

# Ca<sup>2+</sup> signaling and Target Binding Regulations: Calmodulin and Centrin *In Vitro* and *In Vivo*

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## Abstract

Changes in Ca<sup>2+</sup> concentrations act as a second messenger to regulate biological processes. Ca<sup>2+</sup> sensor proteins transduce these Ca<sup>2+</sup> signals upon binding to protein targets that are involved in the cellular process that is being regulated.

Ca<sup>2+</sup> sensor proteins such as calmodulin and centrin bind Ca<sup>2+</sup> through acidic residues that compose the EF-hand motifs and further bind targets via surfaces that recognize specific motifs on targets. However, in some cases, the binding of a Ca<sup>2+</sup> sensor protein to a target can occur in the absence of Ca<sup>2+</sup>. Upon Ca<sup>2+</sup> binding, Ca<sup>2+</sup>-sensor proteins undergo conformational changes, which lead to the exposure of surface that interacts with target. Moreover, Ca<sup>2+</sup> binding and the binding of Ca<sup>2+</sup> sensors to targets induce conformational changes that drive regulation. Comparisons of the Ca<sup>2+</sup>-sensor proteins calmodulin and centrin provide information on regulatory processes.

**Keywords:** Calcium; Calmodulin; Centrin; Conformational changes

## Introduction

### Changes in Ca<sup>2+</sup> concentration act as cellular signals

The divalent cation Ca<sup>2+</sup> is one of the most abundant elements on earth and it is a major cellular second messenger [1]. Ca<sup>2+</sup> exhibits two functions in living organisms: a structural function (e.g., in bones and teeth), which involves 99% of total calcium [2], and a regulatory function (e.g., enzyme activator) [3]. As a regulator, Ca<sup>2+</sup> accomplishes several intra- and intercellular tasks in an organism. Furthermore, Ca<sup>2+</sup> signals are translated into cellular processes via Ca<sup>2+</sup>-binding proteins such as calmodulin and centrin, which act as Ca<sup>2+</sup> sensors. High levels of Ca<sup>2+</sup> are toxic to cells. Cytosolic and nuclear Ca<sup>2+</sup> concentrations are maintained between 50 and 100 nM. Plasma-membrane Ca<sup>2+</sup>-ATPases regulate intracellular Ca<sup>2+</sup> concentrations in eukaryotes and are involved in the maintenance of Ca<sup>2+</sup> homeostasis [3]. Passive influx of Ca<sup>2+</sup> into cytosol takes place through Ca<sup>2+</sup>-channels although its efflux is an active process that is mediated by Ca<sup>2+</sup> pumps.

In animals and plants, changes in Ca<sup>2+</sup> concentrations occur in response to both biotic and abiotic stimuli and are detected by Ca<sup>2+</sup>-sensor proteins [for review, see refs. 4,5]. The cellular signal has to be transduced to other proteins that are involved in the particular pathway being regulated. Ca<sup>2+</sup>-binding proteins are the intermediates that transfer the signal to other proteins via protein-protein interactions. Animals respond to external stresses with behavioral changes conversely plant respond to stress through changes in gene expression. Thus, Ca<sup>2+</sup> regulate many genes in Arabidopsis [6].

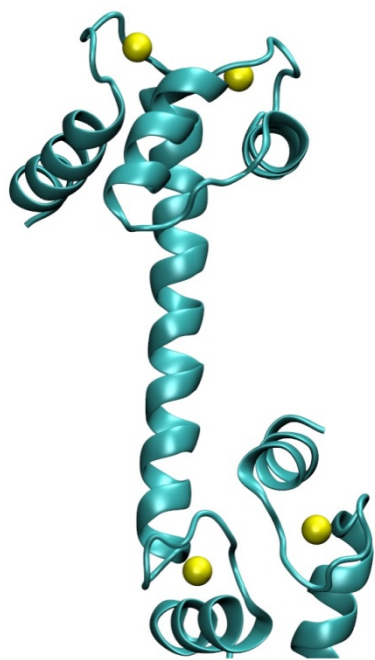
### The Ca<sup>2+</sup>-sensor proteins calmodulin and centrin

Ca<sup>2+</sup>-binding proteins comprise CaM, CaM-like proteins, S100 proteins, centrans [for review, see ref. 7]. Calmodulin and centrin are the most studied Ca<sup>2+</sup>-sensor proteins. Calmodulin and centrin are small (~20 kDa), acidic and stable proteins that are ubiquitous and highly conserved throughout evolution. Several genes encode calmodulin and centrin isoforms, some of which are ubiquitously expressed, whereas others show tissue-specific expression. Three genes (CALM1, CALM2, CALM3) encode calmodulin in humans. In Arabidopsis, seven genes encode four calmodulin isoforms. The number of genes that encode centrin varies between organisms. Indeed, some eukaryotes have only one gene, such as green algae and yeast, whereas higher eukaryotes have several genes. Three genes encode human centrin (Cen1, Cen2, Cen3) [8], and a supplementary gene exists in mouse [9].

### Ca<sup>2+</sup>-sensor proteins bind Ca<sup>2+</sup> through EF-hand motifs

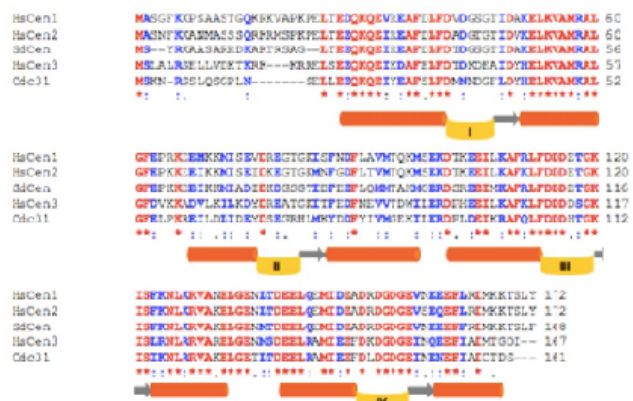
Ca<sup>2+</sup>-binding proteins bind Ca<sup>2+</sup> via the EF-hand motif, which consists of a "helix-loop-helix" structure. The EF-hand motif was first described in the X-ray structure of parvalbumin in 1973 [10].

Calmodulin is composed of two globular domains that are connected by a  $\alpha$ -helix region (Figure 1) [11]. Each globular domain contains a pair of EF-hand motifs, and Ca<sup>2+</sup> binds all four EF-hand sites of calmodulin. NMR structures of apo-calmodulin revealed that calmodulin adopts a globular conformation in the absence of Ca<sup>2+</sup> or target peptide, known as the "closed" conformation [12,13]. Upon Ca<sup>2+</sup> binding, calmodulin undergoes conformational changes and adopts an "open conformation", exposing hydrophobic surfaces for Ca<sup>2+</sup> - dependent interactions with target proteins.



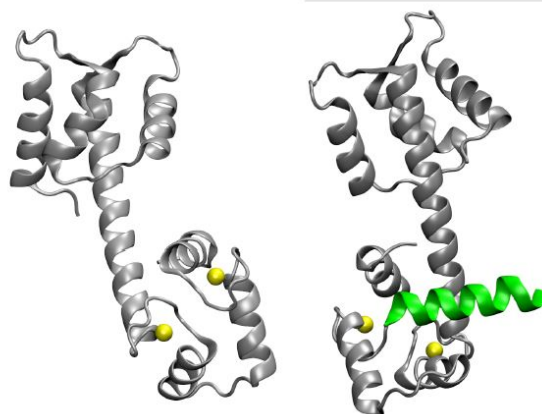
**Figure 1:** Structure of human CaM. CaM contains a pair of EF-hand with Ca<sup>2+</sup> (yellow) in N-terminus and C-terminus (PDB code: 1CLL) [13].

Centrin is composed of two independent domains, each of which contains a pair of EF-hand motifs (Figure 2). Figure 2 shows the sequence alignment of centrin from various organisms. The C-terminal domain of centrin is highly conserved. The Ca<sup>2+</sup> loops are composed of acidic residues, which bind Ca<sup>2+</sup>. However, loop I and loop II which are located in the N-terminal do not bind Ca<sup>2+</sup> conversely loop III (D114-N125) and loop IV (D150-E161) which are located in the C-terminal bind Ca<sup>2+</sup>.



**Figure 2:** Centrin sequences with EF-hand motifs. Centrin alignment showing the four EF-hand loops of *Saccharomyces cerevisiae*, *Scherffelia dubia* and human centrin.

Figure 3 shows the structure of human centrin 2 [14]. The Ca<sup>2+</sup> site of the loop III consists of five oxygen atoms belonging to the side-chains of residues D114, D116, T118, and N125 and the backbone of K120 of the protein and by a sixth oxygen from a water molecule. The Ca<sup>2+</sup> site of the loop IV consists of six oxygen atoms belonging to the side-chain of residues D150, D152, D154, E161 or the backbone E156 of the protein and by a seventh oxygen from a water molecule. NMR structures of the C-terminal domain of human centrin 2 revealed that Ca<sup>2+</sup> binding modifies the conformation of this protein [15].



**Figure 3:** Structure of human centrin 2 and human centrin 2 in complex with XPC peptide. Structure of centrin 2 showing Ca<sup>2+</sup> bound to a pair of EF-hand in C-terminus (left). Structure of centrin in complex with XPC peptide (right) (PDB code: 2GGM) [31].

Protein	EF-hand motif
<b>CaM</b>	
Loop I	21-DKDGNGTITTKE-32
Loop II	57-DADGNGTIDFPE-68
Loop III	94-DKDGNGYISAAE-105
Loop IV	130-DIDGNGENNYEE-141
<b>HsCen2</b>	
Loop III	113-FDDDETGKISFKNL-126
Loop IV	149-ADRDGDGEVSEQEF-162
Residues (red) of centrin which bind Ca <sup>2+</sup> .	

**Table 1:** CaM and Centrin EF-hand motifs reports the sequence of the four EF-hand motifs calmodulin and the two EF-hand motifs centrin showing the presence of acidic residues, which bind Ca<sup>2+</sup>.

### Interaction of calmodulin and centrin with their cellular targets

CaM/centrin targets	CaM/Centrin binding motif of targets
<b>Centrin</b>	
Human XPC	COOH <sup>863</sup> -RKLRRERILLGKALLKWN-843 <sup>NH2</sup>
Human Sfi1R17	NH <sub>2</sub> <sup>641</sup> -RADLHHQHSLVLRALQAWV-660 <sup>COOH</sup>
Human Sac3	NH <sub>2</sub> <sup>1225</sup> -IFQTAKETLQELQCFCKYLQRWR1247 <sup>COOH</sup>
Transducin β	NH <sub>2</sub> <sup>323</sup> -DMAVATGSWDSFLKIWN-340 <sup>COOH</sup>
<b>CaM</b>	
M=Murine Myosin V (IQ motif)	NH <sub>2</sub> <sup>769</sup> -ACIRIQKTIRGWLLRKRY-786 <sup>COOH</sup>
Human MLCKII	NH <sub>2</sub> <sup>566</sup> -KRRWKKNFIAVSAANRFKISS-587 <sup>COOH</sup>
Rat CaM-dependent Kinase II	NH <sub>2</sub> <sup>293</sup> -FNARRKLKGAILTTLAT-310 <sup>COOH</sup>
Residues (red) of target which bind centrin or CaM.	

**Table 2:** Binding motif of calmodulin targets, centrin targets reports the target-binding motifs for calmodulin and centrin.

### Calmodulin targets

Calmodulin targets have been extensively reviewed [16] and include calmodulin-dependent kinase (CaMK), calmodulin-dependent phosphatase, death-associated protein kinase (DAPK), nitric oxide synthase (NOS), plasma-membrane Ca<sup>2+</sup>-ATPase (PMCA), calmodulin-activated adenylyl cyclase, and exotoxin secreted by *Bacillus anthracis*. The calmodulin-binding sites of calmodulin targets share several characteristics, including helical propensity, positive charge and hydrophobic residues. Calmodulin binds targets containing IQ-motifs (IQxxxRGxxxR) in binding mode 1-5-10 or 1-14. Rhoads and Friedberg [17] have studied the calmodulin-binding motifs of targets. Three recognition motifs exist for calmodulin interaction: one for Ca<sup>2+</sup>-independent binding and two for Ca<sup>2+</sup>-dependent binding (1-8-14 and 1-5-10 based on the position of conserved hydrophobic residues). The IQ motifs found in calmodulin targets have been reviewed [18]. Several examples of Ca<sup>2+</sup>-dependent or Ca<sup>2+</sup>-independent interactions of calmodulin with targets are listed below.

#### Calmodulin-CaMBD (small-conductance Ca<sup>2+</sup>-activated potassium channels (SK2))

To explore how target proteins may affect the affinity of calmodulin for Ca<sup>2+</sup>, the X-ray crystal structure of calmodulin in complex with the CaM-binding domain (CaMBD) from small-conductance Ca<sup>2+</sup> activated potassium channels (SK2-b) has been determined at a resolution of 1.9 Å [19]. The splice variant SK2-b is less sensitive to Ca<sup>2+</sup> than SK2-a. This study revealed that target proteins could change the conformation of calmodulin and regulate the affinity of calmodulin for Ca<sup>2+</sup>. Comparisons between SK2-a and SK2-b reveal differences in the C-lobe and in the linker of calmodulin bound to SK2. In addition, the four Ca<sup>2+</sup>-binding sites of calmodulin in complex with SK2-b are occupied, although the C-lobe of calmodulin in complex with SK2-a is free of Ca<sup>2+</sup>.

#### Calmodulin-Protein kinase

Calmodulin-dependent protein kinases catalyze the transfer of phosphate from the gamma position of ATP to the hydroxyl side chains of Ser and Thr residues of protein. Certain calmodulin-

dependent protein kinases are known to act on several substrates (e.g., CaMKI, CaMKII, and CaMKIV), whereas others act on specific substrates (e.g., CaMKIII, phosphorylase kinase, myosin light-chain kinases). Calmodulin-dependent protein kinase kinases have also been identified [20].

#### Calmodulin-Myosin Light-Chain kinase (MLCK)

Calmodulin-dependent smooth muscle myosin-light chain kinase is involved in the activation of smooth muscle contraction. The binding of calmodulin to MLCK creates a surface domain containing Lys30 and Gly40 and residues from the C-terminus of calmodulin, which leads to MLCK activation [21].

#### Calmodulin-Protein phosphatase (calcineurin)

Calcineurin is Ca<sup>2+</sup>-calmodulin-dependent Ser/Thr phosphatase. Calcineurin is a heterodimer (chain A and chain B); chain A contains the catalytic domain, and chain B consists of a regulatory domain that includes a calmodulin-binding region and an autoinhibitory domain. At low Ca<sup>2+</sup> concentrations, calcineurin exists in inactive form in which the auto inhibitory domain is bound to the active site. Upon increases in Ca<sup>2+</sup> concentrations, calmodulin binds Ca<sup>2+</sup> and in turn binds to the regulatory domain of calcineurin, causing conformational changes that lead to the release of the auto inhibitory domain and the activation of the phosphatase [22].

#### Calmodulin-Myosin V

Myosins comprise a family of ATP-dependent motor proteins that are known for their role in muscle contraction [23]. Myosins (ATPases) move along actin filaments by coupling the hydrolysis of ATP to conformational changes. Cells contain various myosin isoforms. For example, myosin II (conventional myosin) generates muscle contractions, and myosin V (unconventional myosin) transports cargo in non-muscle cells. Myosin V consists of a globular motor domain that binds actin and hydrolyses ATP, which is connected to an elongated α-helical lever arm that contains six calmodulin-binding IQ motifs (IQxxxRGxxxR). Ca<sup>2+</sup> activates the ATPase activity of full-length myosin V but not truncated myosin [24]. In the absence of

Ca<sup>2+</sup>, calmodulin binds myosin V, which adopts a folded inactive conformation. Low Ca<sup>2+</sup> concentrations unfold and activate myosin, while higher Ca<sup>2+</sup> concentrations dissociate calmodulin and inhibit myosin activity. The structure of apo-calmodulin bound to the first two IQ motifs of myosin V [25] has been solved and shows that the C-terminal lobe adopts a semi-open conformation when bound to the first part (IQxxxR) of the IQ motif, although the N-terminal lobe adopts a closed conformation when bound to the second part (GxxxR) of the IQ motif.

The CaM complexes structures, which are deposited in the Data Bank (PDB), have been reviewed [16]. The analysis revealed diverse binding modes and highlighted the conformational flexibility of CaM. Shukla et al. [26] constructed Markov state models from molecular dynamics simulations of the C-lobe of CaM in the Ca<sup>2+</sup>-bound and unbound regimes to explore contributions of a single domain of CaM to binding diversity.

### Centrin targets

At least five proteins (XPC, Sfi1, Sac3, POC5, Transducin  $\beta$ ) have been shown to bind centrin. Thus, centrin is involved in several cellular processes via its targets: DNA repair via XPC [27], spindle pole body duplication via Sfi1 [28], RNA export via Sac3 [29] and transduction via transducin  $\beta$  [30]. The hydrophobic pocket of centrin binds targets through a typical hydrophobic helix, W<sup>1</sup>xxL<sup>4</sup>xxxL<sup>8</sup>. However, two sequence orientations of the centrin-binding motif exist, namely, W<sup>1</sup>xxL<sup>4</sup>xxxL<sup>8</sup> for XPC, and the reverse orientation, L8xxxL4xxW1, which is considered to be the amino acid sequence orientation for Sfi1 and transducin  $\beta$ . This is a unique characteristic compared with calmodulin.

Crystal [31-34] and NMR structures [35,36] of centrins in complex with truncated targets or target-derived peptides (i.e., XPC, Sfi1, or Sac3) have been solved. In all cases, the target-binding pocket is located in the C-terminal domain of centrin, and the F113 residue of human centrin is the main residue involved in target binding.

### XPC

The structure from [36] shows that only residues in the C-terminal domain of human centrin 2 are involved in the interaction with the XPC peptide. Nine non-polar residues in human centrin 2 (L133, L112, M145, F113, M166, L126, A109, E105 and V129) form contacts with residues in the XPC peptide. The F113 and M145 residues form the pocket where the W residue of XPC binds.

### Sac3

The structure of the CID region (proximal CTD-Interacting Domain) of Sac3 in complex with Sus1 and Cdc31 has been solved by X-ray crystallography [33,37]. In this complex, the W802 residue of Sac3 appears to play a central role in the interaction with the hydrophobic cavity of the centrin Cdc31. The F105 residue of Cdc31 in turn plays an important role in the association with Sac3.

### Sfi1

A crystal structure of the centrin Cdc31 in complex with truncated Sfi1 has been solved by X-ray crystallography [34]. Both the C-terminal and N-terminal domains of Cdc31 form contacts with Sfi1. The structure also reveals several centrin-centrin interactions. These interactions suggest that a filament of several centrin molecules is

formed by centrin-centrin interactions and that Sfi1 stabilizes this filament through Sfi1 repeat-centrin interactions. An NMR structure of human centrin 2 in complex with the R17-Sfi1 peptide has also been solved [14]. Figure 3 shows the NMR structure of the C-terminal centrin 2 in complex with Sfi1.

Comparison of this structure with the NMR structure of the C-terminal domain of human centrin 2 in complex with P17-XPC peptide [35] led to the proposal that, the centrin 2 residue E148 discriminates between XPC and Sfi1 [14].

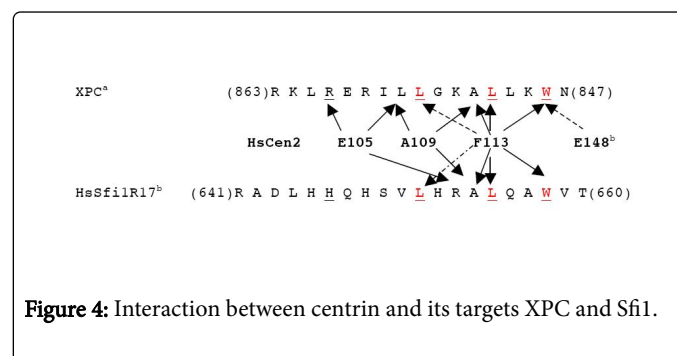


Figure 4: Interaction between centrin and its targets XPC and Sfi1.

Figure 4 summarizes the residues (E105, A109, F113) of centrin, which bind the hydrophobic triad L8xxxL4xxW1 of Sfi1 or the reverse triad W1xxL4xxxL8 of XPC. As for CaM, Ca<sup>2+</sup> binding to centrin can affect the binding of centrin to its targets. Thus, Figure 5 summarizes the effect of Ca<sup>2+</sup> on centrin binding to its targets.

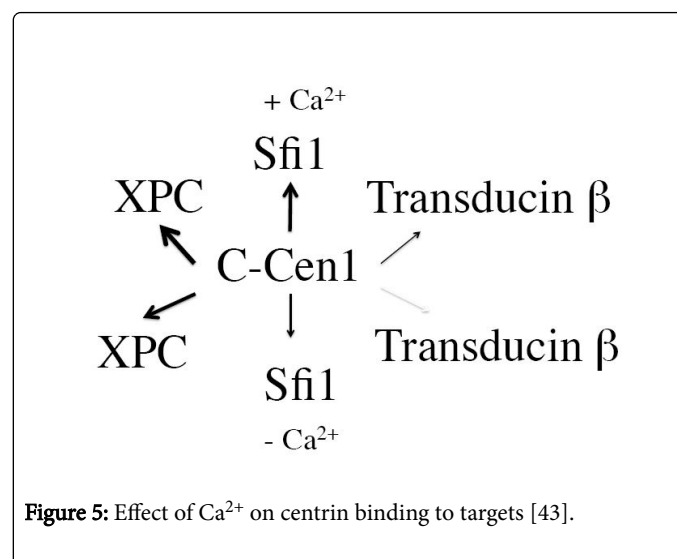


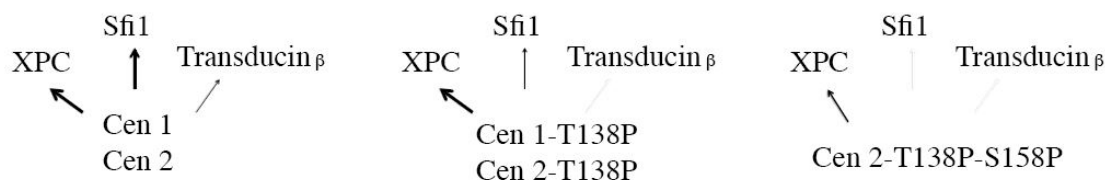
Figure 5: Effect of Ca<sup>2+</sup> on centrin binding to targets [43].

### Other regulated processes: phosphorylation, ubiquitination, SUMOylation

Ca<sup>2+</sup>-dependent structural changes are modulated by other levels of regulation such as the posttranslational modification of proteins (e.g., phosphorylation, SUMOylation, ubiquitination).

Centrin is phosphorylated by MPS1 at residue T138, by CK2 at residue T138 and by Aurora-A at residue S170. The localization of centrin 2 to the centrosome is dependent on the availability of phosphorylatable T118 and functional calcium binding EF-hands [38]. Phosphorylation of human centrins 1 and 2 regulates the interactions

with Sfi1 and transducin  $\beta$  [39]. Figure 6 summarizes the effect of centrin phosphorylation on target binding.



**Figure 6:** Effect of phosphorylation of centrin on binding to targets [43].

Centrins 1 and 2 are substrates of SUMO conjugation, and SUMOylation of centrin 2 is required for its efficient localization to the nucleus. XPC also undergoes SUMOylation, but the posttranslational modifications of XPC and centrin 2 are dependent on different factors [40]. SUMOylation of centrin 2 is also required for efficient interaction with XPC. The SUMOylation and ubiquitination of XPC drive DNA repair [41]. The Sfi1 C-terminal domain harbors phosphorylation sites for Cdk1 and the polo-like kinase Cdc5. Phosphoregulation of Sfi1 by Cdk1 has the dual function of promoting SPB separation for spindle formation and preventing premature SPB duplication [42]. The protein phosphatase Cdc14 has the converse role via phosphate group removal on Sfi1 [43].

## Conclusion

Ca<sup>2+</sup> regulates cellular functions via interactions between Ca<sup>2+</sup>-sensor proteins and targets that are involved in cellular processes. In addition, posttranslational modifications also regulate cellular processes. On some occasions, calmodulin, as well as centrin, bind to some of their targets in the absence of Ca<sup>2+</sup>. However, if a first step is Ca<sup>2+</sup> independent, a second binding step can be Ca<sup>2+</sup>-dependent.

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**Abbreviations** Sfi1: Suppressor of fermentation-induced loss of stress resistance protein1; SPB: Spindle pole body; XPC: Xeroderma pigmentosum group C protein; Sac3: Is the central component of the transcription and mRNA export (TRES-2) complex; CID: Proximal CTD-Interacting Domain; CaM: Calmodulin; MLCK: Myosin light-chain kinase.

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