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MINI-REVIEW

The Arf-GAP and protein scaffold Cat1/Git1 as a multifaceted regulator of cancer progression

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ABSTRACT

Cool-associated tyrosine phosphorylated protein 1 (Cat1), also referred to as GPCR-kinase interacting protein 1 (Git1), is a ubiquitously expressed, multi-domain protein that is best known for regulating cell shape and migration. Cat1/Git1 functions as a GTPase activating protein (GAP) that inactivates certain members of the ADP-ribosylation factor (Arf) family of small GTPases. It is also a scaffold that brings together several signaling proteins at specific locations within the cell, ensuring their efficient activation. Here we will discuss what is known regarding the classical role of Cat1/Git1 in the regulation of cell morphology and migration, as well as highlight some more recent findings that suggest this interesting signaling/scaffolding protein may also contribute in unexpected ways to oncogenic transformation.

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Cat1/Git1 negatively regulates Arf small GTPase activity

Cells respond to cues that they receive from their surroundings by inducing signaling events that often culminate in the activation of small GTP-binding proteins (GTPases), a diverse family of proteins that are defined by their ability to cycle between an inactive GDP-bound state, and an active GTP-bound state. When properly regulated, small GTPases elicit signaling events that mediate a broad spectrum of cellular processes including changes in cell attachment and shape, migration, intracellular vesicle and protein trafficking, gene expression, cell growth, and even apoptosis. However, dysregulation of the signaling capabilities of these same proteins has been shown to hijack "normal" cellular processes, giving rise to pathological conditions such as cancer. Thus, understanding how small GTPases are regulated and function in different contexts continues to be a major research emphasis.

There are 2 major classes of proteins that directly impact small GTPase activity; specifically, guanine nucleotide exchange factor proteins (GEFs) and GTPase activating proteins (GAPs). GEFs function to activate small GTPases by catalyzing GDP-GTP exchange, while GAPs terminate their signaling activity by stimulating the hydrolysis of GTP to GDP. A rather interesting example of a GAP is Cat1/Git1. This ~95 kDa protein has an N-terminal GAP-domain containing a consensus zinc finger motif (Fig. 1, *GAP Domain*) that is required for the ability of Cat1/Git1 to catalyze the hydrolysis of GTP to GDP on certain members of the Arf family of small GTPases, namely Arf1 and Arf6.[1,2]

Arf1 is localized to the Golgi apparatus where it controls the trafficking of transport vesicles containing proteins that will be secreted between the different compartments of this organelle, as well as between the endoplasmic reticulum and the Golgi apparatus. The GTP-bound form of Arf1 recruits coat protein complex 1 (COP1), adaptor proteins (APs)-1,3, and 4, and Golgi-adaptin ear domain homology, Arf-binding domain (GGA), to initiate the budding of transport vesicles, promoting their movement within Golgi compartments.[3,4] GAP-mediated GTP hydrolysis of Arf1 terminates these trafficking events by causing the dissociation, or uncoating, of COP1 from the vesicles.[3] On the other hand, Arf6 is expressed primarily along discrete regions of the plasma membrane and is associated with some endosomes.[3,4] Consistent with these locations, Arf6 has been shown to play an important role in promoting the endocytosis of cell surface receptors.[3] GTP-bound Arf6 stimulates endocytosis by increasing the phosphatidylinositol-4-phosphate 5-Kinase (PIP5K) dependent production of phosphatidylinositol 4,5-bisphosphate (PIP_2)

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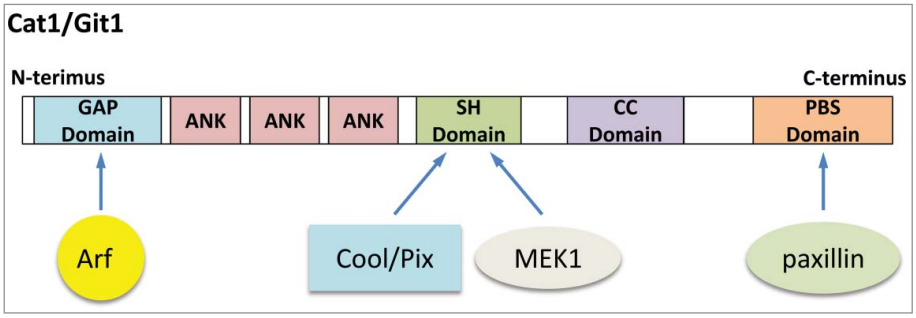

Figure 1. Diagram showing the different structural domains in Cat1/Git1. Starting at its N-terminus, Cat1/Git1 has a GAP domain, followed by 3 ankyrin repeats (ANK), a Spa-2 homology (SH) domain, a coiled-coil (CC) domain, and a paxillin binding sequence (PBS) domain. Also, included on the diagram are some of the major protein partners of Cat1/Git1 (i.e., Arf, Cool/Pix, MEK1, and paxillin) and where they bind.

at the cell surface,[3,5] which promotes the recruitment of endocytic proteins such as AP-2 and clathrin to newly formed sites of endocytosis.[3] Inactivation of Arf6 limits endocytosis and even promotes the recycling of some endosomes, together with their corresponding cargo, back to the plasma membrane.[3]

Increases in Arf1 and Arf6 expression and activation have been observed in several different types of human cancer.[6-9] For example, Arf1 expression is elevated in high grade and aggressive gastric tumor samples, compared with lower grade tumors or normal tissue.[6] The ectopic expression of Arf1 in lower grade gastric cancer cell lines that express relatively low amounts of Arf1 was sufficient to cause these cells to acquire an aggressive phenotype, including enhanced growth, migration, and invasive activities. Similar findings have been reported for Arf6. The levels of this small GTPase were found to be highly upregulated in several aggressive and metastatic cancer cells such as breast, brain and prostate cancer cells.[10-12] Knocking-down its expression in these cells using siRNAs, or introducing a mutant form of Arf6 that is unable to bind GTP, suppressed their invasive and metastatic behaviors.[10,11] These findings, when combined with the fact that ectopically expressing activating mutants of Arf1 and Arf6 together in non-transformed NIH3T3 fibroblasts induces their ability to form colonies in soft agar (i.e. anchorage-independent growth),[13] an *in vitro* read-out of tumorigenicity, suggest that increasing the expression and/or activation of at least some Arf family members potentially play important roles in several different aspects of cancer progression.

Based on these findings, it is reasonable to speculate that proteins that inactivate Arf1 and Arf6, such as GAPs like Cat1/Git1, would function to limit the cancer promoting actions of these small GTPases. Indeed, this seems to be at least partially correct, as expression of a point mutant of Cat1/Git1 that interferes with its ability to act as a GAP (Cat1/Git1 R39A) in HeLa cervical carcinoma cells not only led to increased levels of activated, GTP-bound Arf1 and Arf6, but also strongly enhanced their ability to form colonies in soft agar, compared with the parental HeLa cell line.[13] However, there also appears to be more to the story, as immunohistochemistry performed on 80 cervical cancer sections and 20 normal cervical tissue samples revealed that an overwhelming majority (i.e., ~95%) of the tumor samples overexpressed Cat1/Git1.[13] Increases in the levels of Cat1/Git1 has also been observed in several different advanced-stage breast cancer cell lines.[14] We discovered that depleting Cat1/Git1 expression in cervical cancer cells, or in fibroblasts that have been transformed through the expression of an activated form of the small GTPase Cdc42 (Cdc42 F28L), using siRNAs, caused both cell types to lose their colony forming capabilities.[13] Moreover, knocking-down Cat1/Git1 expression in several cancer cells that were selected for their highly invasive and metastatic activity was shown to revert these phenotypes.[14-19] Thus, these findings highlight 2 critical points that will be the focus of the remainder of this review. First, Cat1/Git1 must have roles other than simply functioning as an Arf GAP. Second, the regulation and functional consequences of Cat1/Git1 in malignant transformation is currently not well understood and will likely be complex and context dependent. At least in some cases, Cat1/Git1 helps maintain oncogenic transformation by potentially impacting several different aspects of cancer progression, such as promoting invasive and metastatic activity, as well as enhancing cell growth and survival. These findings will be highlighted. However, other lines of evidence suggest that Cat1/Git1 may also act to limit transformed characteristics, i.e., possibly when functioning as Arf GAP. Determining whether Cat1/Git1 helps to maintain or prevents the formation of different types of cancers, as well as identifying the mechanisms

through which these effects are mediated, will undoubtedly be a point of emphasis going forward.

Cat1/Git1 is a protein scaffold

In addition to its N-terminal GAP domain, several other domains have been identified in Cat1/Git1 (Fig. 1), including ankyrin repeats (denoted as *ANK*), a Spa2-homology domain (denoted as the *SH Domain*), a coiled-coil domain (denoted as the *CC Domain*), and a C-terminal paxillin binding sequence domain (denoted as the *PBS Domain*). Each domain has been shown to carry-out out a distinct function. The ankyrin repeats help localize Cat1/Git1 to endosomes,[20] while the coiled-coil domain allows for the formation of Cat1/Git1 homodimers.[21,22] However, the Spa-2 homology and paxillin binding sequence domains, which have been extensively studied, serve as binding sites for proteins.[20,23-26] The identification of these distinct regions and the proteins that bind them, has shed some light on the mechanism through which Cat1/Git1 mediate its effects on cellular transformation. In particular, it is now appreciated that Cat1/Git1 can also function as a protein scaffold that brings together several different proteins, ensuring their proper localization and efficient activation. To date, at least 100 Cat1/Git1 associating proteins have been reported, underscoring the potential of this protein to influence several different signaling pathways and cellular processes.[27] Here, we will highlight what is currently known regarding some of the most prominent roles played by Cat1/Git1 as a scaffold. It is important to keep in mind that, although each of the distinct functions of Cat1/Git1 will be described separately, some of them are most likely occurring simultaneously, while others act antagonistically, to mediate specific biologic effects.

The Cool-Cat1/Git1 complex- Cat1/Git1 was initially identified in our laboratory almost 20 y ago as a tyrosine phosphorylated binding partner for a GEF called cloned-out-of-library (Cool), hence the name Cool-associated tyrosine phosphorylated protein, or Cat.[23] The same protein and additional family members were identified by Premont and colleagues (Git),[28] Turner and colleagues (Pkl for paxillin kinase linker),[26] and Di Cesare and colleagues (p95-APP for p95-ADP ribosylation factor GTPase-activating protein).[20] Cool, which is also referred to as p21-activated kinase-interacting exchange factor (Pix), is a GEF for Cdc42 and Rac,[29,30] 2 members of the Rho family of small GTPases. It binds to Cat1/Git1 through the Spa2-homology domain,[24] and Cool-Cat1/Git1 complexes are localized throughout the cytosol, along the cell membrane, and especially to focal adhesions, distinct structures that physically connect a cell to its extracellular environment (focal adhesions will be discussed in more detail later).[31] Because both Cool and Cat1/Git1 can form homodimers, it is thought that Cool-Cat1/Git1 complexes assemble into large oligomeric complexes in cells as a mechanism to promote signal amplification.[27,32,33]

The localization of Cool-Cat1/Git1 complexes to the cell surface has been shown to be important for mediating G-protein coupled receptor- and growth factor receptor-induced activation of Cdc42 and Rac, as well as for the regulation of Arf GTPase activation.[27,31] Activation of these signaling pathways lead to cytoskeletal rearrangements which are required for promoting cancer cell migration and invasion, and enhancement of cell growth.[27,31] The ectopic expression of a mutant form of Cat1/Git1 that is unable to associate with Cool abolishes these effects, highlighting the importance of this protein-protein interaction.[34,35]

The MEK1-ERK1/2-Cat1/Git1 complex- Cat1/Git1 has also been shown to promote the activation of the mitogen activated protein kinase ERK1/2.[36] This is an outcome of a complex that can form at focal adhesions between Cat1/Git1, MEK1.[36] (i.e., the kinase that phosphorylates ERK1/2), and ERK1/2.[37] Similar to Cool, MEK1 binds to the Spa-2 homology domain of Cat1/Git1, an interaction that is enhanced when Cat1/Git1 is phosphorylated by the non-receptor tyrosine kinase c-Src.[36,37] While it is not entirely clear how Cat1/Git1 binds to ERK1/2, this interaction is augmented following the treatment of cells with epidermal growth factor (EGF).[36] Interestingly, knocking-down Cat1/Git1 expression levels in vascular smooth-muscle cells, or human embryonic kidney 293 cells, using siRNAs was sufficient to block EGF- and c-Src-mediated ERK activation and cell migration.[36,37] On the other hand, the ectopic expression of Cat1/Git1 in these same cells was found to extend EGF-induced ERK activation.[36] These findings suggested for the first time that Cat1/Git1, at least in certain contexts, could function as a signaling scaffold that connects growth factor receptor signaling to the ERK pathway. They also raised the intriguing possibility that the ability of Cat1/Git1 to promote ERK activation might also contribute to tumor expansion, since the Ras-ERK pathway is known to stimulate cell growth. Indeed, it was recently shown that Cat1/Git1 mediated activation of ERK was responsible for increasing the rates of tumor formation in a liver cancer model.[16,38]

The Paxillin-Cat1/Git1 complex- Focal adhesions are unique and dynamic structures found along the plasma membrane.[39] They use integrins to physically connect the cell to the extracellular matrix and consist of several additional proteins which are important for helping maintaining cell attachment.[39] Focal adhesions also can assemble and disassemble, or turnover, in a coordinated

manner. This is one of the principal mechanisms underlying cell migration.[39] However, focal adhesions also serve as signaling hubs that transduce signals initiated by cellular interactions with the extracellular environment.[40] In addition to regulating cell attachment and motility, these signaling events have been shown to increase the rates of cell growth and survival.[41]

Paxillin is one of the major constituents of focal adhesions and is known to regulate cell spreading and cell migration.[42] It functions as a scaffold/adaptor protein and is responsible for the recruitment of several different proteins to focal adhesions via their association with one of paxillin's 5 N-terminal leucine-rich (LD) motifs and 4 C-terminal double-zinc finger motifs, referred to as LIM domains.[42] The fourth LD domain of paxillin (i.e., the LD4 domain) was shown to bind to the C-terminal region of Cat1/Git1.[26] The resulting paxillin-Cat1/Git1 interaction causes Cat1/Git1 to localize to focal adhesions, while ectopic expression of mutant forms of Cat1/Git1 that lack the paxillin binding domain are no longer enriched in these structures.[43,44]

In a series of eloquent studies performed by several different groups, it was shown how interfering with the ability of Cat1/Git1 to localize to focal adhesions had a dramatic effect on cell attachment and migration. In one such study, the ectopic expression of a Cat1/Git1 mutant defective in its ability to bind paxillin, while retaining its other activities, in CHO.K1 fibroblasts, was found to be sufficient to cause aberrant cell spreading[43] and blocked the ability of cells to migrate into a wound.[45] Expression of a mutant form of paxillin that is unable to bind Cat1/Git1 in cells yielded similar results.[43,45] Based on these findings, it was thought that the exclusion of Cat1/Git1 from focal adhesions might affect localized signaling events that are important for mediating these effects. This idea turned out to be correct, as subsequent discoveries showed that by failing to associate with paxillin, Cat1/Git1 is not able to help mediate the Cool/Pix dependent activation of Cdc42 and Rac1.[43,46-49] This would in turn compromise the activation of the serine-threonine kinase p21-activated kinase (PAK) at focal adhesions,[50,51] which is a key event for triggering cytoskeletal rearrangements and promoting cell motility.[50-54] It is also worth noting that immunofluorescent experiments have revealed that both the Cool-Cat1/Git1 complex and the MEK1-ERK1/2-Cat1/Git1 complex are enriched in focal adhesions.[37] Thus, it is interesting to consider the possibility that the localization of Cat1/Git1 to these structures enables it to mediate the efficient and localized activation of signaling events needed to elicit biologic effects (i.e., cell attachment and migration).

The mTOR-Cat1/Git1 complex- Deregulation of the phosphatidylinositol-3-kinase (PI3 kinase)/AKT signaling pathway is a common occurrence in human malignancies.[55,56] This pathway is initiated when the activated form of PI3 kinase generates phosphatidylinositol 3,4,5-trisphosphate (PIP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2) along the cell surface. Phosphoinositide-dependent kinase 1 (PDK1) is then recruited to the plasma membrane where it activates AKT. AKT, in turn, prevents tuberous sclerosis complex 2 (TSC2) from acting as a GAP for the small GTPase Ras homolog enriched in brain (Rheb), leading to increases in the levels of the GTP-bound form of Rheb. Activated Rheb binds and activates the mechanistic target of rapamycin (mTOR), specifically, when it is interacting with Raptor and PRAS40 as part of a protein complex referred to as mTOR complex 1 (mTORC1). Activation of mTORC1 induces the phosphorylation of p70S6 kinase, resulting in the synthesis of proteins that stimulate cell growth and survival. It is also worth noting that mTOR can interact with an additional set of proteins, including Rictor and mSIN1, to form mTOR complex 2 (mTORC2).[56] This protein complex is functionally distinct from mTORC1 and is best known for its ability to mediate cytoskeletal rearrangements through the activation of protein kinase C-α (PKCα), as well as members of the Rho family of small GTPases. mTORC2 has also been shown to promote cell survival by phosphorylating AKT.[56,57]

Cat1/Git1 was recently identified in a screen as a novel mTOR-binding partner in astrocytes.[58] The authors showed that the ability of Cat1/Git1 to co-immunoprecipitate with mTOR was dependent on AKT activity, and that the interaction between these 2 proteins was important for mediating the survival of astrocytes. However, what made the findings from this study especially intriguing was the condition under which the interaction between Cat1/Git1 and mTOR appears to occur. Rather than co-immunoprecipitating with mTOR as part of either mTORC1 or mTORC2, Cat1/Git1 interacts with mTOR to form a unique complex that does not include the core components of either mTORC1 or mTORC2, specifically Raptor and Rictor. These findings suggest that Cat1/Git1 can play an important role in regulating the PI3 kinase/AKT/mTOR signaling pathway by forming a novel protein complex in at least some cell types (i.e., astrocytes).

Cat1/Git1 in cancer progression

The protein levels of Cat1/Git1 are frequently upregulated in high grade and aggressive forms of several different types of cancer cells including cervical,[13] breast,[14,18] liver,[59] lung,[15] kidney[60] and oral cancers.[17] At least one mechanism which can account for these changes in Cat1/Git1 expression has been discovered and it involves

microRNAs. To date, 4 distinct microRNAs that target Cat1/Git1 have been identified. Each of these microRNAs is expressed at relatively high levels in normal cells and low-grade cancer cells, and function to keep Cat1/Git1 levels in check. However, in high grade cancer cells with invasive and metastatic activities, the microRNAs targeting Cat1/Git1 are downregulated and lead to increases in Cat1/Git1 expression.[14,17-19] Reversing this effect in some highly aggressive breast cancer, oral squamous cancer and lung cancer cell lines, through the introduction of siRNAs or shRNAs that specifically target Cat1/Git1, caused the cells to lose their invasive and metastatic capabilities.[14,17-19] Since cancer cell invasion and metastasis rely heavily on cell migration, and because Cat1/Git1 is a well-established promoter of cell motility, one can appreciate how this protein exerts a major influence on the aggressive behavior of cancer cells.

However, there is also a growing number of recent findings that implicate Cat1/Git1 as an important contributor to cancer cell growth and survival. For example, Cat1/Git1, functioning as a scaffold for MEK1 and ERK1/2, was recently shown to be essential for mediating the activation of ERK1/2 by methionine adenosyltransferase 2B (MAT2B) in liver and colon cancer models.[16,38] The authors went on to show that the formation of this complex had important consequences on tumor growth. Namely, liver tumors with increased levels of Cat1/Git1 and MAT2B had more ERK1/2 activity and grew faster, compared with those tumors without elevated levels of these proteins.[16,38]

We also have discovered Cat1/Git1 plays an essential role in promoting the transformed phenotype of HeLa cervical carcinoma cells and fibroblasts transformed through the expression of an activated Cdc42 mutant, i.e., Cdc42 F28L.[13] Knocking-down Cat1/Git1 expression from either of these cell types using siRNAs strongly inhibited their ability to form colonies in soft agar. While introduction of an siRNA-insensitive form of wild-type Cat1/Git1 in HeLa cells expressing Cat1/Git1 siRNA completely rescued this effect, a siRNA-insensitive mutant form of Cat-/Git1, defective in its ability to bind paxillin, was ineffective. Because paxillin has been shown to mediate the growth of some cancer and transformed cell lines,[61] we initially suspected this meant that the formation of paxillin-Cat1/Git1 complexes, most likely at focal adhesions, might recruit and activate a unique set of proteins essential for the growth of cancer/transformed cells.

However, in our most recent study,[62] where we set out to learn more about how paxillin-Cat1/Git1 interactions mediate cellular transformation, we discovered that our initial idea was incorrect. Instead of the formation of a complex between paxillin and Cat1/Git1 helping to induce a stimulatory signal that promotes colony formation in soft agar, it appears that the ability of Cat1/Git1 to associate with paxillin prevents it from exerting a negative regulatory effect on this transformed phenotype.

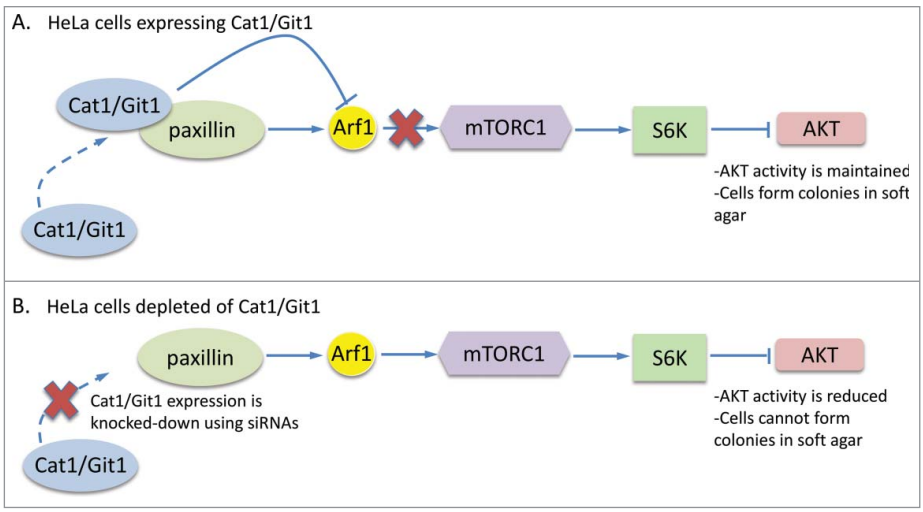

Figure 2. Diagram showing the mechanism through which Cat1/Git1 promotes HeLa cervical carcinoma cell transformation. A) When expressed in HeLa cells, Cat1/Git1 associates with paxillin to prevent it from functioning as a negative regulator of cellular transformation. Specifically, the interaction between Cat1/Git1 and paxillin inhibits the ability of paxillin to activate the Arf1-mTORC1-P70S6 kinase (S6K) pathway, which ensures that the necessary level of AKT activation required for supporting soft agar colony formation is met. B) However, under conditions where Cat1/Git1 expression is knocked-down in HeLa cells using siRNAs, paxillin is now able to stimulate the activation of the Arf1-mTORC1-P70S6 kinase pathway and inhibit AKT activation. These cells are no longer capable of forming colonies in soft agar.

The first indication that this was likely to be the case came from an experiment where it was shown that HeLa cells transfected with siRNAs targeting paxillin consistently performed better in soft agar assays (i.e., they formed more colonies), compared with the same cells expressing a control siRNA.[62] Moreover, the inability of HeLa cells, depleted of Cat1/Git1 expression using siRNAs, to form colonies in soft agar could be restored if paxillin expression was also knocked-down in the cells.

We then went on to uncover the mechanism through which Cat1/Git1 helps maintain this transformed phenotype. It involves Cat1/Git1 binding paxillin and, presumably functioning as a GAP, to counteract the ability of paxillin to increase the levels of active, GTP-bound Arf1 in cells (Fig. 2A). As long as paxillin-mediated Arf1 activity is kept in check by Cat1/Git1, it is unable to stimulate the activation of mTOR complex 1 (mTORC1) and its downstream effector p70S6 kinase.[55,56] This, in turn, prevents p70S6 kinase from negatively regulating the PI3-kinase/AKT pathway, thereby ensuring that enough AKT activity is maintained in the cancer/transformed cells to allow them to form colonies in soft agar.[62] However, when Cat1/Git1 expression is knocked-down in cancer cells, a delicate balance is disrupted and paxillin is now capable of activating the Arf1-mTORC1-P70S6 kinase pathway (Fig. 2B). In this situation, sufficient levels of PI3-kinase/AKT activity are not achieved, causing the cancer cells to lose their transformed characteristics. For additional information regarding these findings, please see our manuscript.[62]

Concluding remarks

Cat1/Git1 is a GAP for Arf1 and Arf6, as well as a protein scaffold/adaptor that binds several different protein partners. It is best known for its roles in promoting cell attachment and migration. However, during the past few years, Cat1/Git1 has been attracting a good deal of attention for its ability to impact several unique aspects of cancer progression. For example, Cat1/Git1 is frequently overexpressed in advanced-stage and highly aggressive forms of several types of cancer, where it promotes their invasive and metastatic activities. It is also becoming increasingly clear that Cat1/Git1, at least in certain contexts, contributes to cellular transformation by stimulating the activation of ERK1/2 and AKT, and promoting the anchorage-independent growth and survival of cancer cells.

While our understanding of the contribution of Cat1/Git1 to cancer progression is still in its infancy, it seems clear that, at least in certain contexts, it plays important roles in the development of this disease. Because of the ability of Cat1/Git1 to interact with several different proteins, as well as regulate a wide range of cellular processes, it seems likely that this interesting signaling/scaffold protein will emerge as a major player in cancer biology.

Disclosure of potential conflicts of interest

The authors report no conflict of interest.

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