorage independence in conditions when a certain growth factor or nutrient is limited.

Yet another aspect that makes TL-HMEC system such a versatile tool for cancer gene discovery is a modular principle by which it is built. Thus, low-passage hTERT-HMECs require a combination of three elements – Large T, PI-3 kinase and exogenously introduced *c-myc* – for anchorage-independent growth. Thus, performing a screen for anchorage-independence in an HMEC line which expresses Large T and PI-3 kinase but not *c-myc* is likely to result in a different set of genes than a similar screen in TL-HMECs. Thus, such parallel approach would significantly broaden the spectrum of novel anchorage-independence driver discovery.

The gain-of-function screen described in this work was performed with a library that (1) covers at least a third of a human protein coding genes, and (2) is not biased in favor of one or another functional group of genes. To the best of our knowledge, this is the most comprehensive screen for positive regulators of anchorage-independence that has been performed. Importantly, we were able to confirm that eleven candidate genes strongly stimulated anchorage-independent

colony formation when individually retested in TL-HMECs. Since the primary objective of performing a screen for drivers of anchorage-independence is to identify novel genes causally involved in tumorigenesis, we asked how many of our validated candidates are recurrently altered in tumors. Strikingly, eight out of eleven genes that strongly induced cell proliferation in the absence of anchorage were found to reside within statistically defined areas of copy number gain recurrently seen in solid tumors. Thus, our list is highly enriched for genes that are recurrently targeted for amplification in tumors, which lends an important proof-of-principle to the use of functional genetic screening approaches as a tool for novel cancer gene discovery.

In addition to genetic screens for anchorage-independence inducers, we envision yet another promising direction that this conceptual framework can be taken into. This direction involves performing genetic screens for shRNAs which are synthetically lethal with growth of cells in anchorage-independence conditions. Specifically, libraries of shRNAs can be screened in cancer cell lines which are grown in the absence of attachment and, in parallel, in same cell lines grown in substratum-attached manner, thus identifying shRNAs that selectively target substratum-detached but not substratum-attached cells. This type of a screening approach may lead to the discovery of some unexpected genetic interactions which are synthetically lethal with a growth condition uniquely experienced by a growing tumor. An approach like this has an appealing potential for yielding new potent and selective therapeutic ideas.

PVRL4 is a cell-cell adhesion-dependent driver of anchorage-independent growth

A question of how different types of cell-cell adhesions contribute to tumorigenesis is complicated by the fact that mammalian genomes encode hundreds of cell adhesion proteins with strikingly diverse tropisms, adhesion strengths and signaling capabilities. In addition, many cell adhesion molecules can influence downstream signaling independently of their role in cell-cell attachment. For example, extracellular and cytoplasmic regions of some cell adhesion proteins may function in one capacity when they are anchored at the plasma membrane and acquire additional signaling capabilities after they are proteolythically cleaved and displaced from the membrane.

Contributions of individual adhesion molecules to the process of tumorigenesis have been described and includes both tumor-suppressive functions, such as those mediated by E-cadherin, as well as tumor-promoting, such as those mediated by N-cadherin or EpCAM. Furthermore, antibody-based therapies against the latter two proteins have shown promising results in preclinical and clinical settings [200, 288]. It is worth noting that at least some of the prosurvival effects elicited by N-cadherin and EpCAM are independent of their role in cell adhesion [201, 270].

In this work, we identify the cell adhesion molecule PVRL4 as a potent functional contributor to tumorigenesis. PVRL4 promotes anchorage-independent growth of mammary epithelial cells, as well as anchorage-independent and clonogenic growth of breast cancer cells *in vitro* and xenograft formation *in vivo*. PVRL4 rapidly and potently induces cell-to-cell adhesion via engaging its counter-receptor PVRL1 on a juxtaposed cell, and our data strongly suggest that the interaction of PVRL4 with PVRL1 on the opposite cell is required for the observed prosurvival effect.

In addition to demonstrating the prosurvival role of PVRL4 and its ability to promote cell-to-cell attachment in various cell types, we find, unexpectedly, that the ectopic expression of some known oncogenic drivers in TL-HMECs, namely, RAS^{V12} mutant, constitutively active catalytic subunit of PI-3 kinase or stable depletion of PTEN, potently promotes cell-to-cell attachment phenotype as well. This observation suggests that activation of oncogenic signaling in a cell affect its cell surface adhesion molecule repertoire through unknown transcriptional or post-transcriptional mechanisms. We envision that the question of which cell adhesion molecules become induced in oncogene-expressing HMECs and whether they are functionally involved in promoting anchorage-independence is a promising direction in which this research could be taken. Because cell adhesion proteins are readily accessible by antibodies, identifying oncogene-specific changes in cell surface adhesion molecule repertoire can foster novel therapeutic ideas. For example a monoclonal antibody targeted against a cell adhesion molecule that is specifically induced by hyperactive PI3K pathway can block a putative pro-survival action of this cell adhesion molecule, or it can trigger selective elimination of oncogene-expressing cells via antibody-mediated recruitment of NK cells and macrophages.

In addition, we performed structure-function analyses of PVRL4 and PVRL1 in order to dissect which parts of the molecules are necessary and sufficient for anchorage-independence. Our analysis revealed that (1) a truncated extracellular region of PVRL4 fails to induce the anchorage-independence phenotype, (2) formation of a PVRL4-PVRL1 cell-to-cell attachment module is essential for anchorage-independence; (3) neither cytoplasmic nor transmembrane regions of PVRL4 and PVRL1 are required for anchorage-independence. An important question that still remains to be explored is whether a soluble extracellular region of PVRL1 can

substitute for PVRL1 depletion in PVRL4-expressing cells, or whether both molecules must be anchored on the membrane for promoting the survival phenotype.

Cytoplasmic regions of all four nectins bind afadin, a large scaffold protein through which nectin-based intracellular signaling events are carried out. Interestingly, and in contrast to PVRL4, loss of afadin has been shown to be strongly associated with poor outcome in breast cancer patients in a number of studies [289, 290]. Whether afadin has negative or neutral effect on the stimulation of survival by PVRL4-PVRL1 interaction remains to be elucidated. Interestingly, we found that deleting four C-terminal amino acids of PVRL4 slightly yet reproducibly enhances its transforming ability which suggests that afadin binding might indeed mitigate some of the protumorigenic effects of PVRL4.

We take further steps towards elucidating the mechanism by which PVRL4-PVRL1 cell-surface *trans*-interaction enables the survival in the absence of anchorage. Specifically, we identify integrin β4 as a *cis*-binding partner of PVRL4 on the cell surface. Integrin β4 has previously been implicated in breast tumorigenesis; furthermore, at least some aspects of its downstream signaling can be activated by forced dimerization of individual integrin molecules on the cell surface. Our analysis of intracellular signaling reveals that clusters of PVRL4-expressing TL-HMECs exhibit marked matrix-independent SFK activation, and that PVRL4-driven anchorage-independence requires intact integrin β4/SHP-2/SFK signaling. How does PVRL4-driven cell clustering potentiate integrin β4 activation? One possibility is that PVRL4-PVRL1 cell surface *trans*-interaction promotes clustering of isolated integrin β4 molecules at the site of cell-cell interaction. Indeed, preceding formation of a *trans*-interacting module between two juxtaposed cells, nectins reside on the cell surface in a form of *cis*-homodimers [236]. One may hypothesize that two cells coming into immediate proximity potentially trigger assembly of

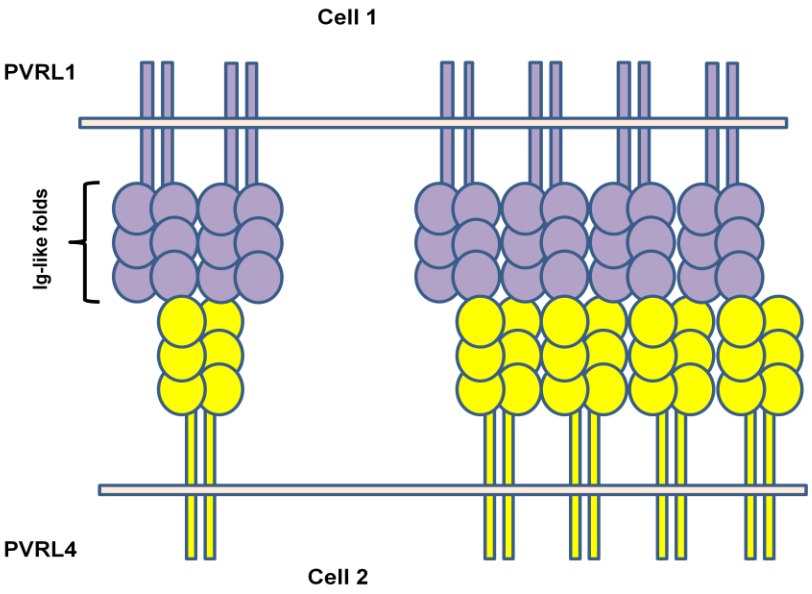


Figure 30. Formation of supramolecular chains of *cis*-interacting dimers at the site of cell-cell contact has been proposed for IgCAMs.

long chains of alternating PVRL4 and PVRL1 *cis*-homodimers, where each *cis*-homodimer engages not one but two *cis*-homodimers on a cell opposite to it (Figure 30). Such mode of interaction has been proposed for a variety of cell adhesion molecules, such as SynCAM3 homotypic *trans*-interaction [291], as well as for *trans*-interaction of PVR and TIGIT cell adhesion molecules [292], with some support from crystal structure data. In case of PVRL4-PVRL1 interaction, assembly of such chains might drive individual integrin β4 molecules into proximity, activating SHP-2/SFK in a manner similar to one seen in artificial dimerization experiments. Further studies on PVRL4-PVRL1-integrin β4-associated signaling, assisted by super-resolution microscopy methods such as TIRF (total internal reflection fluorescence) or STORM (stochastic optical reconstruction microscopy) and crystal structure analysis of PVRL1-PVRL4 interacting heterotetramers may shed more light at the supramolecular arrangement of these molecules at sites of cell-cell contacts.

Intriguingly, PVRL4 is not the only nectin that was shown to physically interact with an integrin. Thus, a *trans*-interaction PVRL3 with PVR was shown to bring αVβ3 integrin to sites of *de novo* forming adherens junctions in adherent MDCK cells [252]. After a junction is

established, PVRL3 recruits PTPμ phosphatase which inhibits interaction of αVβ3 integrin with talin and prevents inappropriate levels of signaling downstream of integrin [253]. Interestingly, while all four nectins were shown to associate with PTPμ phosphatase *in vitro*, integrin β4 lacks binding sites for talin and other focal adhesion complex components. This, in turn, raises an interesting possibility that PVRL4-integrin β4 module may be free of PTPμ phosphatase-based negative feedback regulation mechanism, thus allowing continued potentiation of downstream signaling.

Potential implications of PVRL4-driven anchorage-independence in relation to some aspects of tissue morphogenesis

Even though the role of PVRL1, PVRL2 and PVRL3 in morphogenesis of various tissues has been described, little is known about where, and how, PVRL4 functions in normal tissues. Mutations of PVRL4 were identified in certain mild cases of ectodermal dysplasia [244-247]. In particular, patients with PVRL4 mutations have sparse fragile hair, syndactily and malformation of teeth. Immunohistochemical examination of PVRL4 distribution in skin epidermis and hair follicles from healthy donors revealed that protein expression was strongest in intermediate suprabasal layers, whereas little or no staining was seen in the topmost, terminally differentiated (cornified) layer as well as in the basal layer, which is least differentiated and is the only epidermal layer composed of cells directly anchored on the basement membrane [244]. Even though the skin of individuals with loss-of-function PVRL4 mutations was normal, perhaps as a result of another nectin being able to compensate for loss of PVRL4, their hair growth was profoundly affected.

Considering the possibility that PVRL4 mode of action in tumorigenesis parallels its role in morphogenesis of normal tissue, one may hypothesize that in stratified epithelia, such as skin

epidermis and hair follicles, PVRL4-PVRL1 interaction on surface of neighboring cells may serve to protect suprabasal layers of stratified epithelia from premature physiologic anoikis, thus serving to instate and maintain the stratified architecture of skin and hair follicle. Intriguingly, mammary epithelial cells initiate a squamous-like differentiation program as an alternative form of anoikis in suspension [142], and we have demonstrated that expression of both full-length PVRL4 as well as cytoplasmic region deletion construct alleviates this phenotype.

Interestingly, β4 integrin, paired with α6 subunit, is a major structural integrin in skin epidermis. Mutations in β4 integrin cause junctional epidermolysis bullosa, a severe skin blistering disorder [293]; and conversely, suprabasal overexpression of β4 integrin enhances chemical carcinogen-induced skin tumor formation in mice [294]. Taken together, it would be of particular interest to further explore the contribution of PVRL4 and, potentially, integrin β4/SHP-2/SFK axis to the process of morphogenesis of stratified epithelia via, potentially, delaying premature differentiation of suprabasal epidermal layers.

PVRL4 shows promise as a therapy target in solid tumors

Several lines of evidence obtained from breast, lung and ovarian tumors suggest that PVRL4 expression is upregulated in tumorigenesis and is strongly associated with adverse prognosis in patients suffering from breast and lung cancer. In breast cancer in particular, PVRL4 expression is associated with aggressive tumor characteristics - such as basal-like expression subtype and invasion of cancer cells into multiple lymph nodes. We used blocking antibodies against PVRL4 in a preclinical *in vivo* xenograft model and demonstrate that growth of PVRL4-dependent xenografts is potently inhibited by anti-PVRL4 antibody therapy. Our demonstration of the efficacy of anti-PVRL4 therapy lends a proof-of-principle to a novel

concept of using a therapeutic antibody to block a cancer-specific cell-to-cell attachment to suppress tumor growth.

We also take steps to address three important caveats that arise from this experiment. First of all, we show, via *in vitro* ADCC assay, that PVRL4 antibody does not induce tumor lysis through recruitment of NK cells, supporting that the observed inhibitory effect may be a consequence of blocking PVRL4-PVRL1 interaction and signaling. Second, we show that even though PVRL4 antibody disrupts cell-cell contacts in treated SUM190 xenografts, the cell-cell contact disruption does not affect expression of key epithelial (E-cadherin) and mesenchymal (vimentin) markers, at least at the population level, even though SUM190 cell line is markedly E-cadherin-positive. Third, we show that PVRL4 antibody, though raised against a human epitope, recognizes mouse PVRL4 as well, yet does not trigger acute adverse effects in a mouse. Taken together, our work begins to address some aspects of both efficacy and safety of the proposed therapeutic principle.

It remains to be investigated further, whether in PVRL4-dependent cancer cell lines, such as SUM190 and SUM185 cells, PVRL4 also acts by activating integrin β4/SHP-2/c-Src signaling axis. We began addressing this by demonstrating that the clonogenic growth of SUM190 cells is sensitive to both chemical inhibition of Src and RNAi-mediated depletion of SHP-2 phosphatase, which are key downstream effectors of integrin β4.

It would be further important to explore the relationship between PVRL4 expression in tumors and their sensitivity to chemical inhibitors of c-Src. As gene expression and copy number analysis from human breast cancers suggests, both PVRL4 and integrin β4 expression is associated with a basal-like expression signature, which is characteristic for triple-negative breast cancers (TNBCs). Intriguingly, TNBC tumors display higher levels of activated c-Src in

comparison to other breast cancer subtypes [295]. Since we have shown that PVRL4-driven cell-cell contacts facilitate Src activation it would be further interesting to explore whether positivity for PVRL4 could be a predictor for tumor sensitivity to Src inhibitors, such as dasatinib (Sprycel). Intriguingly, c-Src activation is a predominant mechanism of resistance of HER2-positive breast tumors to trastuzumab therapy [296], which raises an interesting possibility that anti-PVRL4 therapy may be a viable option for treating trastuzumab-resistant tumors.

In addition to treating primary tumors, another utility of PVRL4-targeted antibodies is in preventing metastatic dissemination or colonization by directly targeting circulating tumor cells. Indeed, circulating tumor cells are known to form clusters in the bloodstream as well as to attach to a variety of cell types in order to sustain their survival and promote metastasis. Multiple lines of evidence suggest that PVRL4 is overexpressed in a large fraction of breast tumors and is associated with metastasis; for example, one study showed that among breast cancer cases with 1-3 cancer-positive lymph nodes, over 70% were PVRL4-positive, whereas those cases with four or more positive lymph nodes, 100% were positive for PVRL4. Furthermore, PVRL4 can induce cancer cell attachment to other cell types via PVRL1, as demonstrated by PVRL4-dependent attachment of SUM190 cells to primary human microvascular endothelial cells from the lung, an organ of primary tropism for breast cancer metastasis. Thus, the possibility that PVRL4 may be involved in enabling survival of cancer cells in lymphatic and blood circulation needs to be further explored, including its potential therapeutic implications.

Using histology and two-photon microscopy imaging, we have observed a striking dissolution of cell-cell contacts in anti-PVRL4 antibody-treated tumors. We envision that, besides blocking the cell-matrix mimetic signaling that is associated with PVRL4-PVRL1 interacting module, disrupting cell-cell contacts within a tumor can elicit a number of additional,

less-specific, but no less important, antitumor effects. For example, in the context of an anti-PVRL4 therapy combined with another antitumor antibody or small molecule drug, disruption of cell-cell contacts may have an added benefit of improving the access of therapeutic agents deeper into the tumor core. Similarly, disruption of cell-cell contacts in tumors can allow entry of macrophages and T-cells into deeper areas of the tumor. Finally, cell-cell contact disruption can mitigate tumor hypoxia, acidic pH and interstitial fluid pressure within tumors, which are key contributors to angiogenesis, matrix remodeling and invasion, as well as resistance to therapies [297-299]. Thus, anti-PVRL4 therapy may serve the purpose of normalization of tumor microenvironment in susceptible tumors.

In conclusion, our work uses interdisciplinary approaches, combining high-throughput mammalian cell genetics, analysis of genomic and expression data from human tumors, molecular structure-function studies, exploration of cellular signal transduction and the use of preclinical *in vivo* tumor models, to discover and characterize a novel driver of tumorigenesis, elucidate its mode of action and its effect on intracellular signaling and demonstrate its potential efficacy and safety as a target for antibody-based therapy of cancer.

Appendix I

Supplementary Tables

Supplementary Table 1. PVRL4 deletion series.

Construct	Amino Acids
Full-length	1-510
4 aa C-terminal deletion	1-506
Cytoplasmic region deletion	1-373
Extracellular region	1-342
Extracellular region deletion	341-510
Cytoplasmic region	374-510

Supplementary Table 2. Sequences of shRNAs used in the study.

shRNA	Sense Sequence
FF2 shRNA	CCGCCTGAAGTCTCTGATTAA
PVRL1 shRNA-1	CCCAAGTCACTGGTGCAATAA
PVRL1 shRNA-3	TCCCAGGTGGTCCAGGTGAAC
PVRL4 shRNA-1	CACGGTGAGGGAGATAGAAA
PVRL4 shRNA-3	CAGAGAAGGAGCTATGCTCAA
PVRL4 shRNA-4	CAGGTCACTGTGGATGTTCTT

Supplementary Table 2 (Continued).

shRNA	Sense Sequence
PVRL4 shRNA-5	GTAGCTGCTCTGTGATGAGTGA
PTEN shRNA-1	GAGGCGCTATGTGTATTATTA
PTEN shRNA-2	TGCAGTATAGAGCGTGCAGAT
ITGB4 shRNA-1	ACCTCCAAGATGTTCCAGAA
ITGB4 shRNA-2	AGCACTGTCCTGGTGCACAA
SHP-2 shRNA-1	CGCTCATGACTATACGCTA
SHP-2 shRNA-2	GGCACGAATATACAAATAT

Supplementary Table 3. Sequences of qPCR primers used in the study.

Gene Name	Left	Right
PVRL4	CTGCATTCCCATCACACG	GGCTCTCAGCCCTACACTCTC
PVRL1	ACGGTCATCAGCCGCTAC	AGGCCAAGGACTGCTGGT
PTEN	GGGGAAGTAAGGACCAGAGA	TCCAGATGATTCTTTAACAGGTAGC
TGM1	GGAACTACGGCCAGTTTGAC	GCCGGTCCAGGATGTATAAG

Appendix II

List of candidate ORFs from anchorage-independence screen

Gene Symbol	BC_NUM	ORF_Length	Description
ADCK5	BC031570	510	aarF domain containing kinase 5
AKTIP	BC001134	879	fused toes homolog (mouse)
ALDH1B1	BC001619	1554	aldehyde dehydrogenase 1 family, member B1
ATP5H	BC032245	414	ATP synthase,mitochondrial F0 complex, subunit d
ATPIF1	BC009677	321	ATPase inhibitory factor 1, transcript variant 1
BET1	BC000899	357	BET1 homolog (S. cerevisiae)
C10orf49	BC018068	465	chromosome 10 open reading frame 49
C16orf52	BC027604	276	hypothetical protein LOC146174
C17orf47	BC022189	1713	hypothetical protein FLJ40121
CDC42SE1	BC012796	240	small protein effector 1 of Cdc42
COCH	BC007230	1485	coagulation factor C homolog, cochlin
CSTF2	BC017712	1734	cleavage stimulation factor, 3' pre-RNA, subunit 2
CSTF3	BC009792	312	cleavage stimulation factor, 3' pre-RNA, subunit 3
DCLRE1B	BC029687	1599	DNA cross-link repair 1B
ELAVL2	BC030692	1041	ELAV-like 2 (Hu antigen B)
ERGIC2	BC000887	1134	PTX1 protein
EXOSC3	BC008880	828	exosome component 3
GPR172A	BC002917	1338	putative G-protein coupled receptor GPCR41
GPR177	BC007211	1326	putative NFkB activating protein 373
HIST1H4E	BC012587	312	histone 1, H4e
HSDL1	BC018084	993	steroid dehydrogenase-like
KIAA1609	BC023251	1371	KIAA1609 protein
LPCAT1	BC020166	1170	hypothetical protein FLJ12443
MGC21675	BC013775	276	hypothetical protein MGC21675
MRPS28	BC010150	564	mitochondrial ribosomal protein S28
NPM1	BC002398	885	nucleophosmin (nucleolar phosphoprotein B23)
NTRK3	BC013693	1839	neurotrophic tyrosine kinase, receptor, type 3
PVRL3	BC017572	306	poliovirus receptor-related 3
PVRL4	BC010423	1533	poliovirus receptor-related 4
RAB14	BC006081	648	RAB14, member RAS oncogene family
S100B	BC001766	279	S100 calcium binding protein, beta (neural)
SCRG1	BC017583	180	scrapie responsive protein 1, mRNA
SFRP2	BC008666	888	secreted frizzled-related protein 2
SULF2	BC020962	450	sulfatase 2
TAGLN2	BC009357	600	transgelin 2
TM2D2	BC004878	450	TM2 domain containing 2, mRNA
TMEM65	BC032396	537	hypothetical protein BC017881
TRIP11	BC002656	243	thyroid hormone receptor interactor 11
TRMT1	BC002492	1980	hypothetical protein FLJ20244
UXT	BC000720	474	ubiquitously-expressed transcript

Appendix III: Exploration of potential effects of PVRL4 on antioxidant defense in conditions of anchorage deprivation

Background and Rationale

The understanding of the mechanism and significance of tumor-specific metabolic adaptations have been a focus of intense exploration in the field of cancer biology in the recent years (reviewed in [300]). Profound metabolic changes that tumors undergo include utilization of glucose via aerobic glycolysis (a phenomenon known as Warburg effect) as well as an increased flux through pentose phosphate pathway, a major provider of a cell's NADPH pool. The increased ratio of NADPH to NADP is thought to benefit cancer cells in a number of ways. First, it provides antioxidant capacity to counteract the oxidative stress which is frequently encountered by tumor cells as they are being challenged by constitutively active oncogenes and hypoxic microenvironments. In addition, increased NADPH provides reducing equivalents for a number of complex anabolic processes that are required for proliferation, most notably, *de novo* lipid and cholesterol biosynthesis - molecules which are crucial for membrane formation. The unique metabolic state of cancer cells was shown to be driven by activated oncogenes and is therefore is thought to arise in response to an unchecked pro-growth signaling input experienced by a transformed cell.

Interestingly, interaction of epithelial cells with the extracellular matrix has also been found to profoundly modulate cellular metabolism [131]. In particular, culturing normal mammary epithelial cells in the absence of substratum attachment not only suppressed their proliferation but also caused a profound defect in glucose uptake, reduced the flux through pentose phosphate pathway and decreased the cellular NADPH/NADH ratio. Moreover,

substratum-detached cells were found to contain higher levels of reactive oxygen species. Importantly, the observed defects were shown to be corrected by the ectopic expression of ERBB2 oncogene. Furthermore, supplementing the culture medium with antioxidant compounds such as N-acetylcysteine or Trolox (a vitamin E derivative) was found to be sufficient to rescue the proliferation defect induced by the substratum detachment.

We asked whether TL-HMECs similarly accumulate high levels of reactive oxygen species when cultured in the absence of attachment. Furthermore, having shown that PVRL4 enables colony formation of TL-HMECs in anchorage-independent conditions, we wanted to test a possibility that at least some aspects of PVRL4 action in anchorage-independence can be carried out through the modulation of oxidative stress and NADPH concentrations in a detached cell.

Results

We sought to assess the effect of PVRL4 expression on detachment-induced oxidative stress by staining live TL-HMECs with carboxy-H2DCF-DA, a cell-permeable ROS marker. Strikingly, ROS levels were decreased two-fold in PVRL4-expressing HMECs, as measured by carboxy-H2DCF-DA signal normalized to total protein concentration (Figure 31a, b). Similarly, PVRL4 depletion by RNAi increased ROS levels in SUM190 cell line in suspension (Figure 31c). We next asked whether the observed differences in ROS levels were paralleled by changes in NADPH/NADP ratio, since NADPH is a major supplier of a cell's antioxidant capacity, and NADPH/NADP ratio has been previously shown to be affected by the attachment deprivation. Indeed, we have found that the expression of PVRL4 in TL-HMECs potently increased both total NADP as well as NADPH/NADP ratio (Figure 31d), suggesting that PVRL4 may exert at least

some of its protective role on growth of cells in suspension via mitigating the metabolic defects experienced by epithelial cells upon their detachment from the matrix.

However, restoring the antioxidant capacity of TL-HMECs via exogenous supplementation of medium with antioxidant compounds, NAC and Trolox, failed to protect TL-HMECs from suspension-induced growth suppression (Figure 31e). This result suggests that restoring the antioxidant capacity of TL-HMECs alone is not sufficient for restoration of their proliferation in anchorage-deprived conditions, which is contrary to what has been previously observed in MCF10A cells. Further studies aimed at exploration of the significance of the effect of PVRL4 on cellular oxidative status and NADPH content, as well as the mechanism of this effect, are needed. Besides its role in maintaining the antioxidant capacity of the cell (such as through replenishing reduced glutathione), NADPH is also a molecule critical in biosynthesis of a number of macromolecules, and it would be of particular interest to explore whether PVRL4 expression affects these anabolic processes inside the cell as well.

Figure 31. PVRL4 suppresses suspension culture-induced oxidative stress. a, TL-HMECs expressing PVRL4 or vector control were cultured in the absence of attachment for 96 hours. Cells were stained with carboxy-H2DCF-DA dye to measure reactive oxygen species (ROS) abundance. Dye fluorescence was quantified and normalized to total protein content. Values are expressed as relative to empty vector control. Assays were performed in triplicate (error bars ± SD). **b,** Carboxy-H2DCF-DA-stained cells from **(a)** were photographed to provide a visual representation of the ROS signal. **c,** SUM190 cells expressing anti-PVRL4 shRNA-3 and shRNA-4 or control shRNA were cultured as in **(a)** and relative ROS abundance was similarly determined. Values are expressed as relative to control shRNA sample. Assays were performed in triplicate (error bars ± SD). **d,** TL-HMECs expressing PVRL4 or vector control were cultured in the absence of attachment for 96 hours. Cell lysates were used for determining reduced NADPH and total NADP values for each sample. NADPH/NADP ratio was calculated using the following formula: NADPH/(total NADP – NADPH). **e,** TL-HMECs expressing PVRL4 or vector control in presence of solvent control or indicated antioxidants were assayed for viability under conditions of ECM deprivation by measuring total ATP content in cells cultured on ultra-low attachment plates for 72 hours. Values were normalized to empty vector-transduced sample. Assays were performed in triplicate (error bars ± SD).

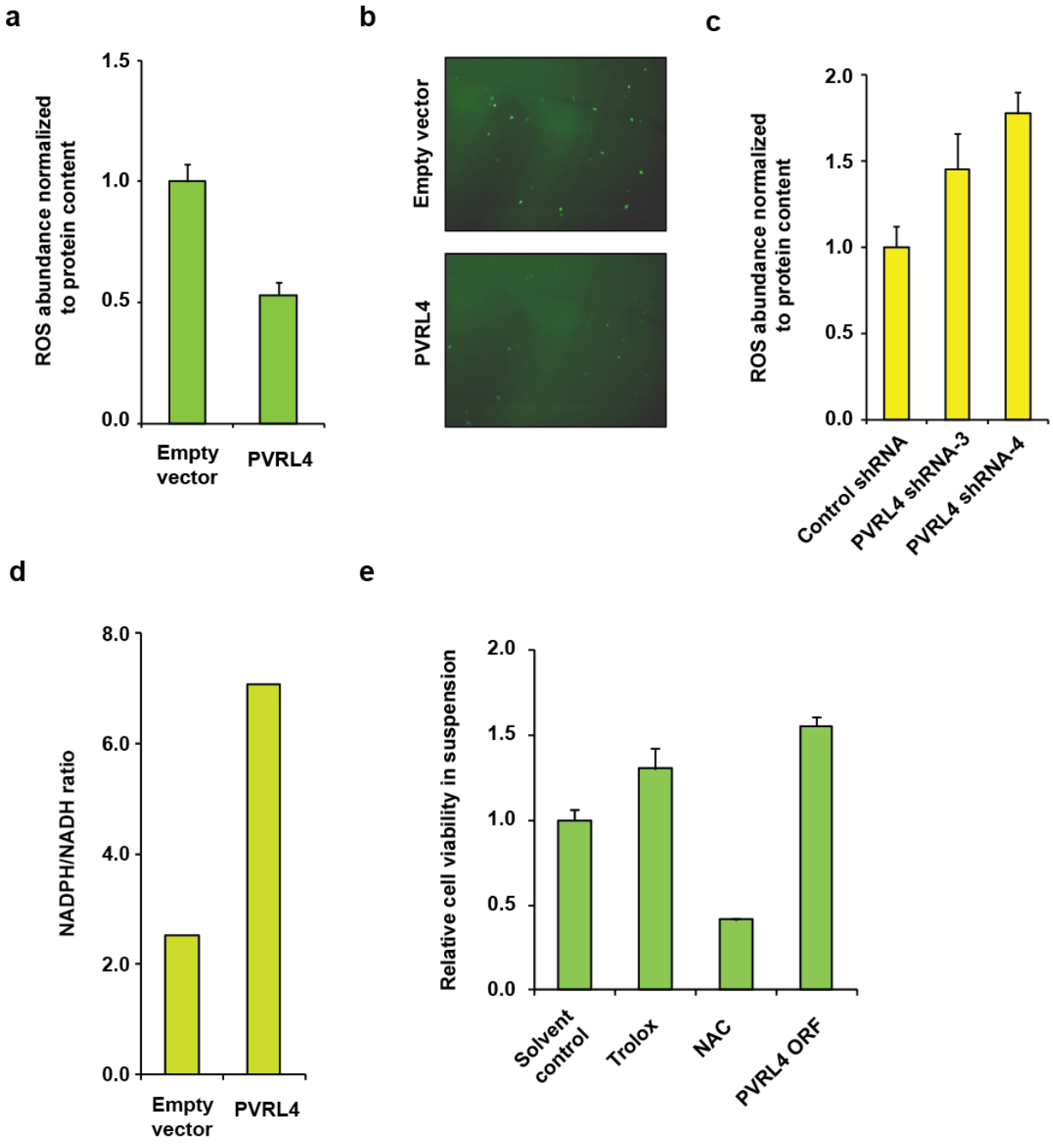


Figure 31 (Continued).

Materials and Methods

ROS measurement. TL-HMEC or SUM190 cells transduced with indicated constructs were cultured on ultra-low attachment plates in their respective complete media with 1% methylcellulose (a thickening agent) for 96 hours. Cells were harvested and washed three times with cold PBS three times. A fraction of cells was used to determine total protein concentration via BCA assay (Pierce), and another fraction was labeled with carboxy-H2DCF-DA ROS sensor dye (C-400, Invitrogen). Carboxy-H2DCF-DA-stained cells were photographed and their fluorescence signal was measured with Victor X5 plate reader. A background fluorescence reading from "no cells" control was subtracted from the raw values, and the resulting numbers were normalized to total protein content.

NADPH measurement. NADPH and total NADP levels from suspension-cultured TL-HMEC lysates transduced with indicated constructs were determined using NADPH/NADP assay protocol (ab65349, Abcam) and normalized to protein content. NADPH/NADP ratio was calculated using the following formula: NADPH/(total NADP – NADPH).

Anoikis assays in the presence of antioxidants. For anoikis assays, cells were cultured on ultra-low attachment plates (Corning) in reduced growth factor MEGM with 1% methylcellulose. Where indicated, 1 mM N-acetyl-cysteine (A7250, Sigma) or 50 μM Trolox (648471, EMD Biosiences) were added. Total ATP measurements were performed using CellTiterGLO reagent (Promega) according to the manufacturer's protocol after 72 hours of growth in suspension, and luminescence values were read with Victor X5 plate reader.

Appendix IV: Alternative strategies for inducing TL-HMEC clustering and their effect on anchorage independent colony formation

Background and Rationale

We have discovered that PVRL4 drives anchorage independent growth of TL-HMECs in a manner that is dependent on its ability to potentiate cell-cell contacts via its counter-receptor PVRL1, whereas the transmembrane as well as cytoplasmic parts of the receptor and its counter-receptor are dispensable and can be substituted by an unrelated transmembrane domain sequences. Furthermore, we have identified integrin β4 as an interacting partner of PVRL4. Importantly, RNAi against integrin β4 negatively affected (albeit not abolished) PVRL4-driven anchorage-independence, suggesting the functional relevance of integrin β4 –PVRL4 interaction to the observed phenotype. However, this finding does not rule out the possibility that PVRL4 may affect cellular survival in a more than one way, in addition to recruiting integrin β4. Notably, as we also show, a number of distinct oncogenic perturbations induce cell-cell clustering. One intriguing possibility is the existence of a generalized prosurvival effect that the increased propensity for cell-to-cell attachment in suspension might convey. Such prosurvival effect can be mediated by, for example, local accumulation of growth factors, facilitation of extracellular matrix component deposition or creation of flattened surfaces in an otherwise rounded cells. To test whether the facilitation of cell-to-cell attachment by itself is sufficient for enhancing anchorage-independent survival, we sought alternative ways of potentiating cell clustering in TL-HMECs and assessed their effects on colony formation.

Results

We tested two distinct strategies to induce cell-to-cell attachment. First, we used a monoclonal antibody against CD44 protein (clone H4C4) that was previously shown to induce self-aggregation of lymphocytes [301]. Similar to lymphocytes, TL-HMECs readily formed multicellular clusters in presence of H4C4 antibodies (Figure 32a). Plating a single-cell suspension of TL-HMECs into semi-solid medium in the presence of H4C4 resulted in a dramatic increase in anchorage-independent colony formation as compared to control IgG (Figure 32b). A caveat to this experiment is that CD44 protein itself can affect signaling events and therefore it is possible that H4C4 antibody perturbs cellular signaling in a manner that is not directly related to cell-cell clustering. Therefore, we decided to test whether a purely artificial inducer of cell-cell clustering will have an effect on anchorage-independence. We created a synthetic construct in which two tandem mutant FKBP dimerization domains (DmrD) with a propensity to form trans-dimers even in the absence of the ligand [302] were fused in frame into a construct with an N-terminal signal sequence and a C-terminal transmembrane domain derived from CD8 (Figure 32c). The DmrD-CD8tm construct was stably expressed in TL-HMECs and its membrane localization was confirmed by immunofluorescence (data not shown). DmrD-CD8tm promoted both cell clustering and anchorage-independent growth of TL-HMECs (Figure 32d, e), thus providing an additional demonstration that potentiation of cell-cell interaction is sufficient for inducing transformation. Taken together, these results suggest that the potentiation of cell-to-cell attachment per se may be sufficient for at least some protection against suspension-induced death or growth arrest.

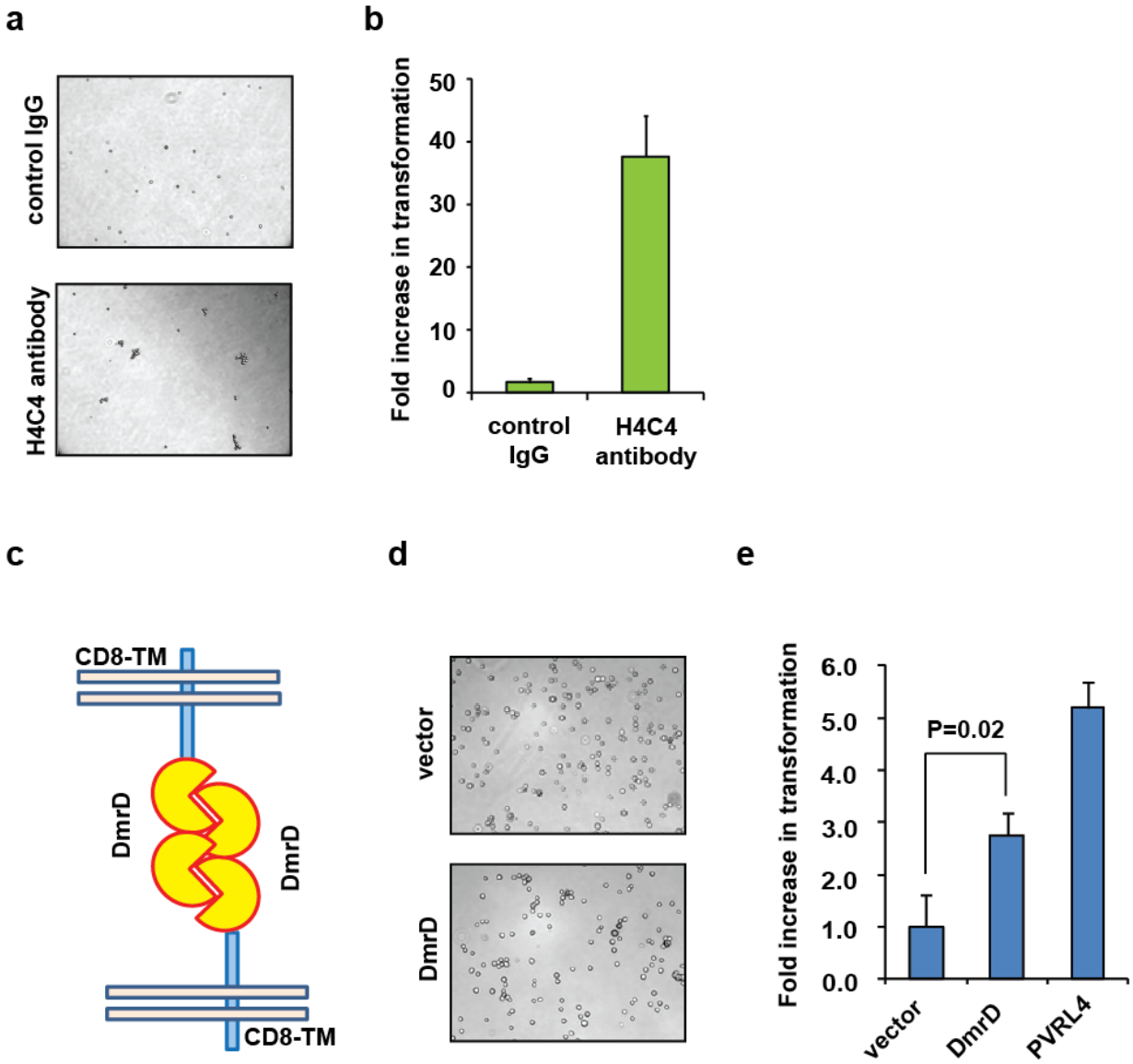


Figure 32. Alternative cell-cell clustering approaches and their effect on anchorage-independent growth. a, TL-HMECs were assayed for cell-cell clustering in presence of H4C4 (anti-CD44 mouse anti-human antibody (University of Iowa Developmental Studies Hybridoma Bank) antibody or control IgG at 4 μg/mL. Representative images are shown. **b,** Transformation of TL-HMECs was assayed in presence of H4C4 (anti-CD44) antibody or control IgG. Colony numbers were normalized to control sample. Transformation assays were performed in triplicate (error bars ± SD). **c,** A scheme depicting the DmrD-CD8tm construct design and binding strategy. **d,** TL-HMECs were transduced with empty vector or the DmrD-CD8tm construct and assayed for clustering. Representative images are shown. **e,** Transformation of TL-HMECs transduced with virus representing the empty vector, or virus expressing DmrD-CD8tm and PVRL4 was assayed as before. Colony numbers were normalized to the control sample. Transformation assays were performed in triplicate (error bars ± SD).

Materials and Methods

To create a DmrD-CD8tm construct, a prHom-Nuc1 plasmid (iDimerize Reverse Dimerization System, Clontech) was used as a template in a PCR reaction with a forward primer containing CD8 signal sequence followed by a FLAG tag and a reverse primer containing CD8 transmembrane domain sequence followed by a STOP codon. The resulting product was cloned into pQCXIN vector.

Appendix V: A whole genome loss-of-function genetic screen for suppressors of TL-HMEC transformation

Background and Rationale

In addition to gain-of-function screens, TL-HMEC transformation system can be used as a discovery tool for identifying negative regulators of transformation and, thus, potential tumor suppressors. Focused libraries of shRNAs have been successfully used by our group for identification of novel tumor suppressors REST [98] and PTPN12 [99] . We therefore set out to perform a whole-genome screen for negative regulators of anchorage-independent growth, taking advantage of an expanded, third-generation shRNA library in miR-30 context. This library provides a comprehensive coverage of 22,000 human genes with each gene targeted with 12 individual shRNAs. The library is divided into 12 pools of equal complexity, each targeting approximately 1,800 genes.

Results

Each pool was screened separately in quadruplicate, with 100-fold library representation for each replicate and the multiplicity of infection of 0.2. For the high-throughput bioinformatic readout of shRNAs that become enriched among resulting colonies and thus are likely to be directed towards transformation suppressor, we took advantage of NextGen Illumina sequencing. To do this, we pooled all of the colonies that were formed within each of the four screen replicates and isolated total genomic DNA. To correct for the relative abundance of each shRNA in the starting material, we also isolated genomic DNA from the corresponding library-infected pools of cells prior to their plating into methylcellulose. For each genomic DNA sample, we

have recovered the antisense arm of the shRNA hairpin by PCR, followed by adding p5 and p7 Illumina adapters in a second round of PCR (for the PCR strategy schematic, see Figure 33a). Resulting PCR products were then used as independent barcodes that we proceeded to sequence following the Illumina protocol (Figure 33b).

For each of the four replicates of each of the twelve pools, we then calculated the enrichment score for each shRNA. Specifically, for each shRNA, we have determined the ratio of the number of sequencing reads in the end sample (represented by shRNAs recovered from anchorage-independent colonies) to the number of reads for the same shRNA in the start sample (represented by the library-infected cell population prior to introduction into anchorage-independent conditions). As expected, a very small subset of shRNAs showed massive enrichment over the start sample, while the majority of shRNAs showed little or no enrichment. In order to be included on the list of potential candidates for the phenotypic validation, we have decided to apply a 10-fold enrichment cutoff for individual shRNAs recovered in each replicate. The reasoning behind this was that since each shRNA should be theoretically present in a 100 cells in the start sample, a 10-fold enrichment in the end sample would correspond to a colony composed of 1000 or more cells.

As a next step, for each gene from the library, we computed a so-called “gene score”, represented by the number of times each of the twelve shRNAs targeting a given gene appeared in four independent screen replicates plus the number of times any of the twelve shRNAs against this gene appearing across all four replicates. With this approach, we treated each of the twelve shRNAs as independent gene-specific events which allowed us to further expand the library representation and increase the discovery power of the screen.

We then have taken several further steps to refine the candidate list. First, we have performed expression profiling of TL-HMEC transcriptome in both attached and suspension cultures and excluding those genes that were not expressed in either condition from the candidate list. Second, we searched several publicly available copy-number, expression and mutation datasets for those genes from the list that had evidence of being altered in various tumors. Finally, we have performed literature searches to identify those genes that were previously shown to be involved in regulation of cellular growth and survival. In total, our list of putative candidate transformation regulated contained 697 candidate genes.

We then began individual validations of the anchorage-independence phenotype with the candidate shRNAs that met the cutoff standards listed above. The absolute colony numbers from validation experiments that have been performed so far are shown in Figure 34. The current direction of this project is to carry out the individual validations of all the genes from the list of candidates with at least two shRNAs.

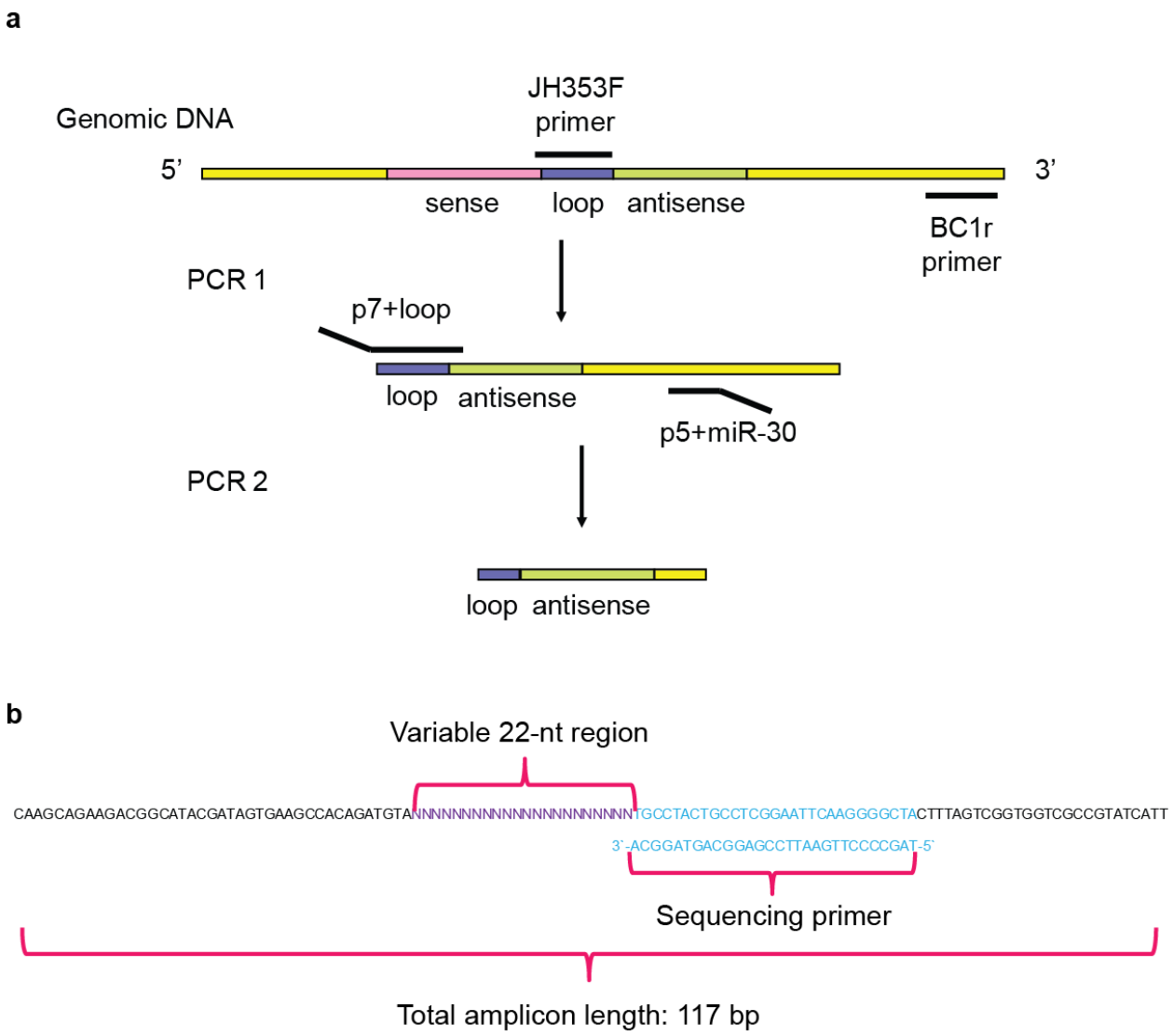


Figure 33. Illumina sequencing strategy. a, A schematic of a two-stage PCR protocol for the recovery of an antisense arm of an shRNA from genomic DNA of virus-transduced cells (PCR1) followed by the addition of p5 and p7 adaptor arms for Illumina sequencing (PCR2). **b,** A detailed structure of the PCR2 amplicon. The variable (antisense) part is highlighted in purple and the sequence to which Illumina sequencing primer miR-30-EcoRI anneals is highlighted in blue.

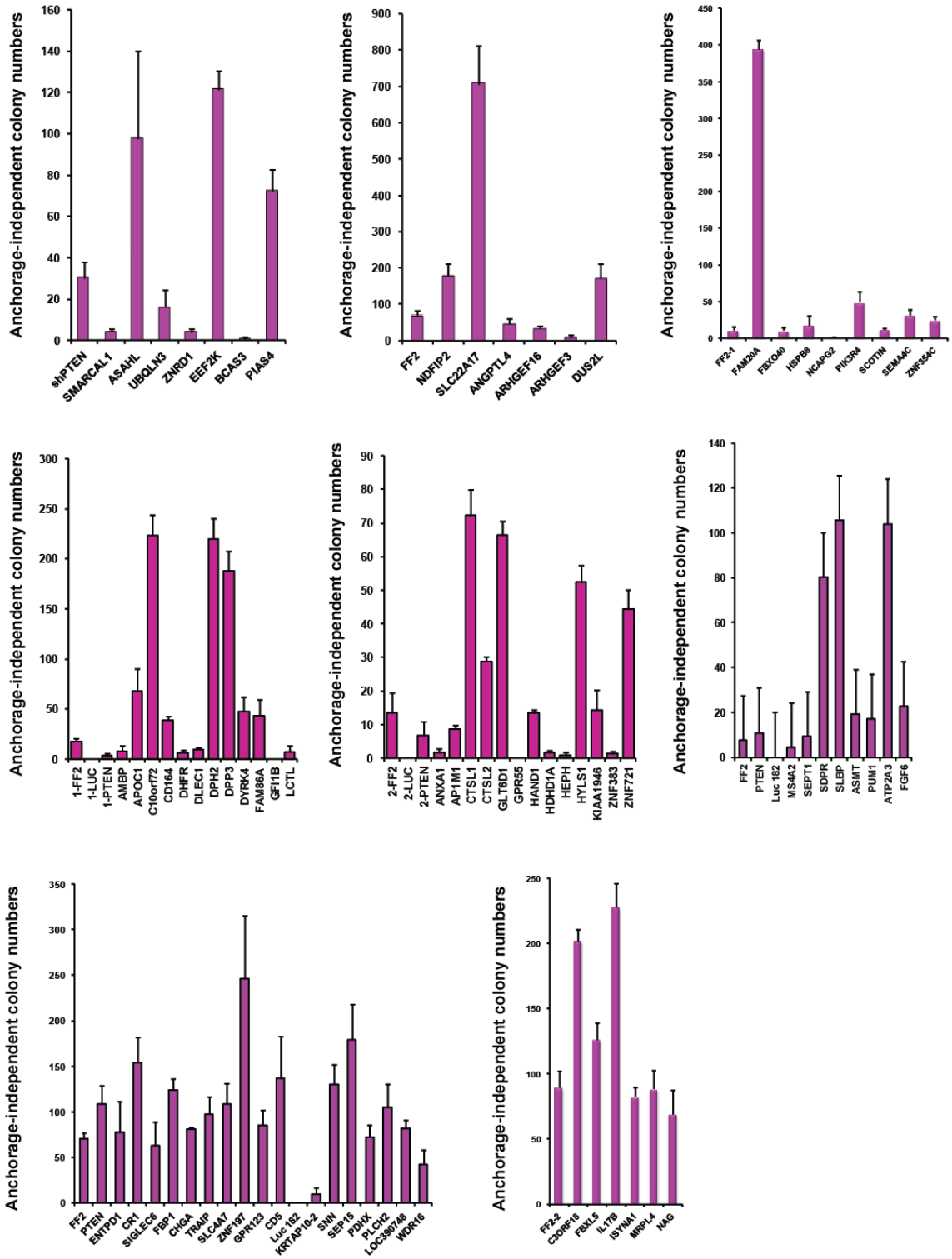


Figure 34. Validation of candidate anchorage-independence suppressors. Candidate shRNAs were de novo synthesized and subcloned into miR-30 context vector. TL-HMECs were transduced with indicated shRNAs or control shRNAs and their ability to form colonies in 2% methylcellulose was assayed exactly as before.

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