Cell Adhesion & Migration 5:2, 150-159; March/April 2011; © 2011 Landes Bioscience

# Diversification of caldesmon-linked actin cytoskeleton in cell motility

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**Key words:** caldesmon, actin cytoskeleton, cell migration, cell invasion, transcription, smooth muscle contraction, cytokinesis

**Abbreviations:** CaD, caldesmon; $Ca^{2+}$, calcium; CaM, calmodulin; TM, tropomyosin; F-actin, filamentous actin; N-terminal, amino-terminal; C-terminal, carboxy-terminal; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SMC, smooth muscle cell; SRF, serum response factor; MRTF, myocardin-related transcription factor; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; VSMC, vascular smooth muscle cell; IGF, insulin-like growth factor; PI3K, phosphatidylinositol-3 kinase; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; ERK, extracellular signal regulated protein kinase; LPA, lysophosphatidic acid; PAK, p21-activated protein kinase; CaMKII, $Ca^{2+}$/CaM-dependent protein kinase II; NPC, neural progenitor cell; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition

The actin cytoskeleton plays a key role in regulating cell motility. Caldesmon (CaD) is an actin-linked regulatory protein found in smooth muscle and non-muscle cells that is conserved among a variety of vertebrates. It binds and stabilizes actin filaments, as well as regulating actin-myosin interaction in a calcium ($Ca^{2+}$)/calmodulin (CaM)- and/or phosphorylation-dependent manner. CaD function is regulated qualitatively by $Ca^{2+}$/CaM and by its phosphorylation state and quantitatively at the mRNA level, by three different transcriptional regulation of the *CALD1* gene. CaD has numerous functions in cell motility, such as migration, invasion and proliferation, exerted via the reorganization of the actin cytoskeleton. Here we will outline recent findings regarding CaD's structural features and functions.

## Introduction

Regulation of cell adhesion and migration is essential, not only for development, immune response and wound healing, but also for various pathological events such as cancer progression.[1-3] During organogenesis and cancer metastasis, cells dissociate from their origin and penetrate into distant target tissues.[2] Cell adhesion and migration are controlled by complex changes in the cytoskeleton, particularly the actin cytoskeleton,[1,4,5] which plays a pivotal role in controlling cell motility, such as cell morphology, adhesion and migration.

During a keen dispute over the $Ca^{2+}$-dependent regulation of smooth muscle and non-muscle contraction, CaD appeared on the cytoskeletal stage as a CaM- and actin-binding protein.[6,7] Subsequent studies on $Ca^{2+}$-dependent regulation in both smooth muscle and non-muscle cells showed CaD to be an actin-linked regulatory protein that functions in the actomyosin contractile system. CaD binds filamentous (F-) actin and inhibits actin-myosin interactions in a $Ca^{2+}$/CaM-dependent manner, as determined by superprecipitation assays and actin-activated myosin ATPase activity.[8-11] CaD is an actin-, myosin-, CaM- and tropomyosin(TM)-binding protein. In vitro, $Ca^{2+}$/CaM's binding to CaD reduces its affinity for actin,[6,12-15] which reverses CaD's inhibitory effect on the actomyosin system. Thus, CaD is a major regulatory component of smooth muscle thin filaments. CaD also enhances the actin binding of TM and stabilizes actin filaments directly by binding along the side of F-actin.[16,17] In vitro reconstitution experiments revealed that CaD competes with fascin, filamin, Arp2/3 complex, gelsolin and cofilin for binding to F-actin.[18-21] CaD's vital roles in various actin cytoskeleton-related cellular processes have been demonstrated in many different cell types, as described below.

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Submitted: 11/01/10; Accepted: 12/06/10
DOI: 10.4161/cam.5.214398

## Structural and Functional Relationships of CaD Isoforms

CaD is expressed by many vertebrates. There are two different molecular weight (*Mr*) isoforms of CaD: high-*Mr*CaD (h-CaD, 120–150 kDa) and low-*Mr* CaD (l-CaD, 70–80 kDa).[22,23] The two isoforms have common amino(N)- and carboxy(C)-terminal domains, but h-CaD has a specific insertion of the central repeating domain containing a lysine-, arginine-, glutamate- and alanine-rich unit (**Fig. 1A**). The human CaD gene (*CALD1*), which maps to 7q33 (**Fig. 1B**), generates at least eight species of CaD mRNA from its 13 exons.[24]

CaD's isoforms are mainly generated by the selective splicing together of exon 1a or 1b, exon 3a or 3ab and/or exon 4. Exons 1a and 1b encode the fibroblast- (fibro-) and HeLa-type N-terminal sequences, respectively.[25] Exon 3b encodes the unique central repeating domain, consisting of the 229 amino acids specific to h-CaD. mRNA transcripts in which exon 3 is represented only

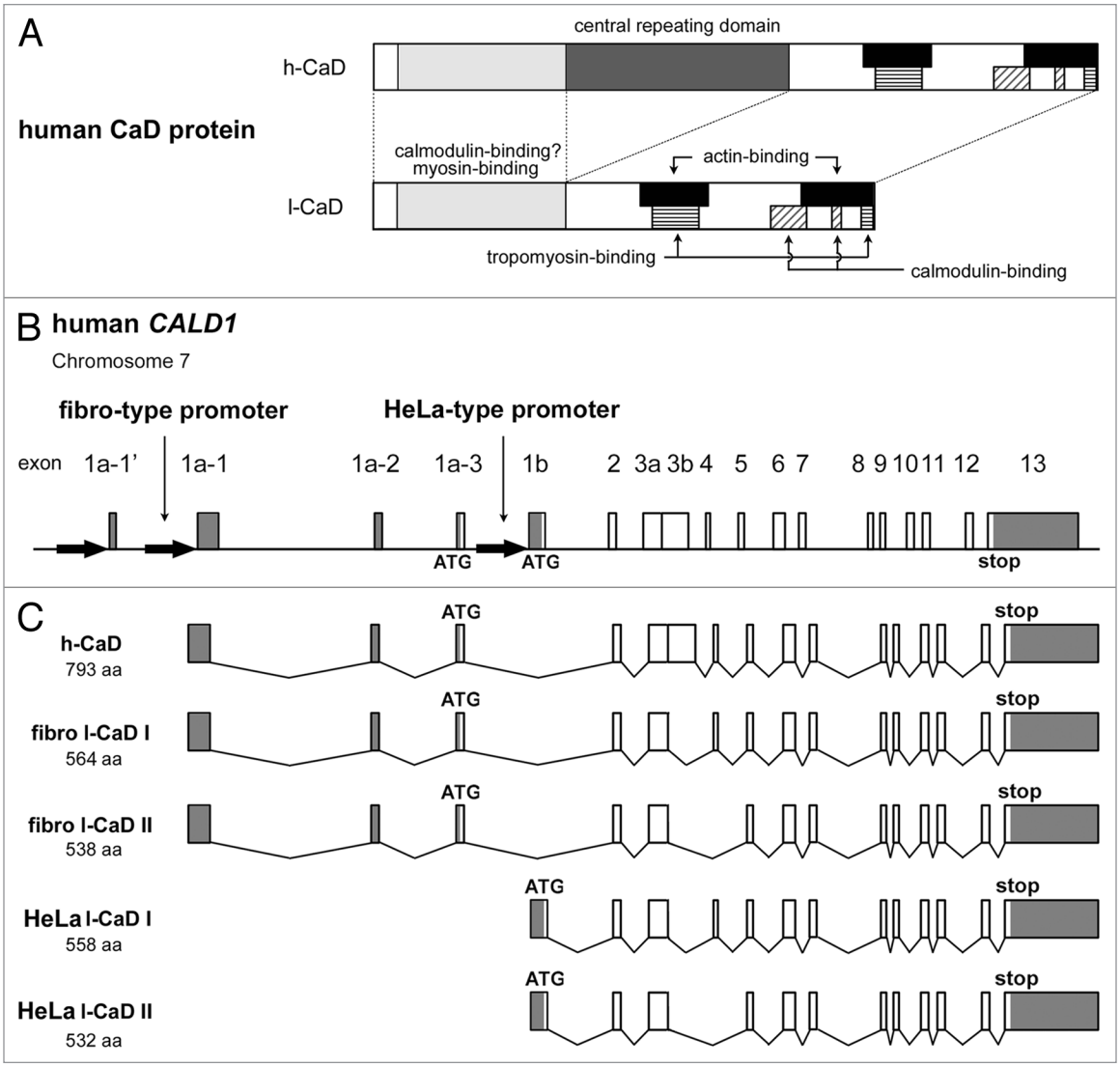


**Figure 1.** The schematic structures of CaD protein isoforms, human *CALD1* gene and the transcripts. (A) The domain structures of h- and l-CaD.[7,115] The myosin-binding site and the potential CaM-binding site are located in N-terminus (light gray). The actin (black)-, TM (horizontal line)- CaM (diagonal line)-binding sites are located in C-terminus. The central repeating domain (dark gray) is inserted in h-CaD. (B) The exon/intron organization of the human *CALD1* gene. The *CALD1* locus is located in 7q33 (chromosome 7). Boxes indicate the exons. The fibro- and HeLa-type promoters are located in the flanking regions of exon 1a-1 and 1b, respectively (black arrows). A potential exon (1a-1′) and the promoter region were identified by a database search of Ensembl (http://uswest.ensembl.org/index.html). (C) The five major mRNA species are shown. CaD isoform diversity is primarily generated by alternative transcriptional initiation from exon 1a or 1b, and by alternative splicing of exon 3a or 3ab and/or exon 4. The h-CaD mRNA containing exon 3ab is specifically generated in differentiated SMCs.

by exon 3a generate l-CaD, whereas those including 3ab encode the longer isoform, h-CaD. Exon 4 encodes 26 amino acids, including an extension of the α-helical motif (**Fig. 1C**).

Both CaD proteins are composed of approximately 50% charged amino acids, such as glutamate, lysine and arginine, which form a particularly prominent cluster in the h-CaD central repeating domain. Owing to its peculiar amino acid composition, h-CaD exhibits slower mobility on SDS-PAGE than predicted simply by deducing the molecular mass from the amino acid sequence.[26] The actin-binding site, one CaM-binding site and the myosin ATPase inhibition site are located within CaD's C-terminal region,[26-28] whereas the myosin-binding and second CaM-binding sites are in the N-terminal region.[29-32] Both CaD isoforms have an elongated shape when viewed by electron microscopy.[30,33]

How has CaD changed evolutionally? Diligent genomic analyses reveal that CaD is ubiquitously distributed among vertebrates (**Fig. 2**). The N-terminal myosin- and CaM-binding regions and the C-terminal actin-, CaM- and TM-binding regions are highly conserved among vertebrate species, whereas several spacers are inserted in the internal region in some species (**Fig. 2A**). CaD has a partial homology totroponin T,[26] which is a critical regulator of $Ca^{2+}$-dependent actin-myosin contraction. These observations may provide a new insight into the evolutional diversity of

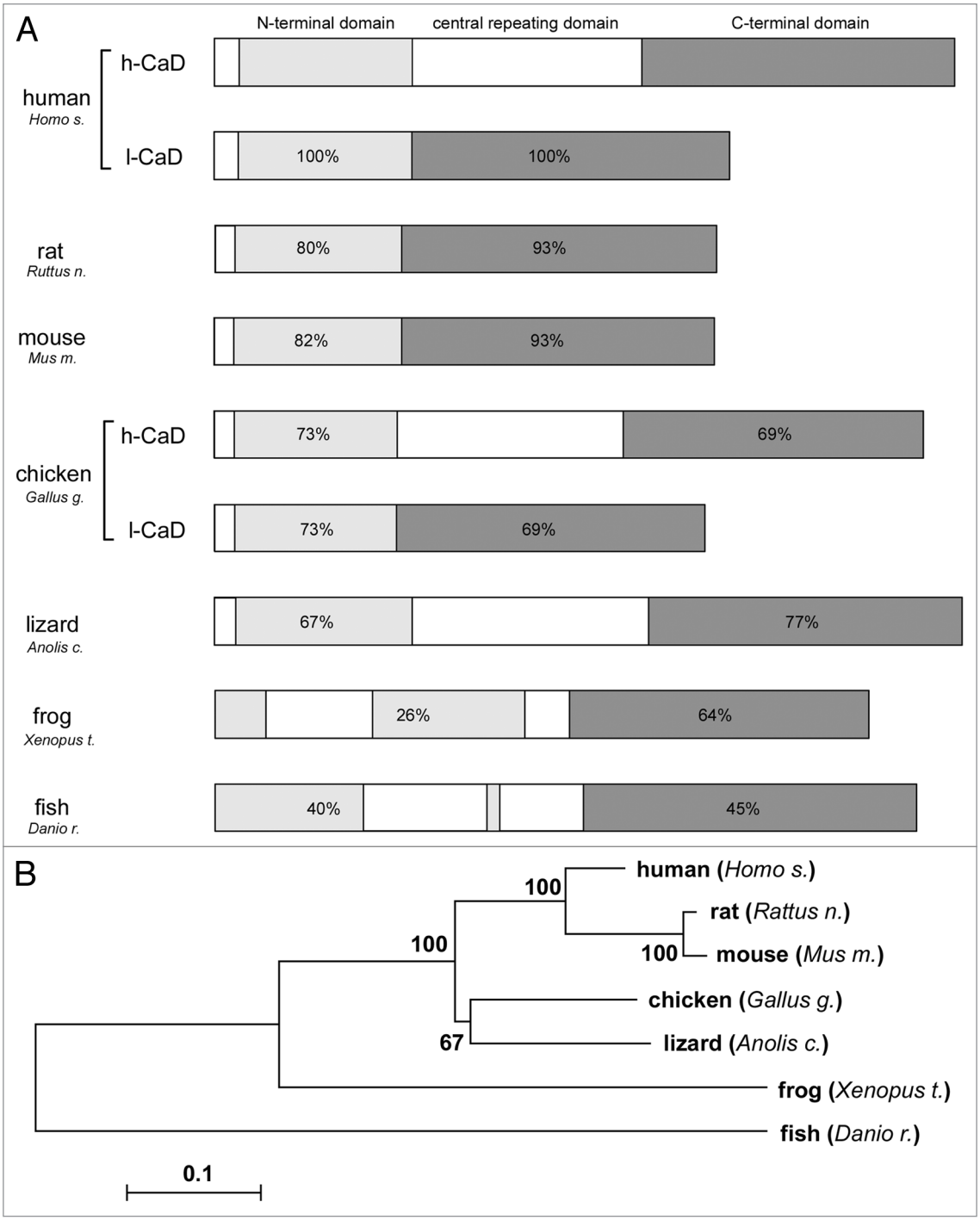


**Figure 2.** The vertebral orthologs of CaD. (A) The domain structures of CaD orthologs among vertebrates. The homology/similarity values to N- or C-terminal domain of humanl-CaD are shown, respectively. Human (*Homo sapiens*) h-CaD [NP_149129.2], fibro l-CaD I [NP_004333.1], rat (*Rattus norvegicus*) l-CaD [AAA68521.1], mouse (*Mus musculus*) l-CaD [AAH19435.1], chicken (*Gallus gallus*) h-CaD [BAA04539.1], l-CaD [BAA04540.1], lizard (*Anolis carolinensis*) CaD [Emsemble ENSACAP00000011703], frog (*Xenopus tropicalis*) CaD [XP_002932341.1], fish (*Danio retio*) CaD [XP_692347.4]. (B) A result of phylogenetic analysis using MAGA4 software based on ClustalW program from the homologies with the exception of the central inserted domain.[116,117] The branch lengths are proportional to the inferred evolutionary change. The scale bar corresponds to 0.1 estimated amino acid substitutions per site. The bootstrap values are shown at nodes.

skeletal, smooth and cardiac muscle, as well as non-muscle contractile systems.

Although h- and l-CaD display the same biochemical character in vitro, their distribution is mutually exclusive. h-CaD is abundantly and specifically expressed in smooth muscle cells (SMCs), whereas l-CaD is ubiquitously expressed in non-muscle cells.[23] h-CaD is localized to the actomyosin contractile structure in SMCs as an integral component of the thin filaments.[11-13] In

migrating cells, l-CaD is localized to stress fibers, membrane ruffles and lamellipodial extensions.[12,22,34] Stress fibers provide the structural and contractile cytoarchitecture for cell morphology and motility.[35,36] Both CaD and myosin II are localized to stress fibers, but are not in the focal adhesions that provide attachment sites to the substratum at the stress-fiber termini.[37] It is not clear whether CaD isoforms function differently in vivo. Guo and Wang have generated transgenic h-CaD-specific knockout mice without affecting l-CaD expression.[38] The homozygotes are viable, though some newborns die 5–7 h after birth with symptoms similar to an umbilical hernia. It has recently been reported that knockdown of CaD ortholog in zebrafish causes defects in vasculogenesis and angiogenesis, as well as in cardiac organogenesis.[39,40] These organotypic abnormalities may cause serious functional defects in cardiac contractility and blood circulation. Together, these reports suggest that CaD is essential for normal vertebrate development, in particular for development of organs composed of SMCs.

## Transcriptional Regulation of the CaD (*CALD1*) Gene

Three different mechanisms for *CALD1* gene transactivation have been demonstrated. In SMCs and non-muscle cells, transcription of the *CALD1* gene primarily depends on serum response factor (SRF),[41,42] which binds to the $CC(A/T)_6GG$ motif (known as a CArG box) within the *CALD1* promoter region and activates its transcription. There is a conserved CArG element in the proximal site of the fibro-type 1st exon (exon 1a-1),[42] and SMC-specific *CALD1* gene transcription regulated by the coordinated function of a triad of transcription factors: SRF, Nkx homeobox (Nkx 3.2) factor and GATA6.[43] Recently, myocardin was identified as a potent co-factor for SRF-mediated transcription in cardiac and SMC gene expression, as it is restricted to cardiac muscle and SMCs.[44] In non-muscle cells, myocardin-related transcription factors (MRTFs) activate actin cytoskeletal gene transcription, including the *CALD1* gene, via the Rho-MRTF-SRF pathway.[45] Glucocorticoid receptor (GR) mediates a second pathway for activating the *CALD1* gene transcription.[46] In lung adenocarcinoma A549 cells and neural progenitor cells (NPCs) an activated GR binds directly to the two glucocorticoid (GC)-response elements (GREs) located near the transcriptional start site of the fibro-type *CALD1* promoter, and enhances *CALD1* transcription.[46,47] The MRTF/SRF-dependent and GR-dependent *CALD1* transcription pathways are mutually independent,[46] and GC-dependent CaD upregulation is prominent in cells expressing low levels of CaD before stimulation.[46,47] A third regulatory pathway, in which CaD expression is upregulated by p53 was recently reported by Mukhopadhyay et al.[48] The activation of p53 correlates with increased CaD expression. Although a chromatin-immunoprecipitation (ChIP) genome-wide analysis show that p53 binds directly to the *CALD1* gene flanking region,[49] the precise mechanism of this novel transactivation pathway remains to be elucidated.

## Caldesmon as a Molecular Marker for SMC Phenotypes

h-CaD is specifically expressed at high levels in differentiated SMCs, and serves as a marker for these cells. In developing chicken gizzards, a CaD isoform conversion from the l- to the h-form, accompanied by an increase to high expression levels, correlateswith SMC differentiation.[50] Conversely, CaD expression decreases and converts from the h- to the l-form in association with vascular SMC (VSMC) dedifferentiation in primary culture.[50-53] Thus, the change in the CaD isoforms expressed precisely reflects the change in SMC phenotypes.

Differentiated VSMCs are spindle-shaped, show ligand-induced contractility, and are positive for SMC molecular markers, whereas dedifferentiated VSMCs lose these properties.[54] Earlier culture methods produced only dedifferentiated VSMCs. By using the sensitive, phenotype-related CaD isoform conversion and other SMC molecular markers to monitor SMC phenotypes, our research group attempted to establish a primary culture system for VSMCs that preserved the differentiated phenotype (**Fig. 3A and B**). VSMCs cultured on laminin under IGF-I-stimulated conditions are spindle-shaped, have carbachol-induced contractility and express high levels of h-CaD and other SMC markers; i.e., they display a fully differentiated phenotype.[51,55] Using this culture system, Hayashi et al. elucidated the signaling pathways regulating the VSMC phenotypes. IGF-I, IGF-II and insulin can maintain a differentiated phenotype via the PI3K-PKB/Akt pathway, whereas PDGF, EGF, bFGF, angiotensin II and serum induce VSMC dedifferentiation via the coordinated activation of the ERK and p38MAPK pathways.[55] Thus, the VSMC phenotype is determined by the balance of strength between the PI3K-PKB/Akt pathway and the ERK and p38MAPK pathways (**Fig. 3C**).

The phenotypic modulation of VSMCs from differentiated to dedifferentiated is critically involved in the development and progression of atherosclerosis.[56] In particular, early events in atherosclerosis are initiated by the formation of neointima, which is primarily composed of dedifferentiated, proliferating VSMCs.[57] Using this VSMC culture system, we further identified bioactive phospholipids, lysophosphatidic acids (LPAs), which are abundant in human serum, but not in plasma, as potent VSMC dedifferentiation factors.[58] In vivo, unsaturated LPAs induce VSMC dedifferentiation by activating both ERK and p38MAPK, resulting in neointimal formation.[52] Epiregulin, a member of the EGF growth factor family, was further identified as a paracrine factor that is involved in the dedifferentiating of normal VSMCs near neointima.[53] Unsaturated LPAs actually also induce epiregulin expression.[53] Thus, the expression of epiregulin triggered by unsaturated LPAs may be a progression factor during the early onset of atherosclerosis. These findings shed light on molecular mechanisms involved in the early stages of atherosclerosis.

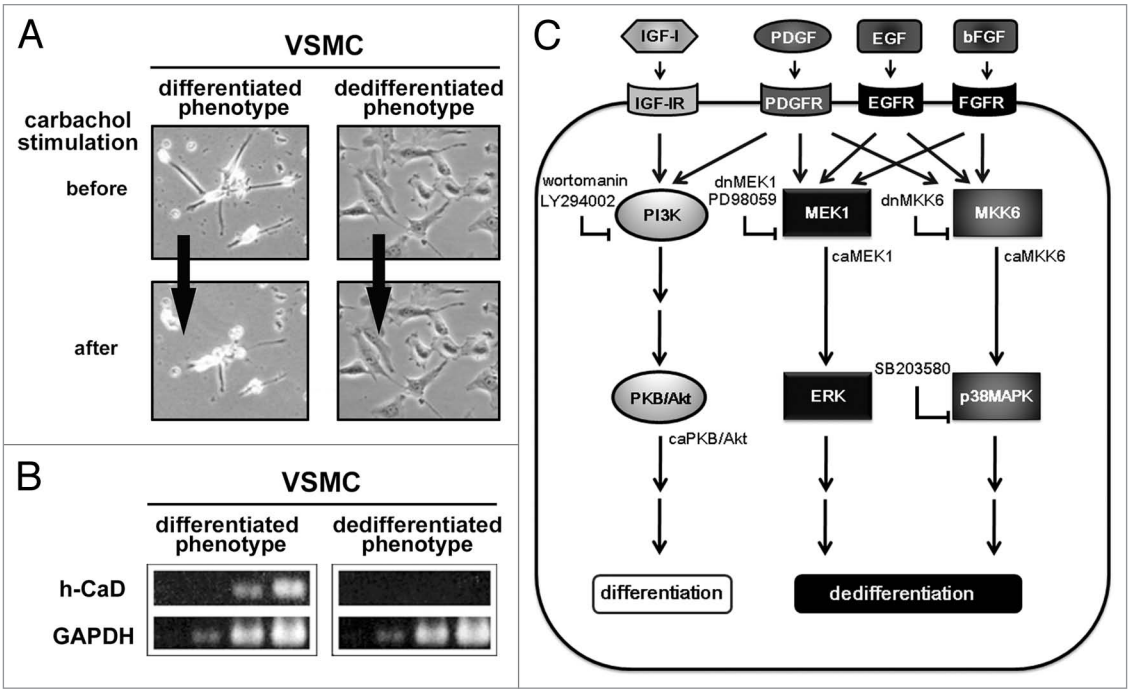


**Figure 3.** The establishment of VSMC culture system exhibiting the differentiated phenotype. (A) The morphological and functional features. The differentiated VSMCs exhibit spindle-like shape and carbachol-induced contraction. The dedifferentiated VSMCs exhibit fibrobrast-like morphology and loss of the contractility. The extracellular factors for differentiation or dedifferentiation are shown above. (B) The restricted expression of h-CaD in differentiated VSMCs. The expressions of h-CaD and GAPDH are detected by RT-PCR. Abundant expression of h-CaD is detected only in differentiated VSMCs. (C) The signal pathways determining differentiated/dedifferentiated VSMC phenotypes. Activation of the PI3K-Akt/PKB pathway is required for exhibiting the differentiated phenotype, whereas activation of the ERK and/or p38 pathway potently induces dedifferentiation of VSMCs.

## CaD's Role in Cellular Functions

**CaD regulates cell morphology and motility.** A number of reports have shown that increased CaD expression in non-muscle cells enhances stress fiber formation and reduces cell motility.[46,59-63] In contrast, depleting CaD by gene silencing impairs or eliminates stress fibers.[46,60] The formation of straight, thick stress fibers is thought to be mediated by CaD's F-actin stabilizing and bundling/cross-linking activities.[6,16,17,64] CaD's binding to F-actin protects the filaments from actin-severing factors such as gelsolin and cofilin, in vitro.[16,21] Furthermore, in vivo, thick stress fibers stabilized by CaD are more resistant to the actin-depolymerizing drug cytochalasin B.[46,63] Myosin ATPase inhibition by blebbistatin, a selective myosin II inhibitor, disrupts stress fibers.[35] These results suggest that the formation of thick stress fibers requires CaD, both to stabilize F-actin and to properly regulate myosin activity.

CaD is phosphorylated by several protein kinases. PAK phosphorylation sites are located close to CaD's CaM-binding sites.[65,66] PAK phosphorylation of CaD reduces its CaM binding ability by about 10-fold.[66] When CaD is phosphorylated by PAK, its affinity for actin-TM is modestly reduced and its inhibition of actin-activated myosin ATPase activity is significantly reduced—especially in the presence of TM.[66] Cells expressing a CaD mutant lacking the CaM binding site exhibit reduced stress fiber formation and motility defects.[61] Eppinga et al. demonstrated that endogenous CaD is phosphorylated by PAK during wound healing and that a PAK-dependent, phospho-regulation-deficient mutant of CaD stops wound-induced migration.[63] CaD phosphorylation by ERK1/2 and/or p38MAPK is involved in cell migration.[62,67] When CaD is phosphorylated by PKC, both its binding to actin and $Ca^{2+}$/CaM and its inhibitory effect on myosin ATPase activity are weakened in proportion to the degree of phosphorylation.[68-70]

CaD phosphorylation sites for various kinases, including PKC, ERK and p38MAPK, are very near the C-terminal region that contains actin-, CaM- and TM-binding sites and inhibits myosin ATPase activity.[70-74] CaD is also phosphorylated by other kinases, such as $Ca^{2+}$/CaM-dependent protein kinase II (CaMKII),[9] cdc2 protein kinase,[75,76] myosin light chain kinase (MLCK),[77] protein kinase A (PKA)[78] and casein kinase II.[79] In addition to serine/threonine phosphorylation, CaD is phosphorylated on tyrosine residues.[80-82] Highly phosphorylated CaD tyrosine residues are located in the N-terminal region, which possesses myosin- and secondary CaM-binding sites. CaD is phosphorylated on tyrosine during the cellular response to viral-type EGFR (v-erbB) activation, which promotes CaD binding to myosin II, Grb2, Shc, Nck, MLCK and PAK.[80,81] Although the role of CaD's interactions with these scaffold proteins has not been elucidated, they are thought to contribute to actin cytoskeletal reorganization and to anchorage-independent growth in EGFR-activated tumor cells.[80] High levels of tyrosine phosphorylation in CaD are observed in zyxin-deficient cells, in which focal adhesion assembly and cell motility are enhanced, further implicating CaD in actin cytoskeletal remodeling and motility.[83]

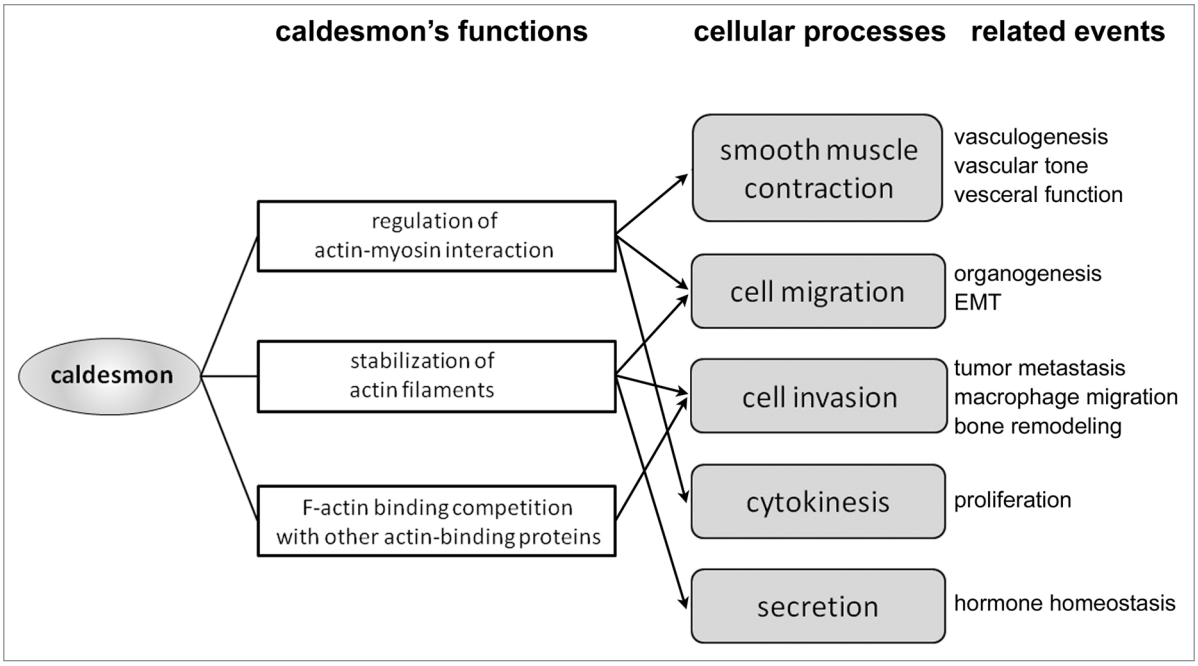


**Figure 4.** Schematic summary of CaD's functions dealing in this review.

**Upregulated CaD mediates glucocorticoid-induced inhibition of cell migration.** In addition to the qualitative regulation of CaD by $Ca^{2+}$/CaM and phosphorylation, quantitative regulation through transcription regulation has been documented. GCs are major stress-response mediators that regulate numerous cellular functions. In the human lung adenocarcinoma A549 cell line, GC-induced CaD upregulation enhances the formation of thick stress fibers and focal adhesions, which suppress cell migration.[46,60] This GC-dependent CaD upregulation also delays neuronal migration during neocortical development. CaD is upregulated by activated GR binding to two GREs in the fibrotype *CALD1* promoter.

It is well documented that stress-triggered GCs, as well as acute or chronic GC treatment, impair the structural and functional plasticity of the brain. The exposure of perinatal animals and humans to excess stress/GCs can affect brain development, leading to altered behaviors in adult offspring in animals, and an increased risk of psychiatric disorders in humans (reviewed in ref. 84). Fukumoto et al. recently showed that excessive GC exposure retards NPC migration by dysregulating actin-myosin interactions via CaD upregulation.[47,84] Migrating NPCs are normally bipolar, with a characteristic long leading process and rear retracting process. GC-treated NPCs become multipolar and CaD, F-actin and myosin IIA are found in the soma and concentrated in the tips of the multipolar processes. As with GC-treated NPCs, CaD-overexpressing cells exhibit a bipolar-to-multipolartransition, with dynamic growth endings at the tips of their multipolarprocesses, and they migrate randomly. These data indicate that an appropriate level of CaD expression is critical for the actin-myosin II interactions in NPCs that maintain their bipolar shape and linear migration. Elucidating the molecular mechanisms underlying the detrimental effect of GCs on neocortical development will expand our understanding of how stress/GCs alter neural network formation and affect behaviors later in life.

**CaD regulates cell morphology and motility in the Rho-dependent MRTF/SRF transactivation pathway.** The Rho-MRTF/SRF pathway plays important roles in numerous physiological processes by upregulating actin cytoskeletal/focal adhesion genes, including CaD. RhoA is a crucial regulator of stress fiber formation, focal adhesion assembly and cell morphology and motility, mediated by the reorganization of the actin cytoskeleton.[85] It is well-documented that the RhoA-ROCK-LIMK pathway increases actin polymerization by inactivating its downstream target, ADF/cofilin, which severs F-actin.[86] Rho's interactions with diaphanous-related formin (DRF) family proteins leads to the nucleation and elongation of non-branched F-actin.[87] Rho activation triggers the translocation of MRTFs to the nucleus, and enhances the MRTF/SRF-dependent transcription of actin cytoskeletal/focal adhesion genes such as CaD, TM, vinculin and zyxin.[45,88] MRTF activation enhances stress fiber formation and focal adhesion assembly, in parallel with actin cytoskeletal/focal adhesion protein upregulation. On the other hand, MRTF inhibition leads to morphological changes, including decreased cell spreading and adherence and fewer stress fibers and focal adhesions.[45] The transcriptional regulation of actin cytoskeletal/focal adhesion genes via the MRTF/SRF-dependent pathway is essential to maintaining and changing cell morphology in response to stimulation and to changes in the extracellular environment.

MRTF/SRF-dependent transcriptional regulation also contributes to epithelial-mesenchymal transition (EMT). EMT is a critical process that occurs during embryonic development, fibrosis and tumor progression. It is defined as a reduction in cell-cell adhesion accompanying decreased epithelial gene expression,

acquisition of motility, cell-shape alteration and increased mesenchymal gene expression.[89-91] The remodeling of the actin cytoskeleton is a major EMT event, and it is part of the alteration from cell-cell adhesion to cell motility.

Transforming growth factor β (TGFβ) is one of the main EMT inducers; it triggers the dissociation of cell-cell contacts and actin cytoskeletal remodeling, permitting adherent epithelial cells to scatter and migrate directionally through the extracellular matrix.[92] In human, mouse and canine epithelial cells, TGFβ1 stimulation causes a series of actin cytoskeletal genes, including CaD, to be upregulated during EMT via the Rho-MRTF/SRF pathway.[93] TGFβ stimulation also increases Snail expression via MRTF/Smad activation, in which Snail is a transcription factor that induces cell-cell detachment by repressing E-cadherin,[94] which leads to cells being released from cell-cell attachments simultaneously with the remodeling of their actin cytoskeleton.[93] In kidney tubule cells as well, the Rho-MRTF/SRF-dependent pathway mediates the disruption of cell-cell contact, inducing EMT.[95] These regulatory mechanisms maybe involved in various pathological events, including cancer metastasis and fibrosis, as well as in development.[96,97]

**CaD is a suppressor protein in podosome/invadopodium formation.** The podosome and invadopodium are highly dynamic cell adhesion structures that degrade the extracellular matrix (ECM) and promote cell invasion.[98-101] While CaD is not localized at focal adhesions, it is a component of the podosome.[37,102] CaD suppresses podosome/invadopodium formation in smooth muscle cells and in transformed or cancer cells.[102,103] CaD depletion results in the increased formation of numerous small podosomes/invadopodia, which have a significantly shorter lifetime and more mobility than control cells.[103] Forced CaD expression, on the other hand, suppresses the formation of podosomes/invadopodia, ECM degradation and cell invasion.[104] CaD suppresses podosome/invadopodium formation by competing with the Arp2/3 complex, which is activated by N-WASP and is required for podosome formation.[103] In addition, CaD's role in podosome formation is regulated by PAK phosphorylation and $Ca^{2+}$/CaM binding.[102,103] These results are consistent with CaD being a potent suppressor of cancer cell invasion.

CaD expression is decreased in certain transformed and cancer cells.[22,37,104] A decrease in the expression of CaD and TM correlates with suppressed MRTF nuclear translocation in Ras- or Src-transformed intestinal epithelial cells, suggesting that MRTF/SRF-dependent transcription is also downregulated.[105] Furthermore, the ectopic expression of active-form MRTFs reverses the inhibition of CaD and TM expression and the associated morphological phenotypes, resulting in less invasive, anchorage-independent growth. In vivo, cancer cells that express active MRTFs form fewer subcutaneous cancer foci and show fewer liver metastases from the spleen.[105] In another metastasis models in which breast carcinoma or melanoma cells were injected into the mouse tail vein, inactivating the MRTF/SRF pathway reduces cell motility and metastasis to the lung, whereas forced expression of active-MRTF-A increased colonization of the lung.[106] This discrepancy may be due to differences in the metastasis model. A recent report shows that p53 activation upregulates CaD expression and suppresses tumorigenic phenotypes;[107] p53 suppresses podosome/in vadopodium formation, promotes stress fiber stability and attenuates cell migration/invasion mediated by upregulation of CaD.[48,107] These results suggest that CaD is a critical target of p53 in preventing invasion and metastasis.

**CaD is a regulatory protein and cytokinesis component.** During mitosis, CaD is phosphorylated by cdc2 kinase, which greatly reduces CaD's binding affinity for actin filaments.[75,76,108] During cytokinesis, CaD is not detected in cleavage furrows mainly composed of contractile elements, such as myosin and actin.[109] A CaD mutation containing alanine substitutions in all seven cdc2 phosphorylation sites inhibits cell division and delays M-phase entry in Xenopus embryos and CHO cells.[110] This mutated CaD inhibits microfilament disassembly during mitosis. These results suggest that the regulation of actin filament assembly/disassembly in cleavage furrows by CaD via cdc2-dependent phosphorylation is important for cell cycle progression.

Manes et al. reported that cdc2 activity increases in cell migration through its specific association with cyclinB2 in the αvβ3 integrin signaling pathway.[111] CaD and cdc2 are co-localized in membrane ruffles in motile cells. Their finding suggests that CaD phosphorylation by cdc2 is required to activate cell motility via the αvβ3 integrin-cdc2-dependent pathway, since a CaD mutation affecting all seven cdc2 phosphorylation sites blocks cdc2-induced motility. Cdc2-dependent CaD regulation may therefore be significant in tumorigenesis, which involves successive cell proliferation and configuration.

**CaD regulates secretion.** Stimulation of adrenal chromaffin cells increases the concentration of intracellular free $Ca^{2+}$, which initiates catecholamine secretion by exocytosis. CaD acts to regulate the organization of actin filaments beneath the cell periphery during the secretory process.[112] CaD also plays a role in GnRH (gonadotropin releasing hormone)-stimulated gonadotropin release from the pituitary.[113] Similarly, GC induces negative feedback regulation of ACTH (adrenocorticotropic hormone) secretion by upregulating CaD to stabilize actin filaments.[59] Further, it is likely that TM and CaD affect intracellular granule movement by regulating the contractile system in response to $Ca^{2+}$ changes inside non-muscle cells.[114] These data indicate that CaD regulates secretion and intracellular granule trafficking in a $Ca^{2+}$-dependent manner.

## Conclusion

It has become clear that CaD is an important regulator of cell motility, such as cell morphology, migration, cytokinesis and secretion as well as of smooth muscle contraction (**Fig. 4**). CaD regulates the organization of the actin cytoskeleton and actin/myosin-dependent contractility. Because these mechanisms are fundamental for cell structure and force generation, they link to numerous cellular processes, such as intracellular trafficking, cell morphology maintenance and changes, cytokinesis, secretion, cell adhesion and migration. CaD's functions are controlled by $Ca^{2+}$/CaM binding and/or phosphorylation,

which provide spatio-temporal control of directed motility. Further, CaD expression levels are regulated by at least three different signaling pathways—the GR-, Rho-MRTF/SRF- and p53-dependent pathways, all of which are all deeply involved in development, stress response and various pathological changes. Although we have made remarkable progress in understanding the physiological roles of CaD, this may be only a partial list of its functions. CaD's ubiquitous distribution and fundamental functions suggest that it is likely to play significant roles in other, as yet unknown, cellular processes.


### Acknowledgements

This work was supported by Grant-in-aid for Scientific Research 20240038 (to K.S.) and Grant-in-aid for Young Scientist 21700352 (to T.M.) from the Japan Society for the Promotion of Science.


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