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Loss of cks1 homeostasis deregulates cell division cycle

Anand Krishnan, S. Asha Nair *, M. Radhakrishna Pillai

Translational Cancer Research Laboratory, Department of Molecular Medicine, Rajiv Gandhi Centre for Biotechnology, Kerala, India

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Abstract

Genetic and biochemical studies have provided considerable insight into the multiple functions of cyclin-dependent kinase subunit (cks)1 in cell division cycle. In addition to enhanced substrate targeting by specific ubiquitin ligases SCF^{skp2} and APC/C, its direct interaction with proteasome components normalizes multiple cell cycle regulators. Importantly, it also acts as a transcriptional regulator. cks1 overexpression reflects poor prognosis in malignancy thus indicating its possible role in tumour diagnosis and management. The present review compiles the multiple functional roles of cks1 in cell division with specific emphasis on its molecular mechanisms. Its docking functions and the possible downstream proteolytic and transcriptional targets are described. The spatial configuration of cks1–cdk2 complex and the structural organization of cks1–p27–skp2 assembly required for p27 ubiquitination are discussed in detail. In addition to enhanced p27 degradation, the possible other mechanisms which underlie its pathological functions in human cancer progression are also discussed. Though there are apparent gaps in information, the turnover mechanism of cks1 is well addressed and presents opportunity to exploit the target for disease management.

Keywords: cks1 • ubiquitin ligase • cell cycle • p27 • cancer

Introduction

Cyclin-dependent kinase subunit (cks)1* was initially identified in 1986 in fission yeast by virtue of its ability to rescue certain temperature sensitive mutants of fission yeast cyclin-dependent kinase (cdc2) [1] and further biochemical studies revealed its vital role in the regulation of cell division cycle [2–3]. Its human homologue was identified in 1987 in complex with cdc2 [4] followed by identification of its budding yeast homologue in 1989. Subsequent PCR analysis, using cDNA libraries from HeLa cells, probed with degenerated oligonucleotides designed based on amino acid sequences of fission yeast and budding yeast cks1 homologues revealed two identical clones in human beings (cks1 and cks2) which show 81% identity among their amino acid sequences [5]. Both fission yeast and budding yeast homologues show 67% amino acid sequence identity with a single difference being the presence of additional 16 consecutive glutamine codons in budding yeast cks1 gene [6]. cks1 and cks2 show 53% and 57% identity, respectively, with both budding yeast and fission yeast homologues [5] whereas the xenopus homologue of cks1 shows 91% identity with cks2 [7]. Expression analysis shows cks1 abundance throughout cell division cycle. It forms a complex with both partial (in absence of regulatory cyclins) and complete forms of cdc2 active kinase which is maintained even when the cells enter into stationary phase and during abnormal cell division states (S phase arrest) [6–8].

*Correspondence to: Dr. S. Asha NAIR,
Scientist E1, Translational Cancer Research Laboratory,
Department of Molecular Medicine,
Rajiv Gandhi Centre for Biotechnology,
Trivandrum, Kerala 695 014, India.
Tel.: +91 471 2529501
Fax: +91 471 2349303
E-mail: sasha@rgcb.res.in

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** To bring more clarity and uniformity, the general names 'cks1' and 'cdc2' is used throughout the manuscript to represent all the cks1 and cdc2 homologues, respectively, in different species.*

Functional analyses of cks1

The essential functions of cks1 for normal cell division and growth have been demonstrated through various genetic and biochemical experiments in different species. cks1 deletion confers mitotic arrest phenotype that eventually lose viability whereas its overexpression produced another abnormal phenotype due to delayed mitotic entry [2, 9, 10]. These observations and the demonstration that cks1 deleted fission yeast could enter mitosis [9] initially suggested the physiological requirement of cks1 exclusive for mitotic exit. However, conditional mutation and functional inactivation experiments have indicated the requirement of proper functions of cks1 for both G1/S and G2/M transitions and maintenance of cell viability [6, 11]. Interestingly, consistent with the production of abnormal mitotic phenotype, cks1 overexpression has induced abnormal effects in meiosis also (production of two spored asci which failed to complete second meiotic nuclear division) underscoring the pathological functions of cks1 when it is expressed at physiologically high levels [2, 10]. Thus, it is evident that cks1 functions at multiple levels, depending upon the cell division status, for normal check point transitions, mitotic progression and mitotic exit. Similarly, it is interesting to observe that both deletion and overexpression of cks1 could confer a similar phenotype (delayed mitotic entry) [6, 7, 11]. The docking functions of cks1 could explain this. Collectively, in addition to the observation that cks1 is associated with mitotic cdk, functional analyses of cks1 indicate that, irrespective of species differences, the following is apparent (*i*) cks1 is essential to maintain cell viability (*ii*) remarkable changes in its expression could break the rhythm of cell division cycle. However, the underlying mechanism that breaks the rhythm of cell cycle at conditions of abnormal cks1 kinetics has remained elusive and formed the basis for further research on cks1.

Cell cycle kinetics depends upon changes in the kinase activity of cyclin-dependent kinases [12, 13]. The influence of cks1 on cell division cycle and its complex formation with mitotic cdks has thus put forth substantial hint for cks1 having critical roles in regulating the kinase activity of cdks. Substantiating this hypothesis, fission yeast studies demonstrated that only 5% of total cdc2, which is found to be in complex with cks1, is active as a kinase [8]. Similarly, cks1 association was observed in both partial and completely active cdc2 kinase complex in budding yeast [6]. Depletion of cks1 from xenopus egg interphase extract had a negative effect on cdc2 kinase activity to perform G2/M transition [7]. However, as in the case of cks1 depleted/overexpressed functional phenotypes, quite distinct observations were obvious. In particular, cks1 deleted cells arrest at M phase with high cdc2 kinase activity [7, 9, 11]. Furthermore, addition of exogenous cks1 to interphase extract had remarkably reduced cdc2 kinase activity and prevents mitotic entry [7]. A probable role of cks1 to inactivate cdc2 kinase activity has also been observed in fission yeast [9]. Overall, these findings suggest that, though in complex with mitotic cdks, cks1 may not be strictly a positive or negative regulator of cdks and could be considered as a cdk modulator at its physiological levels. However questions still remain regarding the specific molecular mechanisms leading to G2 phase arrest when there is excess cks1 or how excess of cks1 at interphase reduces cdc2 kinase activity. Similarly, how its deletion alters cdc2 kinase activity and arrests cells at check point transitions (except in fission yeast: the cells enter mitosis even when cks1 is suppressed), mechanisms that delay the cells from mitotic exit when cks1 is depleted from mitotic extract or how cks1 deletion from mitotic extract shoot up cdc2 kinase activity. The finding that cks1 does not alter cdc2 kinase activity directly [9, 14] had refocused the research on molecules that interacts with cks1, apart from cdc2, and the quest is still on to respond to the queries.

cks1: linking the unlinked

cdc2 is the sole kinase protein which mediates all stages of cell division in yeast [15] whereas in higher eukaryotes it is primarily engaged in mediating G2/M phase transition and mitotic progression [13, 16]. Under normal conditions, cdc2 is maintained inactive during interphase due to inhibitory phosphorylation at tyrosine 15 and threonine 14 residues by wee1 and myt1 kinases and cdc2 activation occurs after dephosphorylation of these residues by cdc25 phosphatase [17]. Once cells have entered mitosis, progression depends upon MPF (M phase-promoting factor [cdc2/cyclin B complex]) dependent activation of a specific ubiquitin ligase (E3) APC/C. In turn, exit from mitosis requires APC/C mediated ubiquitination and subsequent proteasomal degradation of cyclin B [17, 18]. cdc25 becomes active upon phosphorylation whereas phosphorylation negatively regulates wee1/myt1 kinase activity [17].

Molecular mechanistic studies show lack of Tyr 15 dephosphorylation in cks1-depleted interphase extract and this molecular defect correlates with the functional phenotype (lack of mitotic entry) observed in cks1 depleted cells [7]. This indeed indicates the requirement of cks1 activity for Tyr-15 dephosphorylation and correlates well with the role of cks1 in promoting MPF-dependent phosphorylation of cdc25, wee1 and myt1 [19]. Interestingly, addition of excess recombinant cks1 to interphase extract has also showed similar phenotype associated with lack of cdc25 phosphorylation and Tyr-15 dephosphorylation [7]. This reveals the optimum docking theory for cks1 wherein cks1 at normal levels may serve for the interaction/or acts as a docking factor between MPF and cdc25 or Wee1/Myt1 for eliciting normal cell cycle functions (Fig. 1) and higher concentrations of cks1 may disrupt such an interaction [7]. Even though further validation of this optimum docking theory is missing, it could partially resolve the confusions

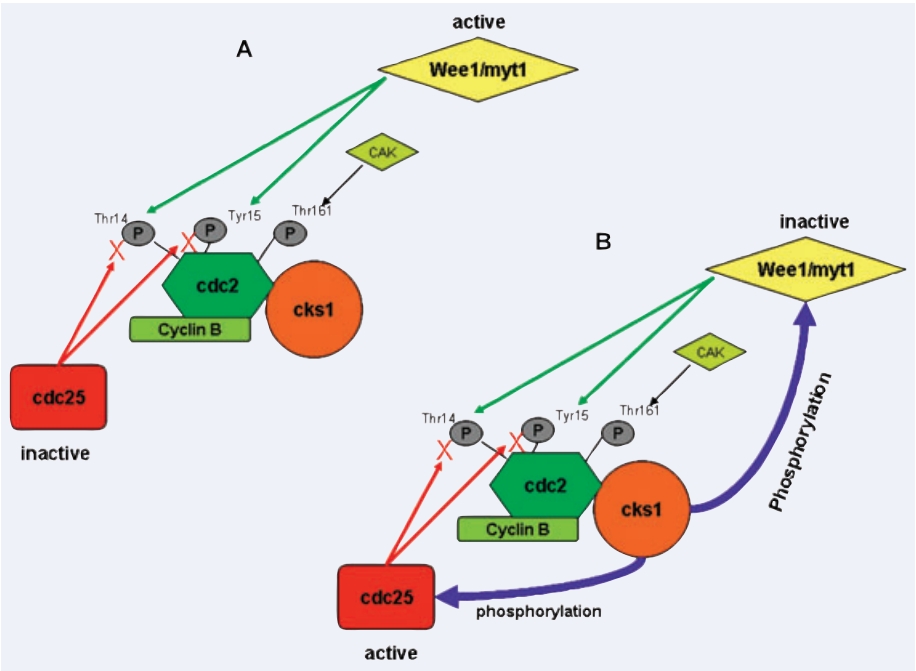

Fig. 1 cks1 in cdc2 activation: (**A**) cdc2 is maintained inactive during interphase due to inhibitory phosphorylations at tyrosine 15 and threonine 14 residues by active wee1 and myt1 kinases. cdc2 becomes active upon dephosphorylation of these residues by cdc25 phosphatase which is maintained inactive during interphase. Activation of cdc2 requires phosphorylation at Thr161 residue also. (**B**) cdc25 becomes active upon phosphorylation whereas phosphorylation negatively regulates wee1/myt1 activation. cks1 promotes MPF-dependent phosphorylation of cdc25, wee1 and myt1 to make them active/inactive (*in addition to this, cks1-dependent transcriptional elevation of cdc2 and cyclin B may also contributes to cks1-dependent cdc2 activation*).

lying around the development of same phenotype under cks1 depleted/overexpressed conditions.

The regulatory role of cks1 on MPF has been initially demonstrated in a heterologous experiment wherein an inhibitory effect of fission yeast cks1 was observed on MPF isolated from cell free-extract of xenopus egg [20]. Initially, it was interesting to observe that cks1 depleted mitotic extracts could accumulate cyclin B which was reversed upon addition of recombinant cks1 [7, 10]. Because cyclin B degradation is essential for proper mitotic exit, the mitotic arrest phenotype observed in cks1 depleted mitotic extract was initially believed to be exclusively due to lack of cyclin B degradation. However, demonstration of cks1 involvement in mediating the degradation of Pds1 [21], an anaphase inhibitor, in budding yeast indicates an additional mechanism involved. Thus, it is evident that in addition to enhance substrate access to MPF, cks1 engages in other activities also in order to elicit its regulatory effect on MPF. Different studies suggest different molecular mechanisms through which cks1 operates the degradation of cyclin B. Studies which found out its docking role in promoting MPF-dependent activation of APC/C (through phosphorylation of its components cdc27 and BIME) [22] and its association with active phosphorylated APC/C complex suggest it is through its interaction with APC/C [23] whereas another group claims that it is through its direct interaction with 19S proteasome components (Fig. 2) as demonstrated from a direct association of cks1 with 19S proteasome components (Rpn3, Rpt1 and Rpt6) [21]. Overall, because MPF-dependent phosphorylation functions of cks1 on wee1, myt1, cdc25, cdc27 and BIME requires less modification of MPF kinase activity, these results suggest cks1 as a master docking agent for the execution of proper cell cycle functions [19, 22, 24].

cks1 acts as a transcriptional regulator

Underscoring its mitotic functions, cks1 has been shown to be involved in the efficient transcription of cdc20 (activator of APC/C). This specific transcription is associated with physical recruitment of proteasome components (Rpt1, Pre1) to the cdc20 promoter region. Though, it remains unclear whether the proteolytic or non-proteolytic functions of proteasomes contribute to cdc20 transcription, the involvement of cks1 in recruiting proteasomes to this specific promoter region is well established. In addition, cks1 is also required for the periodic dissociation of cdc2 from the cdc20 promoter region for eliciting this specific transcription [25]. Therefore it is likely that, in addition to cyclin B degradation, cdc20 transcription is also involved in the basic mechanisms underlying cks1-dependent mitotic regulation. Adding up to its transcriptional roles, cks1–cdc2 complex stimulates GAL1 transcription by recruiting proteasome to the GALI ORF and there are studies (unpublished data) indicating the requirement of cks1 for the efficient transcription of approximately 25% of genes in the yeast genome [26]. Consistent with this, cks1 has been shown to regulate transcription of cdc2, cyclin B and cyclin A in mammalian cells [24, 27].

cks1 turnover

The possible pathological roles of cks1 in tumour progression have made it crucial to address its turnover mechanism. Its mRNA

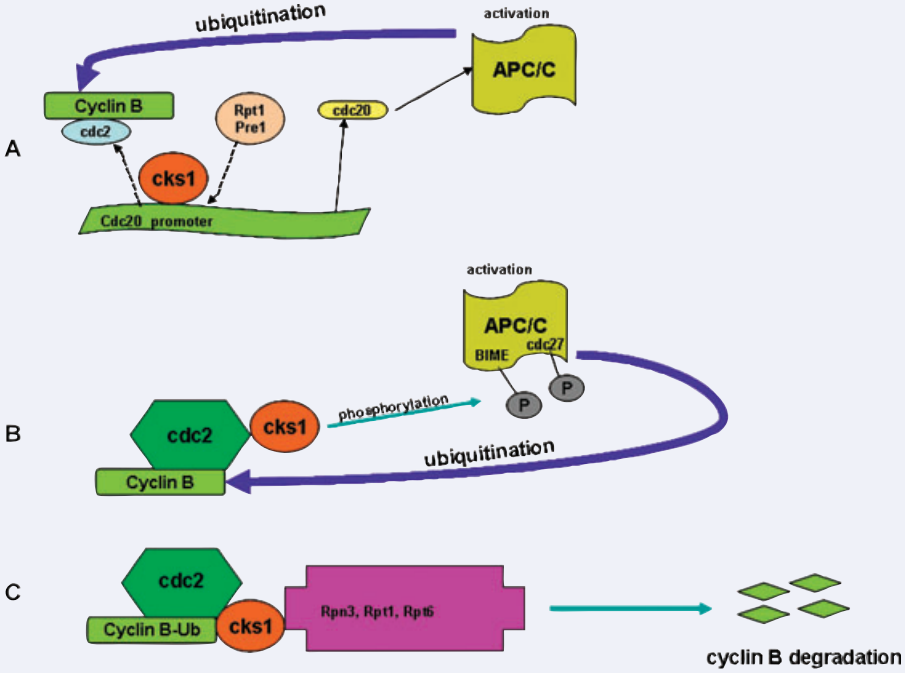

Fig. 2 cks1 in cyclin B degradation: cks1 regulates cyclin B metabolism. (**A**) cks1 elevates cdc20 expression by promoting its transcription. This activity of cks1 involves dissociation of cdc2 from and recruitment 19S and 20S proteasome components (Rpt1 and Pre1, respectively) to cdc20 promoter. cdc20 in turn activate APC/C for cyclin B ubiquitination.(**B**) cks1 stimulates phosphorylation of components of APC/C (cdc27 and BIME) to make it active for cyclin B ubiquitination. (**C**) cks1 directly interacts with 19S proteasome components (Rpn3, Rpt1 and Rpt6) and helps in presenting the ubiquitinated cyclin B to proteasomes.

kinetics reveal two peaks with a small one at G1 to S boundary and a larger one near the end of the cell cycle (G2/M phase) [5, 28]. In comparison, cks1 mRNA levels are low in early G1 cells and increases in G2 to M cells [5, 28, 29]. However, cks1 protein profile shows a considerable decrease at late M and early G1 phases and increases again as cells approach S phase [30]. Thus the protein stability is more observed in cells at G1/S boundary than in cells at late M/early G1 phases [31, 30]. Altogether, cks1 mRNA and protein profile show that the protein expression may be controlled at the transcriptional level during interphase [5, 29–31] and at the same time the variation observed between its mRNA and protein levels at M phase clearly indicates that the protein is regulated at M phase by the protein degradation machinery [30, 31].

Cloning and sequence analysis of cks1 promoter region have shown the presence CDE/CHR tandem element which serves as a transcriptional repressor regulating cks1 gene expression [29]. In addition, deletion and dominant negative studies identified two transcriptional activators, NF-Y and FoX-M1, for cks1gene [29, 32]. Interestingly, c-myc and p53 have been shown to have positive and negative regulatory effect, respectively, over cks1 transcription. However, because cks1 promoter region contains no myc or p53 binding consensus some other transcription factors, the identities of which are unknown yet, are also suggested to be involved in both myc and p53-dependent cks1 regulation [29, 33]. In addition, cks1 transcription is shown to be enhanced by co-stimulation of TCR/CD3-plus-CD28 in T lymphocytes through PI3K- and MEK-dependent pathways [34]. Transforming growth factor-β is another molecule which negatively regulates cks1 transcription [35, 36]. Recently, cyclin D1 and B-RAF-MEK signalling has been implicated in cks1 mRNA regulation [37].

Identification of the ubiquitin ligase involved in the ubiquitination of cks1 has gained considerable attention once it has been established that cks1 is degraded through the proteasome machinery [30, 31]. Initial studies were designed presuming the involvement of skp2, a closely associated E3 ligase, but failed to identify any such association [31]. Follow-up studies by Bashir *et al.* have identified APC/C^{cdh1} as the specific ubiquitin ligase which regulates cks1 degradation independent of cks1 interaction with either cdk2 or skp2 [30]. The observation that down-regulation of APC/C^{cdh1} prevents cks1 degradation and promotes S phase entry again indicates that the effect of proteasome control over cks1 lasts from late M phase (protein instability was more observed at late M phase compared to prometaphase) till G1 phase [30]. However, whether cks1 is a direct substrate or an indirect downstream target of APC/C^{cdh1} needs further validation.

Human cks1

Draetta *et al.* have initially identified human cks1 in complex with cdc2 [4] and after 3 years. Richardson *et al.* isolated two human orthologs of human cks1, cks1 and cks2 [5]. However, there was a minor difference in the molecular weight of these two cloned proteins (9 kD) from the originally identified 13 kD by Draetta *et al.* [4]. Crystallographic studies showed 79 amino acid residues that form a single cks1 domain which is folded into four antiparallel β-strands (β_1–β_4) and two short α-helices. cks1 is primarily monomeric whereas cks2 exists in monomeric, dimeric and

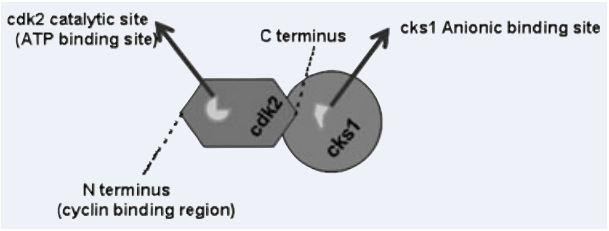

Fig. 3 cks1 as a docking agent for cdk2 and its substrates: cks1 interacts with cdk2 C-lobe and positioned at an opposite side relative to the structurally homologous cdk2 N-lobe to which cyclin binds predominantly. The crystal structure positions cks1 anion binding site on the same side of the cdk2 catalytic site thus forming an extended recognition surface flanking the cdk2 catalytic site. A conserved exposed surface is positioned adjacent to this anion binding site which could probably aid in cks1 binding to a second protein and implicates a structural basis for cks1 to enhance the interaction between cdk2 and its substrates and at least one other phosphoprotein.

hexameric forms. The β-hinge region (residues Glu61 to His65) which forms a β-bend between β_3 and β_4 in cks1 is sequence conserved, but is conformationally different in cks2 where it forms an extended conformation promoting a β-strand exchange that interlocks two subunits into a dimer. The cks1 single domain fold exposes conserved aromatic side chains from β_1, β_2 and β_3 for possible kinase interactions whereas the hexameric cks2 structure sequesters these side chains within an internal channel [38].

While initial co-immunoprecipitation studies revealed cks1 interaction with cdc2, a major breakthrough occurred when cks1 crystal structure resolved in complex with cdk2 [39]. cks1 shows structural homology with N-lobe domain of human cdk2 which extends to the functionally important cdk2 ATP binding site [38]. Earlier, the association of cks1 with cdk2 was observed in xenopus but considered insignificant due to its already highly established mitotic functions [7]. The crystal structure of cks1/cdk2 complex showed the interaction of cks1 with cdk2 C-lobe and positioned it at an opposite side relative to the structurally homologous cdk2 N-lobe and placed 26A far from cdk2 Tyr-15. This binding site is entirely different from that of cyclin located predominantly in the cdk2 N-lobe.

Structural basis for docking functions

The cdk2–cks1 crystal structure positions the cks1 anion binding site on the same side of the cdk2 catalytic site, thus forming an extended recognition surface flanking the cdk2 catalytic site. A conserved exposed surface is positioned adjacent to this anion binding site which could probably aid in cks1 binding to a second protein and implicates a structural basis for cks1 to enhance the interaction between cdk2 and its substrates and at least one other phosphoprotein (Fig. 3). Structural analysis predicted lesser probability of any conformational change for cdk2 upon cks1 binding and therefore the interaction could less likely restrict access of other proteins to cdk2. However, cks1 binding restricts access to cdk activating kinase for the active phosphorylation of cdk2 at Thr160. Consequently, there is a highlighted possibility that cdk2 active phosphorylation at Thr 160 precedes cks1 binding to cdk2 [39].

Strikingly, cdk2 binding regulates the conformational changes (monomer–dimer conversion) in cks1. Higher fluctuations are observed in the back bone nitrogen residues of hinge loop (mediator of cks1 dimerization *in vitro*) in free form of cks1 than in complex form with cdk2. This reduction in flexibility upon complex formation is due to the formation of intermolecular hydrogen bonds between the back bone of His^{60} (cks1) and the side chain of Lys^{237} (cdk2) and between the side chain of His^{60}(cks1) and the back bone of Leu^{174} (cdk2). Thus, in the free form these interactions are missing and the hinge becomes more stable allowing cks1 dimerization *in vitro* [40]. This finding presents molecular evidence for the initial structural studies showing the in efficiency of cks1 dimer to form complex with cdk2 [39]. However, whether cks1 forms dimer *in vivo* has not been substantiated yet. Similarly, Lys^{11}, Arg^{20}, Trp^{54} and Arg^{71} (residues which form the phosphate binding site in cks1) exhibit higher flexibility in the free cks1 form than in complex form with cdk2. Thus, cdk2 binding also facilitates phosphoproteins binding to cks1 by reducing the configurational entropy [40].

Targeting function of skp2–p27 interaction

Independent experiments from two different groups [41, 42] have laid down new foundation for the biological functions of cks1. Spruck *et al.* observed p27 accumulation in $cks1^{-/-}$ mouse cells and follow-up studies demonstrated the essential role of cks1 in promoting skp2 mediated p27 ubiquitination. Skp2 is the F box protein component of the SCF skp2 E3 ligase complex and specifically identifies phospho-p27 (phosphorylated at Thr-187) and promotes its ubiquitination [43, 44]. The association of skp2 and cks1 has been observed long back in transformed human fibroblasts in complex form with cyclin A-cdk2, but the functional significance of such complex was unknown [45]. Further studies had demonstrated a direct interaction of skp2 and cks1 in mammalian cells [46]. Like in $skp2^{-/-}$ murine cells, targeted deletion of cks1 also accumulates phosphorylated p27 in its non-ubiquitinated form [42] and several studies demonstrated that skp2–cks1 interaction is vital for p27 ubiquitination [41, 47].

Initially two models have been put forth to explain this interaction. The allosteric model suggests the induction of a conformational change in skp2 upon cks1 binding which facilitates phospho-p27 access to skp2 [42, 48] and the adaptor model suggests cks1 to act as an adaptor linking skp2 and phospho-p27 and thereby facilitating the interaction [41, 49]. In addition the structural requirement of cyclin E/A-cdk2 complex is also suggested for this biological interaction [50]. Combining all these, a new model has been proposed by Sitry *et al.* in an attempt to identify different binding sites on cks1 which are required for p27-ubiquitination [51]. In this model, which actually is a compiled form of both allosteric and adaptor models, cks1 binding to skp2 creates an

initial substrate binding site for the interaction of C-terminal region of phospho-p27 and at the same time cks1–cdk2 association brings down the p27–cyclin E–cdk2 complex and physically favours such interaction and p27 ubiquitination. The model suggested the requirement of all the three binding sites on cks1 (skp2 binding site, anion binding site and cdk2 binding site) for the efficient p27 ubiquitination by SCF skp2 complex. Follow-up experiments by Ungermannova *et al.* [52] had confirmed the proposed model by demonstrating the requirement of phospho-p27–cyclin E contact for p27 recruitment to SCF^{skp2}. Requirement of this contact was further substantiated and shown that cyclin E binds to N terminus of p27 [53]. Thus, both C and N terminals of p27 are involved in $SCF^{skp2–cks1}$ mediated p27 ubiquitination. It has also been shown that the Thr187 phosphate group in p27 C terminus is recognized by Arg^{306} of skp2 and suggested that this recognition is strengthened by the anion binding site of cks1 [52]. However, the observation that purified skp2, cks1 and phospho-p27 could form complex *in vitro* in the absence of structural association of cyclin E–cdk2 complex [48] indicates that cyclin E–cdk2 complex is required either for strengthening skp2-p27-cks1 interaction or for the orientation of phospho-p27 in the complex for efficient ubiquitin transfer from SCF^{skp2} E3 ligase rather than initiating the formation of skp2–p27–cks1 complex. Overall, the new model suggests direct interaction of cks1 to cdk2–cyclin E–p27 complex and skp2 for efficient ubiquitination of p27 but still lacks clarity in the order of events. Whether cks1 can directly interact with phospho-p27 in absence of skp2 is still controversial wherein one group has already ruled out such possibility [48], the demonstration that ^{35}S-labelled cks1 could bind phospho-p27 in the absence of Skp2 [41] by another group retains the probability of such interaction.

The recently resolved crystal structure of skp1–skp2–cks1 complex bound to a $p27^{Kip1}$ phosphopeptide has given added insights to the model depicting the strong structural requirement of cyclin A/cdk2 complex [54]. Non-catalytic structural requirement of cyclin A/cdk2 complex in p27 turnover has been reported previously [55]. The crystal structure showed that cks1 binds to the leucine-rich repeat domain and C-terminal tail of skp2 whereas the Glu185 side chain in the C terminal binding motif of p27 inserts into the interface between Skp2 and cks1 and interacts with both [54]. Overall, these results reinforce that efficient binding of phospho-p27 to $SCF^{skp2–cks1}$ complex needs the structural requirement of both skp2 and cks1 and confirms the structural requirement of either cyclin E/cdk2 or cyclin A/cdk2 for the efficient ubiquitination of p27 by SCF^{skp2} E3 ligase (Fig. 4).

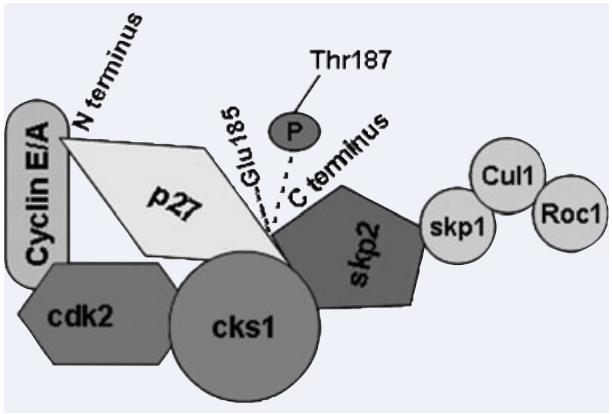

Fig. 4 cks1 in p27 ubiquitination: efficient ubiquitination of p27 needs structural requirement of either cyclin E/cdk2 or cyclin A/cdk2 complex. cks1 binds to the leucine-rich repeat domain and C-terminal tail of skp2. TheGlu185 side chain of p27 C terminus inserts into the interface between skp2 and cks1 interacting with both. In addition, phosphorylated Thr187 side chain of p27 C terminus is recognized by both Arg306 of skp2 and phosphate binding site of cks1. Cyclin E interacts with N terminus of p27.

Other targets of human cks1

Because cks1 has been identified as an adaptor protein linking skp2 with its major substrate p27, various hypotheses linking its involvement in the ubiquitination of other SCF skp2 targets had emerged out. Despite p21 and p130 being identified to be cks1 substrates in mice [56, 57], not many detailed studies have been carried out in this direction in human systems. However, there are still possibilities that these molecules and the other well-known targets of skp2 such as cyclin E [58], p57 [59], B-Myb [60] and hOrc1p [61] can also act as cks1 targets. In addition, the likelihood of cks1 involvement in the degradation of other SCF type E3 ligase (having F box component other that skp2 such as SCF^{Fwd1}) targets such as IκB and β-catenin was also analysed in mice but failed to elicit any association [42]. Recently, cks1 has been shown to be required for cdk2-/cyclin E-dependent phosphorylation of skp2 *in vitro* which has a positive effect on skp2 auto ubiquitination [53]. However, this function of cks1 is limited to certain conditions as cks1 in presence of phosphorylated p27 has a negative effect on skp2 ubiquitination [35, 53]. This deviation might be due to p27 occupying skp2 in presence of cks1 and thereby hindering its ubiquitination. Also, at times of reduced cks1 levels, the inefficiency of p27 to occupy skp2 may prevent its antagonistic effect on skp2 ubiquitination and this may explain why cks1 down-regulation most of the time accompanies skp2 down-regulation [27, 30, 37].

There is strong evidence for cks1 control over cyclin B degradation in yeast and xenopus [7, 10, 21]. Based on this, regulatory effect of cks1 on cyclin B degradation has been studied in human fibroblasts at conditions of a transient block at M phase and hence observed to stimulate the same [28]. However, the exact molecular mechanism by which it stimulates cyclin B degradation in human system has not been identified yet. More recently, cyclin A degradation has been found to be promoted by human cks proteins [62].

Mitotic functions of human cks1

Although p27 ubiquitination has been identified as the key function of mammalian cks1 its possible mitotic functions has never been ruled out in light of the well-established mitotic roles of its homologues in lower eukaryotes. Crystal structure analysis shows that cks1 binds to continuous sequence of residues that forms α helix 5 and loop L14 at the carboxy terminal lobe of the cdk2 which are evolutionarily conserved in a subset of human cdks: cdc2, cdk2 and cdk3 [63]. This indicates its possible association with human cdc2. A reminder at this point is mandatory that cks1 was initially identified in complex with mitotic cdc2 in HeLa cells [4]. In addition, cks1 was found to be present in cdc2-cyclin B immunoprecipitates from human fibroblasts [45].

Determining its importance in mitosis, an interesting study by Hixon *et al.* has demonstrated the development of a polyploidy phenotype in human fibroblasts containing mutated form of p53. p53 mutation in such cell type has up-regulated cks1 expression which in turn enhanced cyclin B degradation. This ultimately has led the cells escape a transient mitotic delay induced by colcemide and resulted in polyploidy. The results were also confirmed in cks1 overexpressed mouse myoblast cells. This indicates the pathological effect of cks1 overexpression during spindle cell cycle check point signals [28]. However, the results are quite contradictory to the finding wherein cks1 knockout cells have been observed to induce polyploidy [24, 42].

The regulatory role of human cks1 on MPF has also been demonstrated indirectly [28]. cks1 kinetics in human fibroblasts shows peak levels at the onset of cyclin B degradation confirming its regulatory role on cyclin B degradation and, in addition, a cell cycle dependent association of cks1 and cdc2 was observed at 40- to 56-hr period following cell cycle entry in fibroblasts. Induction of a transient spindle cell cycle arrest by colcemide resulted in 65% decrease in the amount of such association indicating the requirement of this complex for mitotic progression. Down-regulation of cdc2 kinase activity and induction of G2/M arrest observed in cks1 depleted lung and breast cancer cells further substantiate the possibility of mitotic roles of human cks1 [27, 64]. More recently, cks1 and cks2 double knock out MEF cells have been shown to be arrested at G2 phase accompanied by low transcript levels of cyclin A, B and cdc2 leading to polyploidy [24]. This finding may explain the molecular basis for previously observed polyploidy phenotype in cks1 knock out cells [24, 42].

Human cks1 and cancer

The importance of cks1 in cancer pathology is reflected from its overexpression in tumours that have poor prognosis. cks1 overexpression has been observed in prostate cancer [65] and combined overexpression of cks1 and skp2 has been observed in breast, oral, urothelial, colon, gastric and lung cancers [66–71]. cks1 expression has been observed to be inversely correlated with p27 levels in some cancer biopsies [66, 67, 69, 70] whereas biopsies from other few cancer types showed no such correlation [68, 71]. This indicates that different mechanisms may be involved in cks1 mediated tumour initiation and progression.

Following the identification of cks1 as a growth stimulatory agent, in particular its regulatory role at G1/S transition, researchers have been keen in looking into its involvement in various small molecules'/drugs' pharmacology. Retinoic acid [72], oncostatin M [73] and fluoxetine [74] are some candidates identified that mediate its action via cks1 pathway either directly or indirectly, thus exhibiting their potential as anticancer agents. LY294002 and UO126 are the other potential candidates which inhibit cks1 at the transcriptional level through inhibition of PI3K and MEK signalling, respectively [34]. Further attempts are ongoing to look for new small molecules with potential to disrupt cks1–skp2 interaction.

Future directions

Basic research on cks1 for the last two decades has revealed its critical importance in cell cycle regulation. Findings in yeast and xenopus systems have established multiple functional roles for cks1 with most of them pointing to its specific roles in mitotic entry, progression and exit. In contrast, except for a few studies, most of the studies in human cell lines and tissues establish its specific role in p27 degradation, an essential event associated with G1/S transition. However, its mRNA and protein kinetics as well as knockdown and overexpression studies have clearly suggested its possible mitotic roles in human cells also. Although most of the cks1 overexpressed human tumours negatively correlate with p27 levels there are few cancer types which show no such correlation but still have poor prognosis [68, 71]. These p27-independent pathological roles of cks1 in human tumours might possibly be from its uncontrolled mitotic functions. Development of polyploidy upon cks1 over activity at mitotic stop signals [28] and the polyploidy phenotype observed in cks1 and cks2 double knock out cells strongly supports this hypothesis [24]. However, in spite of identification of its regulatory roles on cyclin A, B and cdc2 turnover [24, 27, 28, 62] and cdc2 kinase activity [64], its implications in cancer progression has not been evaluated in clinical samples.

Another interesting question to be addressed is whether cks1 acts as a tumour initiator or enhances the tumour progression by acting as secondary tumour triggering agent for some primary disease cause. Crystal structure studies proposed the inefficiency of cks1 dimer conformation to form complex with cdk2 [39]. Because p27 ubiquitination requires cdk2–cks1 assembly and also proteasome-dependent cks1 turnover mainly occurs at M phase [30, 31], its possible dimer formation at G1/S transition phase under *in vivo* conditions may serve for its self-regulation in controlling p27 degradation. This indeed indicates that prolonged monomer state of the molecule may stimulate abnormal cell division due to uncontrolled p27 degradation. Therefore, mutational analysis and conformational studies on cks1 in tumour biopsies may provide clarity about its possible primary role in tumour initiation. However, there are multiple reasons to believe its role as a secondary tumour triggering agent. Its link with p53 [28] indicates that it may act downstream of

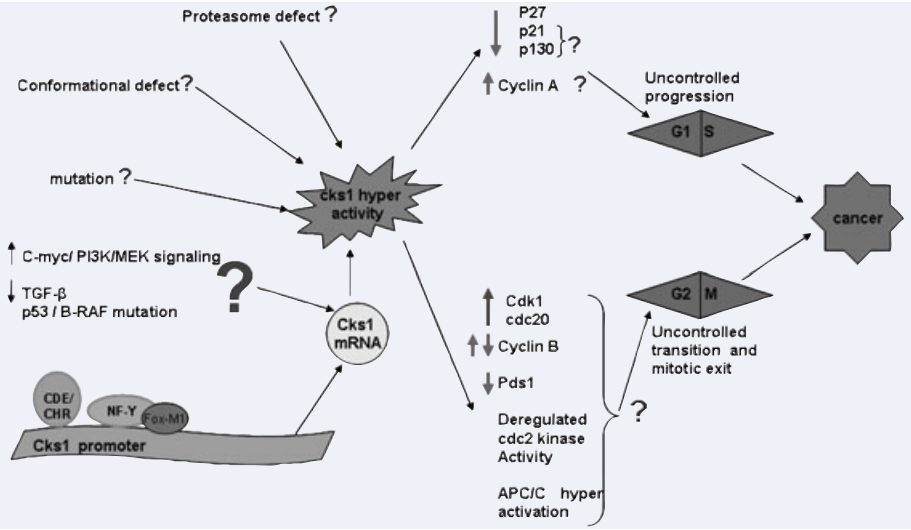

Fig. 5 cks1 in cancer progression: cks1 overexpression in cancer indicates poor prognosis. The figure outlines the possible causes of cks1 overexpression and the functional defects associated with cks1-dependent cancer progression. All the depicted events are well studied (except inherent conformational, mutational and proteasomal defect as a cause of cks1 hyperactivity/ overexpression) and established in mammalian and lower eukaryotic systems. However, this needs validation in human tumours (except p27 down-regulation).

Table 1 Summary of similarities and dissimilarities between cks1 and cks2

Similarities	Dissimilarities
Presence of 815 identical amino acids revealing 81% identity in the amino acid sequence [5]	cks1 is primarily monomeric whereas cks2 exists in monomeric, dimeric and hexameric forms [38]
Phosphate and cdk2 binding surfaces of both cks1 and cks2 are similar and sequence conserved [49]	cks2 does not bind to skp2 whereas cks1 binds [42] *(this may be due to the replacement of Ser41 and Asn45, which participate in the extended hydrogen bond network, with glutamic acid and arginine residues, respectively, in cks2) [54].*
Both of them bind to cdc2 [5]	cks2 knockout cells do not accumulate p27 whereas cks1 knock out cells do [42]
Stable overexpressions of both of them promote polyploidy at conditions of transient spindle cell cycle arrest induced by colcemide [28]	cks2 mRNA increases sevenfold in G2 to M phase cells compared to G1 cells whereas cks1 mRNA increases fourfold [5]
Both of them degrade through proteasome machinery [31]	p53 mutation does not elevate cks2 expression whereas it elevates cks1 expression [28]
Combined deletion of both cks1 and cks2 leads to early embryonic lethality at or before the morula stage indicating some common essential functions for these two orthologues at this stage of development [24]	cks2 is abundantly expressed in mature testes in mice and cks2$^{-/-}$ mice are sterile whereas cks1 is not expressed in mature testis [79]
Both of them are involved in promoting cyclin A degradation [62]	

p53 mutation, but no studies have so far been done to check out this possibility in human tumour samples. In addition, cks1 involvement in fibroblast growth factor receptor kinase [75] and myc signalling warrants an assessment of its implications in cancer progression. Whether cks1 overexpression in tumours is due to abnormalities in the specific ubiquitin ligase for cks1 or defects in the proteasome components need to be assessed. Even if APC/C^{cdh1} is proposed to be the specific ubiquitin ligase responsible for cks1 ubiquitination [30], its inefficiency in promoting cks1 ubiquitination *in vitro* suggests the involvement of additional molecules in this pathway. Therefore, confirmatory evidence of the specific ubiquitin ligase and the accessory molecules involved need to be known in order to analyse whether cks1 overexpression in tumours is secondary to abnormalities in the proteasome machinery.

The molecular biology of cks1 at G1/S and G2/M phases differ and this indicates that cks1 has distinct and specific roles at different time-points of cell division cycle. The efficiency of cks1 to phosphorylate cdc25, wee1 and myt1 and at the same time its inability to phosphorylate the same molecules at times/conditions when it promotes cdc27 and BIME phosphorylation [22] strengthened this hypothesis. Induction of hyper degradation of p27, p21, p130, cyclin B and Pds1 by cks1 when overexpressed is critical in terms of its role in tumour progression as all these functional defects trigger hyper/abnormal cell division from different points

of the cell cycle. In addition, considering the transcriptional roles of cks1 on cdc2, cyclin B, cyclin A and cdc20 it is likely that cks1 overexpressed tumours progress through unregulated expression of these proteins. Therefore, it becomes critical in analysing the levels of all these proteins in cks1 overexpressed tumours to reveal the exact pathological behaviour of cks1 beyond p27 degradation (Fig. 5). The molecular basis of cks1-dependent cyclin B degradation in human system is another area of research interest.

Even though few structural and functional dissimilarities prevail, human orthologues cks1 and cks2 are 81% identical [5]. The observed important similarities and dissimilarities between these two orthologues are presented in a tabular format (Table 1). These findings indicate some critical biological functions for cks2 also in human system independent of cks1 functions. A mutual functioning scenario for a specific biological effect also cannot be ruled out, in particular, at mitotic phase of the cell cycle. Substantiating its mitotic roles, cks2 has been shown to directly interact with the genes and promoters of cyclin B and cdc2 [24] to enhance their transcription. Therefore, future studies with a closer look on to the basic biology of cks2 should also be done in parallel to cks1 in similar systems in order to clearly distinguish the biology of these two orthologues to have better understanding of their regulatory roles in mammalian cell division and cancer pathology.

Does cks1 inhibition alone kill cancer cells? cks1 knockout mice have been shown to be viable with small sized phenotypes [42, 76]. In addition, cks1 null cells retain viability with a slow proliferation rate [42, 77]. Though cks1 disruption up-regulates p27 and disrupts G2/M phase transition, this may not be ultimate for the induction of apoptosis. Interestingly, combined knockdown of both cks1 and cks2 conferred lethality in mouse embryo and induced apoptosis in HeLa cells [24]. More recently, an independent role of cks2 to protect cells from apoptosis has been demonstrated [65]. This indeed indicates that independent targeting of either cks1 or cks2 may be utilized in repairing the disrupted molecular mechanism associated with cell proliferation or apoptosis whereas combined targeting can be adopted to drive cytotoxicity in tumour cells. Interestingly, cks1 has now been presented as a unique model to study the molecular mechanism of poly (Q) aggregation associated with polyglutamine deposition diseases thus extending its biological importance beyond the horizon of cancer [78].

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