Perspective

Activation of calcineurin in cancer: many paths, one hub

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Comment on: Peuker K, Muff S, Wang J, *et al.* Epithelial calcineurin controls microbiota-dependent intestinal tumor development. Nat Med 2016;22:506-15.

Abstract: Calcineurin is a calcium dependent serine/threonine phosphatase that integrates changes in intracellular calcium with downstream signaling pathways. A recent paper by Peuker *et al.* published in Nature Medicine identifies calcineurin as a key mediator of tumor growth and proliferation in response to altered stratification of the microbiota. Peuker *et al.* show that alterations in microbiota stratification activate toll-like receptor (TLR) signaling, which in turn activates calcineurin-NFAT signaling. While this is the first example of calcineurin activation in cancer through the intestinal microbiota, calcineurin is increasingly being recognized as a commonly activated target in cancer. Increases in intracellular calcium in response to hypoxic conditions, inflammation, and vascular endothelial growth factor signaling have all been demonstrated to result in increased calcineurin activation in malignant cells. More recently, cleavage of calcineurin has been associated with increased activity in cancer. Activation of calcineurin is implicated in signaling pathways promoting proliferation, migration, and metastasis. The elevated frequency of calcineurin activation in cancer highlights the importance of this pathway as a potential therapeutic target.

Keywords: Calcineurin; cancer; cleavage; microbiota; phosphatase

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Introduction

Colorectal cancer is the third most diagnosed cancer and the fourth leading cause of cancer-related death worldwide, and the second leading cause of cancer-related deaths in developed countries (1). The worldwide incidence of colon cancer is predicted to rise by 60% within 15 years, in line with observations linking increased colon cancer incidence with increased industrial development (2). Intestinal inflammation is a risk factor for colorectal cancer: with inflammatory bowel disease significantly increasing the risk of colitis-associated colorectal cancer (3). Inflammatory signaling through STAT3 and NF-κB is also activated in colorectal tumors and cell lines (4,5). In their recent publication, Peuker *et al.* (6) identify calcineurin as a promoter of colorectal cancer growth through maintenance and proliferation of cancer stem cells. Calcineurin is a calcium dependent serine/threonine phosphatase that plays a central role in immunity, as demonstrated by the use of calcineurin inhibitors cyclosporine A and tacrolimus (FK506) as immunosuppressants (7).

Calcineurin is composed of two subunits, a catalytic subunit called calcineurin A (CNA) encoded by three separate genes (*PPP3CA*, *PPP3CB* and *PPP3CC*), and a regulatory subunit, calcineurin B (CNB; Cnb) encoded by two genes, *PPP3R1* and *PPP3R2*, with the latter restricted to testis and brain. In the presence of elevated calcium, calmodulin binds to calcineurin, displacing the autoinhibitory domain from the active site, leading to activation of calcineurin and subsequent dephosphorylation of target proteins. Calcineurin substrates include transcription factors, proteins involved in cell cycle and apoptosis, cytoskeletal proteins, scaffold proteins, membrane channels and receptors (*Table 1*) (10).

The best characterized calcineurin substrates are the nuclear factor of activated T cells (NFAT) transcription

Table 1 Targets of calcineurin dephosphorylation

Category	Target
Transcription factors/transcriptional regulation	c-Jun (8)
	DAXX (9)
	Elk-1 (10)
	Hcm1 (11)
	MEF2 (12)
	NFAT (13)
	NFI (14)
	TFEB (15)
Cytoskeleton	MAP2 (16)
	Tubulin (16)
	Tau (16)
Cell cycle/apoptosis	BAD (17)
	pRb (18)
	Drp1 (10)
Scaffold proteins	KSR2 (19)
	DARP-32 (10)
	RACK1 (10)
	Synapsin (20)
	GAP43 (20)
Ion channels/membrane channels	GluA1 receptor (20)
	NMDA receptor (20)
	GABA(A) receptor (20)
	mGluR5 (20)
	Kv4.2 (20)
	TRESK (10)

factors. Four of the five members of the NFAT protein family are regulated by calcium signaling: NFATc1 (NFAT2), NFATc2 (NFAT1), NFATc3 (NFAT4), and NFATc4 (NFAT3). In resting cells, NFAT is highly phosphorylated which precludes exposure of a nuclear localization sequence (21). Following dephosphorylation by calcineurin, NFAT is translocated to the nucleus where it regulates gene expression, including genes encoding cytokines in immune cells.

Peuker *et al.* (6) examine the contribution of calcineurin and its downstream target NFAT to intestinal tumor growth in both a genetic model and a colitis-associated model of colorectal cancer. By specifically deleting the regulatory B1 subunit of calcineurin (Cnb1, encoded by *Ppp3r1*) in the intestinal epithelial cells of the $Apc^{Min/+}$ mouse model of colon cancer, they observe both fewer and smaller intestinal tumors. Similar results were obtained when mice carrying intestinal epithelial cell-specific deletion of *Ppp3r1* were treated with colitis-inducing dextran sulfate along with the carcinogen azoxymethane. In particular, the authors noted that early stage lesions were reduced in their colorectal cancer models, and that the early lesions showed reduced epithelial proliferation and increased apoptosis. Peuker *et al.* (6) further showed that NFATc3 is the main NFAT expressed in normal intestinal epithelial cells, and that tumor formation is accompanied by calcineurin-dependent cytoplasmic to nuclear translocation of NFATc3. Intestinal epithelial cell-specific deletion of *NFATc3* resulted in a phenotype similar to that of *Ppp3r1*–/– mice, albeit attenuated. The full phenotype was restored upon treatment of $Apc^{Min/+}$ mice with a peptide that interferes with calcineurin-dependent activation of all NFATs.

Calcineurin in stem cell proliferation and differentiation

The intestinal epithelium is maintained by continuous renewal through a tightly regulated balance of intestinal stem cell proliferation and differentiation. In Drosophila, high cytoplasmic calcium concentration results in activation of calcineurin and downstream targets, triggering proliferation of intestinal stem cells (22). Silencing of either the regulatory subunit of calcineurin *CanB2* or the catalytic subunit of calcineurin *CanA1* in Drosophila significantly reduces stem cell proliferation, highlighting a crucial role for calcineurin in translating changes in calcium signaling to proliferation of intestinal stem cells.

Paradoxically, in addition to a role in cell proliferation, calcineurin also promotes differentiation. Calcineurin-NFAT signaling is necessary for lineage specification in embryonic stem cells, triggering the transition of these stem cells from self-renewal to differentiation (23). Calcineurin-NFAT signaling also initiates skeletal muscle differentiation (24), alveolar specification of adult lung stem cells (25), terminal differentiation of osteoclasts (26), and stem cell quiescence in keratinocytes (27). In the cardiovascular system, calcineurin is important for cardiomyocyte maturation, valve formation, and vascular development. Loss of calcineurin results in heart defects

and reduced proliferation, whereas calcineurin is necessary for hypertrophic response in adult cardiomyocytes (28,29). In the brain, calcineurin is highly expressed in neurons, and plays an important role in the modulation of synaptic transmission (20). Calcineurin activity is also required for neural induction in the developing embryo through dephosphorylation and inactivation of Smad1/5 to antagonize signaling through the bone morphogenetic protein (BMP) (30).

The immunosuppressive activity of the calcineurin inhibitor cyclosporine A was first described in 1976, and calcineurin inhibitors have been widely used as immunosuppressants since the mid-80's (31). The study of calcineurin inhibitors revealed a vital role for calcineurin in regulating T cell development and activation through the NFAT family (32-34). In T cells, binding of the T-cell receptor results in release of calcium from intracellular stores, which activates sustained Ca^{2+} entry through Ca^{2+} release-activated Ca^{2+} (CRAC) channels (35). As a result, calcineurin is activated and NFAT is dephosphorylated and shuttles to the nucleus where it binds to the promoters of T cell-activated proteins including interleukin 2 (IL-2) and interleukin 3 (IL-3) to induce transcription (36,37). The immunosuppressant action of cyclosporine A and tacrolimus are mediated by inhibiting dephosphorylation of NFAT by calcineurin in immune cells, especially T cells (7,34,37,38).

Calcineurin in cancer

Chronic use of immunosuppressants such as the calcineurin inhibitors cyclosporine A and tacrolimus increases cancer incidence (39). Overall, the transplant population has a two-fold increased risk of cancer, with much higher increases for specific cancers, including nonmelanoma skin cancer and virally linked cancers (40). This increased risk of cancer is linked to three primary mechanisms: (I) increased risk of virally driven malignancy due to immunosuppression; (II) impaired immunosurveillance of transformed cells; and (III) specific effects of drugs used for immunosuppression (39). The first two are directly related to disruption of the immune system. Thus, calcineurin inhibitors can directly promote tumorigenesis in an autonomous manner through modulation of the immune system (41-43).

In apparent contradiction, activation of calcineurin and its downstream targets also increases tumorigenic potential. As observed by Peuker *et al*. and others, calcineurin and downstream signalling pathways are activated in colorectal cancer tumors and cell lines, and inhibition of calcineurin decreases cancer stem cell survival and proliferation (6,44). Similarly, calcineurin is activated in breast cancer, specifically in triple negative breast cancer, and promotes migration and invasion *in vitro* and growth and metastasis *in vivo* (45,46). Analogous findings by others support a pro-tumorigenic role for calcineurin signaling in lung, prostate, bladder, ovarian, pancreatic, and liver cancer, as well as glioblastoma, melanoma and leukemia (14,47-55).

Studies addressing calcineurin activation have focused principally on dephosphorylation and activation of NFAT and NFAT transcriptional targets. NFAT in and of itself is constitutively activated or overexpressed in numerous cancers and can contribute to cancer development and progression (56). Additional calcineurin substrates including myocyte enhancer factor 2 (MEF2), kinase suppressor of ras 2 (KSR2), DAXX, c-Jun and Nuclear Factor I (NFI) have all been shown to have pro-tumorigenic roles (8,9,14,57-60). For example, c-Jun is stabilized by calcineurin dephosphorylation in cervical cancers compared to normal tissue, thereby increasing c-Jun dependent gene expression (8). Calcineurin has a similar effect on the transcription factor NFI, with dephosphorylation by calcineurin increasing its transcription regulatory activity (14).

Activation of calcineurin

Calcineurin can be activated by a variety of mechanisms (*Figure 1*). The newly discovered mechanism reported by Peuker *et al*. (6) appears to be specific to intestinal cancers, and is driven by changes in gut microbiota stratification and composition. This change in microbiota activates toll-like receptor (TLR) signaling which in turn induces calcium entry and calcineurin activation. Although the exact mechanism of action of the gut microbiota was not addressed in their paper, Peuker and colleagues did observe differences in microbial community structure between intestinal tumors and normal intestinal mucosa. The discovery that calcineurin can be activated by alterations in bacterial communities is exciting and a major step forward in our understanding of calcineurin's importance in tumor formation.

The mechanisms driving calcineurin activation in other cancers is less clear. It is a well-known fact that the inflammatory response is activated in many cancers, and that chronic inflammation is a risk factor for tumor development (3). There is also ample evidence showing that inflammation activates calcineurin activity (61-63). Thus, chronic inflammation triggered by environmental

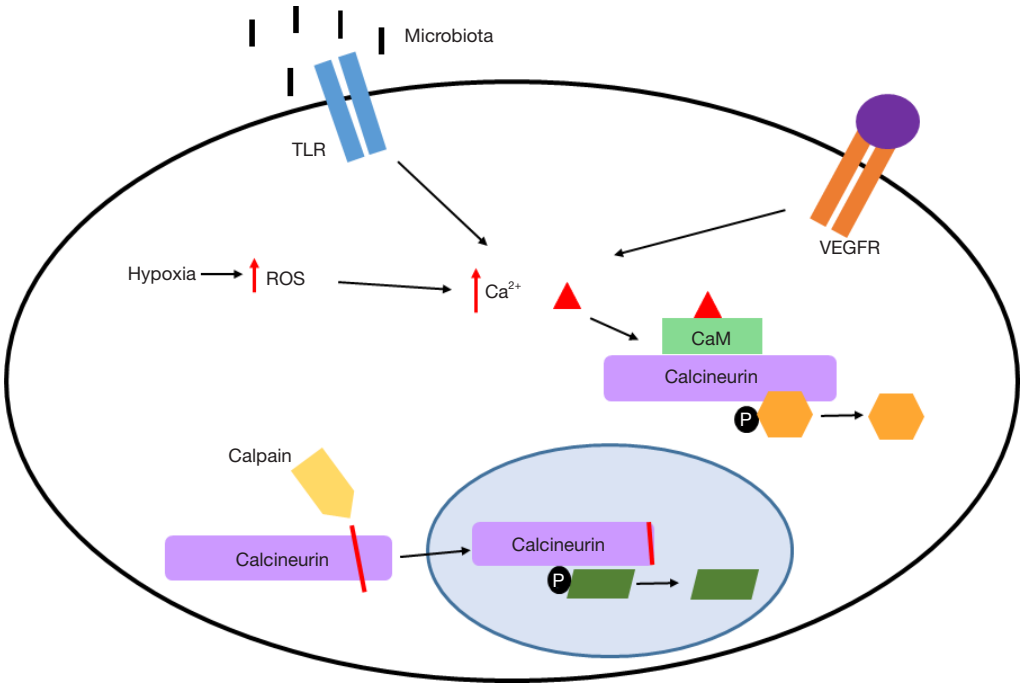

Figure 1 Mechanisms driving calcineurin activation in cancer. Calcineurin can be activated downstream of toll-like receptor (TLR) signaling, vascular endothelial growth factor receptor (VEGFR) signaling, or increased reactive oxygen species (ROS) in response to hypoxia. Cleavage of calcineurin by calpain can also activate calcineurin, and alter calcineurin subcellular localization. P denotes phosphorylated residues on substrates.

factors such as chronic infection, inhaled pollutants, and dietary factors may activate calcineurin activity, thereby contributing to tumor initiation and growth. Necrosis resulting from tumor growth would further contribute to the inflammatory response (64). A well-characterized receptor implicated in calcineurin activation is the vascular endothelial growth factor receptor (VEGFR) whose activation increases cytosolic calcium in endothelial cells to regulate tumor angiogenesis and metastasis (49,65). Hypoxia can also activate calcineurin through increased intracellular calcium by activation of CRAC channels (66), and calcium signaling through CRAC channels promotes proliferation, migration and metastasis in a number of tumors (67,68).

Calcineurin activity is modulated by a number of factors in addition to intracellular calcium including interaction with regulatory factors, subcellular localization, and intramolecular cleavage. Regulators of calcineurin (RCAN1-4) can bind to and inhibit calcineurin activity (69-71). RCANs bind calcineurin at the same site as NFAT and other substrates, and one mechanism of action is via competition for binding (72). Increased expression of RCAN1 (also known as Down's syndrome candidate region-1, DSCR1) inhibits tumor growth in mice, and increased copies of the *DSCR1* gene contributes to decreased cancer rates in Down's syndrome (73)

Calcineurin is predominantly cytosolic in unstimulated cells (74,75). However, in response to calcium signaling, calcineurin can translocate to the nucleus to interact with target substrates (76). A nuclear localization signal (NLS) in the catalytic domain of CNA is necessary for nuclear import of activated calcineurin via importin β1, with a nuclear export signal (NES) located in the C-terminus of CNA (*Figure 2A*). The autoinhibitory domain of CNA regulates nuclear import and export by masking the NLS in inactive calcineurin in addition to blocking the catalytic site (10,75). Subcellular localization is also mediated by interaction with targeting proteins, with the AKAP 79/150 scaffold shown to anchor calcineurin at distinct subcellular locations (77).

The autoinhibitory domain of CNA is located in the C-terminal region (75). In response to stress, calcineurin can be cleaved by the Ca^{2+}-dependent cysteine-protease calpain, resulting in three main products of 45 kDa, 48 kDa and 57 kDa (*Figure 2B*). The autoinhibitory domain is missing

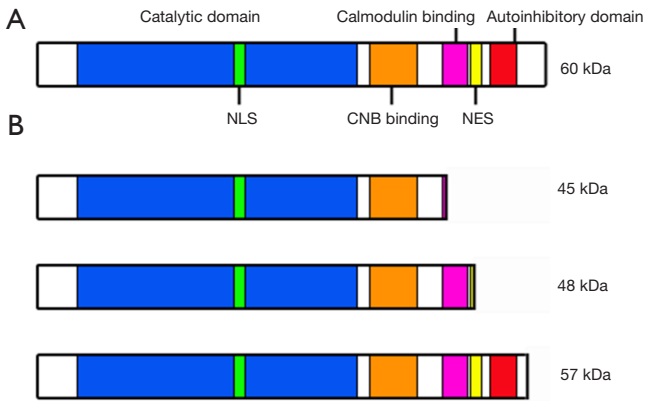

Figure 2 Schematic representation of the different forms of calcineurin A. The different forms of calcineurin A [full length depicted in (A) and cleaved forms depicted in (B)] have different activities and subcellular localizations. Cleaved forms have been detected in pathogenic states including Alzheimer's disease, cardiac hypertrophy and cancer. Domains are color-coded: catalytic domain in blue; nuclear localization sequence (NLS) in green; calcineurin B (CNB) binding domain in orange; calmodulin binding domain in pink; nuclear export sequence (NES) in yellow; autoinhibitory domain in red. Molecular weights are indicated on the right.

from the 45 kDa and 48 kDa isoforms, resulting in constitutively active products that localize to the nucleus (75,78). Cleaved forms of CNA have been observed in cardiac hypertrophy, in the brains of Alzheimer's disease patients, in response to neurotoxicity, and in a glaucoma model (75,79-81). In Alzheimer's disease brains, the 57 kDa cleaved form of CNA which retains the autoinhibitory domain, still requires Ca^{2+} and calmodulin for its activation, but shows increased activity compared to the 60 kDa uncleaved form of CNA (80). The 57 kDa cleaved form of CNA has also been detected in malignant glioma cells, where it localizes to the nucleus, and regulates dephosphorylation and activation of NFI (14). NFI activates expression of the pro-migratory gene fatty acid-binding protein 7 (FABP7) and represses transcription of the tumor suppressors p21 and p53 in malignant glioma (82-85). NFAT is also expressed and localizes to the nucleus in these tumors (50).

Expression, activation, and cleavage of calcineurin are not commonly studied in cancer. Direct measurement of calcineurin activation is difficult: *in vitro* assays of calcineurin activity do not clearly correlate with endogenous activity, and antibodies to calcineurin may not detect cleaved forms. Thus, calcineurin activity is commonly measured by phosphorylation, nuclear localization, or transcriptional activity of NFAT. Peuker *et al.* (6) do not directly examine calcineurin expression, activity, and subcellular localization in their study, but use NFAT nuclear localization in tissue samples as a readout of calcineurin activity. Consequently, the localization and forms of calcineurin expressed in their intestinal tumor models remain unclear. To date, cleavage of CNA in cancer has only been clearly demonstrated in malignant glioma (14). However, immunoblotting of cervical tissue lysates with anti-CNA antibody reveals differences in migration of CNA between normal and tumor tissues in a subset of cases (8). Banding patterns consistent with CNA cleavage are also observed in T-ALL cells (86). Furthermore, CNA has been shown to localize to the nucleus in small cell lung cancer, although immunoblotting was not carried out to examine CNA banding patterns (87). These combined data suggest that calcineurin can be activated by CNA cleavage under different pathological conditions, including cancer. It will therefore be important to examine the subcellular localization of calcineurin as well as detect the forms of CNA present in the intestinal cancers studied by Peuker and colleagues.

Cleavage of CNA and nuclear localization of calcineurin in response to pathogenic stimuli also modulate substrate dephosphorylation. Nuclear localization of calcineurin alters substrate accessibility, resulting in decreased dephosphorylation of cytoskeletal and membrane-associated substrates, thereby modulating downstream signaling pathways. Subcellular localization of calcineurin has also been shown to regulate dephosphorylation of the pro-apoptotic Bcl-2 family member BAD (17) and NFAT (88).

Targeting calcineurin activation for the treatment of cancer

Numerous kinases and their phosphorylated substrates play critical roles in cancer formation and progression. Convergence of varied inputs resulting in calcineurin activation in different types of cancers implies a central role for the calcineurin phosphatase and its dephosphorylated substrates in malignancy. Activation of calcineurin by altered microbiota stratification in colorectal cancer builds on previous observations of calcineurin activation in cancer. Activation through a variety of mechanisms in both solid tumors and lymphoid malignancies further underlines the importance of this pathway in cancer, and suggests that dephosphorylation

of calcineurin substrates transcends tissue-specific factors associated with tumor progression.

Preliminary studies have examined the use of calcineurin inhibitors as potential anti-cancer agents. Cyclosporine A treatment induced apoptosis in lymphoma and leukemia cell lines, and treatment of mouse models of T-cell acute lymphoblastic leukemia with cyclosporine A or tacrolimus increased mouse survival and resulted in rapid tumor clearance (54). Similarly, cyclosporine A and tacrolimus decreased proliferation and migration in both bladder and prostate tumor cells *in vitro*, and bladder and prostate xenografts *in vivo* (89,90). Tacrolimus treatment in a mouse model of breast cancer reduced tumor growth, and angiogenesis within these tumors *in vivo*, and decreased migration of both breast cancer and endothelial cells *in vitro* (91). In patients with acute myeloid leukemia who have an internal tandem duplication of Fms-related tyrosine kinase receptor 3 (FLT3), a retrospective analysis shows a promising correlation between inclusion of cyclosporine A in salvage therapy and increased survival (92).

As alluded to earlier, there are caveats to targeting calcineurin for cancer treatment. For example, in breast cancer, calcineurin can interact with the plasma membrane calcium ATPase 2 (PMCA2), which can sequester calcineurin to the membrane, reducing activation of the calcineurin-NFAT pathway. However, specific disruption of the PMCA2-calcineurin interaction with a small peptide increases NFAT activity, and reduces cell viability in breast cancer cell lines, resulting in increased apoptosis and sensitivity to paclitaxel (93). Treatment with calcineurin inhibitors can also increase the risk of cancer, as observed in transplant patients. Furthermore, cyclosporine A itself is also directly implicated in tumor growth as it can increase TGFβ production (41), activate Ras (43), and suppress PTEN expression, increasing activation of AKT (42).

More specific targeting of specific components of calcineurin activation may alleviate issues associated with long term treatment with cyclosporine A and tacrolimus. For example, inhibitors targeting interaction with specific substrates, or specific sets of substrates may help to focus growth inhibitory effects on cancer cells. If additional work in the field demonstrates that altered cleavage and/or nuclear localization of calcineurin is a frequent event in cancer, altering cleavage or nuclear localization would present a tumor-specific way to target activated calcineurin in cancer cells, while preserving normal immune function.

In conclusion, phosphorylation represents one of the most, if not the most, well-studied and widespread mechanism regulating protein function in both normal and cancer cells. There is emerging evidence suggesting that dephosphorylation of proteins by the phosphatase calcineurin may also play a critical role in tumor formation and progression. Calcineurin's activity is regulated by surprisingly diverse mechanisms, ranging from the gut microbiota and inflammation, to intramolecular cleavage and subcellular localization. Further work on the role of calcineurin in cancer may reveal a fundamental pathway that is commonly altered to promote cancer formation.

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Footnote

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