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Predicting CK2 beta-dependent substrates using linear patterns

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ABSTRACT

CK2 is a constitutively active Ser/Thr protein kinase deregulated in cancer and other pathologies, responsible for about the 20% of the human phosphoproteome. The holoenzyme is a complex composed of two catalytic (α or α′) and two regulatory (β) subunits, with individual subunits also coexisting in the cell. In the holoenzyme, CK2β is a substrate-dependent modulator of kinase activity. Therefore, a comprehensive characterization of CK2 cellular function should firstly address which substrates are phosphorylated exclusively when CK2β is present (class-III or beta-dependent substrates). However, current experimental constrains limit this classification to a few substrates. Here, we took advantage of motif-based prediction and designed four linear patterns for predicting class-III behavior in sets of experimentally determined CK2 substrates. Integrating high-throughput substrate prediction, functional classification and network analysis, our results suggest that beta-dependent phosphorylation might exert particular regulatory roles in viral infection and biological processes/pathways like apoptosis, DNA repair and RNA metabolism. It also pointed, that human beta-dependent substrates are mainly nuclear, a few of them shuttling between nuclear and cytoplasmic compartments.

1. Introduction

CK2 is a constitutive and ubiquitous Ser/Thr protein kinase that functions as a global regulator of cell survival often found deregulated in cancer and other complex diseases [1–4]. The CK2 consensus sequence, [pS/pT]-{P}-x-[E/D] or [pS/pT]-{P}-x-pS, constitutes a small motif found so far in more than 300 experimentally determined CK2 substrates among different organisms and viral proteins [3]. Such promiscuity connects CK2-dependent phosphorylation with key cellular biological processes and pathways involved in DNA repair [5], apoptosis [6], survival/proliferation [7], and viral infection [8].

The mammalian CK2 holoenzyme is a heterotetramer composed of two catalytic (CK2α and/or CK2α′) and two regulatory subunits (CK2β) with a α2-β2 stoichiometry (α2-β2, α′2-β2 or α1α′1-β2) [9, 10]. The heterotetramer can dissociate under certain conditions with evidences indicating the presence of individual subunits [11]. The free catalytic monomers maintain its activity against a range of substrates while the regulatory subunits might display CK2-independent functions targeting DNA repair, cell cycle and protein kinases as A-Raf, c-Mos and Chk1 [12]. In the holoenzyme, CK2β is a substrate-dependent modulator of kinase activity. In accordance, Pinna grouped the CK2 substrates in three classes based on the subunit composition of active enzyme [4]. Class-I includes CK2 substrates modified either by CK2α or the holoenzyme, poorly influenced by the CK2β subunit [13]. Class-II, substrates such as calmodulin (CALM), phosphorylated by CK2α alone with holoenzyme formation inhibiting kinase activity [14]; this regulatory effect is mitigated with the addition of polycationic molecules (e.g., histones, polylysine and polyamines) [15]. Class-III substrates are beta-dependent and their phosphorylation relies on the integrity of the N terminal acidic loop of this subunit (sequence D^{55}LEPDEELED^{64}). This is true for Rev [16, 17] and eIF2β [18] where electrostatic contacts between basic residues on the substrates and the acidic loop enhance kinase-substrate binding.

The classification of CK2 substrates based on active enzyme subunit composition provides a framework for understanding the regulatory function of CK2β subunit. However, few experiments exist that functionally explore such classification [17–23]; this reflects practical limitations of elucidating kinase specificity. In this regard, high-throughput methods provide an alternative to traditional techniques for phosphosites identification (e.g., ELISA and phospho-specific antibodies). For instance, mass spectrometry-driven approaches figure at the most promising high-throughput techniques with a subsequent increase in the use of *in*

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silico high-throughput methods for data interpretation. Currently, *in silico* methodologies enclose motif-based identification of phosphorylation sites, structural information integration, integration of phosphorylation site structural context, phospho-clusters modeling, integration of Protein–Protein Interaction Network (PPIN) information, and multi-organisms prediction [24, 25]. Here, we propose four linear patterns for identifying class-III CK2 sites. These patterns were constructed manually based on literature and database information describing well known class-III substrates.

2. Materials and methods

2.1. CK2 substrates

The information of class-III CK2 substrates for motif design was obtained by mining PubMed for functional relationships using RLIMS-P [26] and Chilibot [27] text mining tools (Fig. S1). The experimentally determined CK2 substrates were obtained from PhosphoSitePlus resource [28] and the literature. The PhosphoSitePlus information was extracted from the **"Substrates of"** CK2 protein, selecting both **"CK2A1"** and **"CK2A2"** entries (S1 File). Literature information was gathered from the works of Meggio and Pinna, 2003 [3] and Bian et al., 2013 [29] (S1 File) in the form of phosphorylated peptides and UniProt entries, respectively. The phosphorylated peptides reported by Pinna et al., were mapped to UniProt identifiers using BLAST program, set to default values.

2.2. Class-III substrate prediction

The class-III substrates prediction (S2 File) was performed with the ScanProsite tool [30] using the four designed linear patterns and the UniProt identifiers obtained from PhosphoSitePlus resource and the literature (S1 File). The parameters selected for the run were Option 3 (i.e., submit protein sequences and motifs to scan them against each other) and match mode "greedy, overlap, includes" as pattern options.

2.3. CK2 substrates three-dimensional structures

The PDB IDs corresponding to the predicted class-III substrates were retrieved from UniProt (S3 File). The three-dimensional structures of the experimentally determined and predicted class-III substrates were retrieved from PDB database [31] (S3 File) for interactive visualization and analysis using Chimera program [32]. The predicted class-III phosphopeptides were mapped to the PDB sequences in FASTA format using PeptideMatch from PIR [33].

2.4. Sequence alignment

The sequence multiple alignment of Vpu sequences from HIV1 was performed using Clustal Omega program [34].

2.5. Functional classification of predicted class-III substrates

The functional classification of human predicted class-III substrates was performed with DAVID [35], GeneCoDis [36] and NetVenn [37] enrichment analysis tools based on GO biological process, cellular component, cancer genes and/or KEGG pathway annotations. The default parameters were selected and the significance level was set at 0.05 ($P < 0.05$ considered significant). For DAVID analysis, the background list was selected as the CK2 substrates identified in Bian et al. study. The protein function and the sequence related annotations were extracted from UniProtKB [38] and from the literature using Chilibot [27], RLIMS-P [26] and GoPubmed [39] information retrieval and text mining tools.

2.6. Network analysis

Network representation and analysis were performed with NetworkAnalyst tool [40] using the protein list functionality and the predicted class-III substrates from Bian et al. dataset as the input list. The network, 3509 nodes (193 seeds) and 8032 edges obtained from InnateDB interaction dataset was trimmed to keep only the seed nodes (class-III substrates predicted from Bian et al. dataset) and their first neighbors. The obtained network, 607 nodes (193 seeds) and 2801 edges, fitted the recommended size (200–2000 nodes) to avoid dense network and facilitate subsequent analyses. Network nodes were functionally explored based on KEGG pathway annotation and on their degree.

3. Results and discussion

3.1. Linear pattern design for class-III substrate prediction

Pattern-matching is a useful approach for deciphering kinase–substrates relationships at phosphosite level. Here, we designed four linear patterns for matching CK2 class-III sites based on available information of known class-III substrates. First, we used text mining for extracting information on class-III substrates. As a result, we found significant mentions on: Rev protein from HIV1

Table 1
In vitro CK2 class-III substrates retrieved from the literature.

Protein name (UniProt ID)	Class-III sites/basic cluster	Maximum distance[a]
Rev (REV_HV1H3, REV_HV1B1)	**S^{5}GDSDED**LLKAVRLIKFLYQSNPPPNPEGTRQA**RR**N**RRRR**^{44}	29
eIF2β (IF2B_HUMAN)	**S^{2}GDE**MIFDPTMS**KKKKKKKK**^{20}	8
Nrg1 (NRG1_YEAST)	**R^{152}K**Q**R**TDPRNTL**SDEE**^{166}	7
Fdx1[b] (ADX_HUMAN)	**H^{56}**LIFEQ**H**IFE**K**LEAI**TDEE**NDMLDLAYGLTD**RSR**LGCQICLT**K**^{98}	12
CFTR (CFTR_HUMAN)	**R^{1446}**V**K**LFP**HR**NSS**K**CKSKPQIAALKEE**TEEE**^{1474}	13
Olig2 (OLIG2_HUMAN)	**S^{84}STSS**AAASST**KK**D**KK**QMTEPELQQLRLKINS**RE****RKR**^{120}	27
SIX1 (SIX1_HUMAN)	**R^{110}VRRK**FPLPR**TIWD**GEETSYCFKEKSRGVLREWYAHNPYPSP**RE****KR**^{155}	5/28
Pdx1 (PDX1_HUMAN)	**R^{197}**RMKW**KK**EED**KKR**GGGTAVGGGGVAEPEQDCAV**TSGEE**^{234}	30
En2 (HME2_CHICK)	**S^{150}SDSDSS**QAGSNAGNQPMLWPAWVYCTRYSDRPSSGP**RS****RK**P**KKK**^{194}	33

[a] Maximum number of amino acids in the substrate from CK2 consensus sequence ([pS/pT]-{P}-x-[E/D] or [pS/pT]-{P}-x-pS, underlined text) to a mapped basic cluster. For Nrg1, Olig2, SIX1, Pdx1, and En2 substrates the basic clusters were selected considering Rev's basic cluster position as reference.

[b] The position of the phosphosite Thr-71 refers to the mature protein and differs from the position reported in UniProt where the residue corresponds to Thr-131.

virus [16] and eIF2β human translation factor [18], two well-studied CK2 substrates (Table 1). We also detected the yeast transcriptional regulator Nrg1 [41], the bovine ferredoxin Fdx1 [42], the human transporter CFTR [43], the human homeoprotein SIX1 [19], the human transcription factor Olig2 [20], the human pancreas/duodenum homeobox protein 1 [21,22], and the chick homeobox protein En2 [23] as beta-dependent substrates (Table 1).

Second, we found relevant for holoenzyme-mediated phosphorylation the interaction between a cluster of basic residues in Rev and eIF2β substrates (Rev: R^{38}RNRRRR^{44} and eIF2β: K^{14}KKKKKK^{20}) and the acidic loop of CK2β (Table 1). The basic cluster in both substrates is located relatively far from the phosphorylatable residue (Table 1) and the acidic loop has been described as the binding region for polybasic substances [14]. The physical association of these determinants mitigates the intrinsic down-regulatory function adjudicated to the acidic loop, allowing the phosphorylation of the substrates [16]. For example, Rev and CFTR-derived peptides lacking the basic cluster lose class-III behavior [16,17,43]. In addition, polycationic effectors inhibits holoenzyme-dependent Rev phosphorylation indicating a competitive mechanism in which binding of the acidic loop to a basic cluster in Rev is imperative for substrate phosphorylation [16]. Similarly, polylysine has a negative effect on eIF2β phosphorylation. For instance, the addition of polylysine to the eIF2β 1-22 peptide, which contains the basic cluster, prevents peptide binding to the CK2β acidic loop decreasing its affinity for the holoenzyme [18].

CK2 phosphorylates eIF2β protein at Ser-2 residue and Rev protein at Ser-5 and Ser-8. In these substrates, the basic cluster is positioned down-stream the consensus sequence to a maximum distance of 8 and 29 amino acids, respectively (Table 1). Interestingly, we found that the other selected class-III substrates also contain a basic cluster relatively far from the phosphoacceptor site at a maximum distance of 33 residues (Table 1). This basic cluster primarily locates down-stream the modified residue(s). However, for Fdx1, CFTR, SIX1 and Pdx1 proteins a basic cluster was found up-stream the modified residue (Table 1) allowing us to speculate that this determinant might act equally up-stream or down-stream to regulate beta-dependent phosphorylation.

Since little information is available regarding CK2 substrates structure and the phosphoacceptor sites spatial coordinates, we considered only elements from the primary structure. In correspondence, we designed two linear phosphorylation patterns starting with the CK2 consensus sequence (positions 0−+3). Next, we included the minimum and the maximum number of amino acids (x) allowed between CK2 consensus sequence and the acidic cluster, five and 33, respectively. The lower limit is in agreement with the minimum number of residues that in an extended conformation fit the distance between CK2α active site and the acidic loop in CK2β (Fig. 1). In this case we considered Cα-Cα distance as ~3.8 Å [44] and the hydrogen bond distance between catalytic Asp-156 and phosphorylable residue of ~3.0 Å. The upper limit was fixed based on the maximum number of amino acids from consensus sequence to the basic cluster in the validated class-III substrates (Table 1). Finally, we included at least three basic residues to match the basic cluster (Table 1). This element fits the basic cluster of mentioned substrates except for Fdx1 and CFTR proteins. The basic residues of these substrates are spaced irregularly compared with the others from which the former pattern can be deducted (Table 1).

The defined linear phosphorylation patterns are:

1. [ST]-{P}-x-[ED]-x(5,33)-[KR]-x-[KR](2) or [ST]-{P}-x-[ED]-x(5,33)-[KR](2)-x-[KR]
2. [ST]-{P}-x-S-x(5,33)-[KR]-x-[KR](2) or [ST]-{P}-x-S-x(5,33)-[KR](2)-x-[KR]

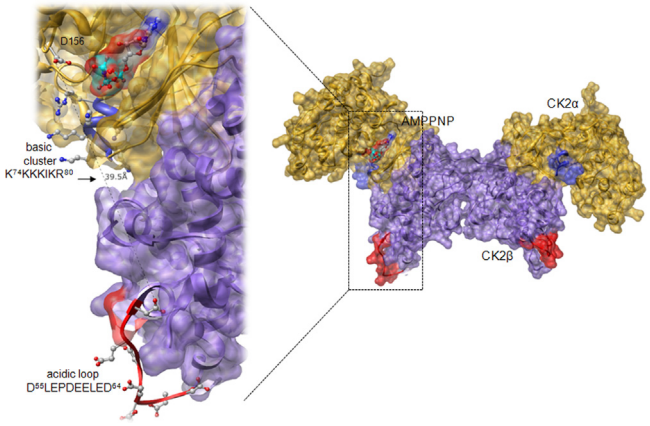

Fig. 1. Surface representation of the three-dimensional structure of the human CK2 holoenzyme (PDB ID: 1JWH). Catalytic (CK2α) and regulatory (CK2β) subunits are colored in yellow and purple, respectively. In one of the catalytic subunits, the active site is occupied by the ATP analog adenylyl imidodiphosphate (AMPPNP). In a close view, residues of two important functional regions, the basic cluster in CK2α and the acid loop in CK2β, are denoted as balls-and-sticks in colors blue and red, respectively. The minimal distance between the catalytic residue Asp-156 and the acidic loop in CK2β (Asp-55 residue) is indicated with a dashed line (39.5 Å).

We also considered pattern variants where the basic cluster is located up-stream the CK2 consensus sequence. The up-stream linear patterns are:

3. [KR]-x-[KR](2)-x(5,33)-[ST]-{P}-x-[ED] or [KR](2)-x-[KR]-x(5,33)-[ST]-{P}-x-[ED]
4. [KR]-x-[KR](2)-x(5,33)-[ST]-{P}-x-S or [KR](2)-x-[KR]-x(5,33)-[ST]-{P}-x-S

3.2. CK2 class-III substrates prediction

A global classification of CK2 substrates based on the subunit composition of active enzyme is an ambitious and demanding experimental task that will further increase our knowledge on this kinase function. As previously reported, the use of *in silico* tools is widely-accepted for kinase motif prediction and mapping of phosphorylation sites [30,45]. Here, we applied phosphorylation-patterns to predict class-III phosphorylation sites from sets of experimentally determined CK2 substrates. As a result, those proteins containing at least one match were considered tentative CK2 class-III substrates (S2 File). Next, we functionally analyzed the obtained results based on GO biological process, cellular component and KEGG pathway annotations.

The class-III pattern-driven prediction was performed on 913 experimentally determined CK2 substrates, 1745 sites, retrieved from PhosphoSitePlus database and the literature. This set includes data on CK2 phosphoacceptor sites for several organisms such as human, mouse and rat (Table 2). The work reported by Meggio and Pinna summarizes the information of phosphoacceptor sites of 33 different organisms and virus [3]. Here, we selected 13 of them to match those represented in the PhosphoSitePlus resource also considering the information available in the literature relative to CK2 phosphorylation.

The prediction shows that 327 substrates, 467 sites, out of the total matched the described phosphorylation patterns (Table 3). Here, the number of substrates analyzed for prediction was 883 instead of 913 given that the ScanProSite tool eliminated 30 proteins from which the corresponding UniProt entries were either merged or deleted (Table 3). The rabbit, chicken, and African clawed frog (*Xenopus laevis*) sets stood out with more than 50% of

Table 2
CK2 *in vitro* substrates analyzed for class-III site prediction.

Source	Organism	No. proteins	No. phosphosites
PhosphoSitePlus	Chicken	4	9
	Cow	17	40
	Human	199	406
	Mouse	48	94
	Rabbit	12	17
	Rat	37	86
	Dog	3	6
Meggio and Pinna[2]	Bovine	9	14
	Fruit fly	7	15
	Epstein-Barr virus	2	4
	Chicken	2	4
	Hepatitis delta virus	1	1
	Human	78	135
	HIV1	3	5
	Mouse	9	12
	Rabbit	7	13
	Rat	11	22
	Baker's yeast	4	7
	SV40	1	3
	African clawed frog	2	3
Bian et al. [29]	Human	575	986

Table 3
Class-III substrates and phosphosites predicted based on the designed linear patterns across several organisms and viruses.

Organism	No. proteins	No. Class III proteins (%)	No. phosphosites	No. class III sites (%)
Human	734	274 (37)	1380	392 (28)
Mouse	50	16 (32)	98	25 (26)
Rat	39	11 (28)	90	16 (18)
Bovine	20	7 (35)	51	10 (20)
Rabbit	14	7 (50)	28	7 (25)
Fruit fly	7	2 (29)	15	3 (20)
Chicken	4	2 (50)	11	4 (36)
Baker's yeast	4	1 (25)	7	1 (14)
HIV-1	3	2 (67)	5	3 (60)
Epstein-Barr virus	2	2 (100)	4	2 (50)
African clawed frog	2	1 (50)	3	1 (33)
Hepatitis delta virus	1	1 (100)	1	1 (100)
SV40	1	1 (100)	3	2 (67)
Dog	2	–	6	–
Eliminated [a]	30	–	45	–
Total	913	327	1745	467

[a] CK2 substrates eliminated from the analysis by ScanProsite tool since the corresponding UniProt entry was either merged or deleted from the database.

the proteins having at least one class-III site; contrastingly, in other organisms such as human, mouse and fruit fly the matches represent around one third of the corresponding proteins (Table 3). Nevertheless, a more systematic annotation of CK2 phosphoacceptor sites that provide comparable data among organisms is needed.

3.3. Structural considerations

The sequence between the phosphosite(s) and the basic cluster forms an extended region in eIF2β and a structural HLH (helix–loop–helix) motif in Rev [16–18] (Fig. 2). Rev basic cluster localizes at a greater distance compared with eIF2β (Table 1) but the existence of a HLH motif might bring closer these determinants due to loop flexibility (Fig. 2) [16,17]. The deletion of the loop in Rev protein reduces holoenzyme phosphorylation efficiency [16]. This illustrates the importance of considering information of protein

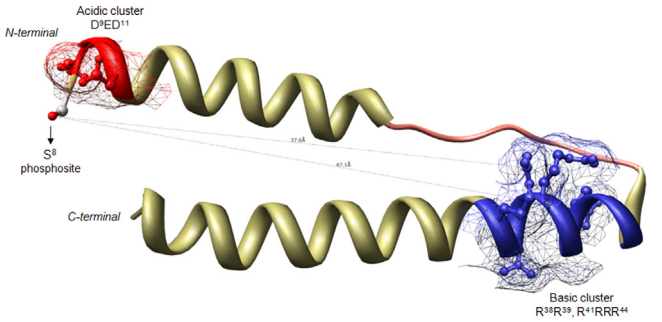

Fig. 2. Ribbon representation of CK2 holoenzyme substrate Rev helix–loop–helix motif (PDB ID: 3LPH). The phosphosite Ser-8 is shown as balls-and-sticks. The acidic residues in the vicinity of Ser-8 and those within the basic cluster are colored in red and blue, respectively. The helixes and the loop of the HLH motif are depicted in yellow and pink, respectively. The distances between Ser-8 phosphosite and the first (Arg-38, 47.1 Å) and last amino acid (Arg-44, 37.6 Å) of the basic cluster in the substrate are represented in dashed lines.

secondary structure when analyzing beta-dependent phosphorylation. However, experimental information is missing on known class-III substrates that allow us to comprehensively describe the vicinity of the phosphosites in a structural basis. For example, among the other experimentally determined class-III substrates only Fdx1 and SIX1 have structural information available; in both cases the sequence between the phosphosite and the basic cluster forms an extended region (Fig. 3).

Based on Rev and eIF2β information, we searched for the occurrence of phosphopeptides located in a HLH motif or in an extended region. First, we retrieved the PDB sequences in FASTA format of the predicted substrates with associated PDB entries

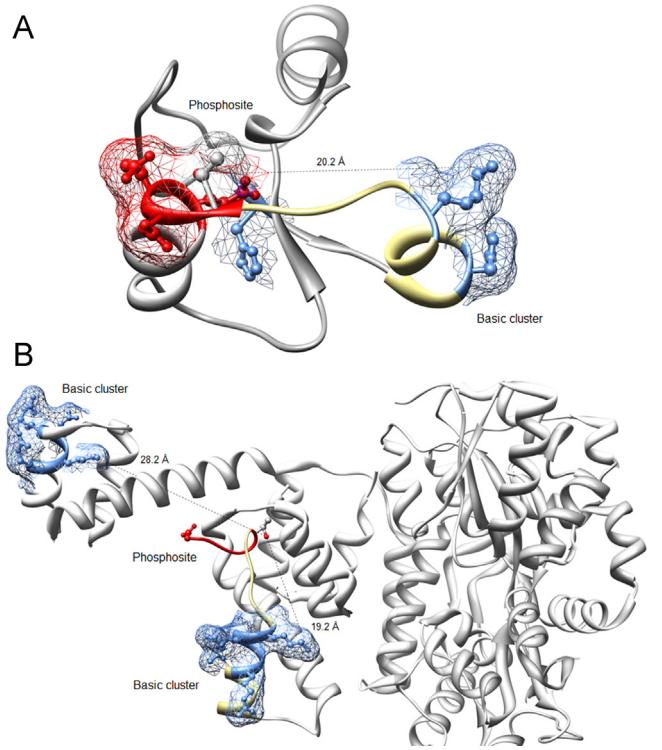

Fig. 3. Ribbon representation of class-III CK2 substrates A) Fdx1 (PDB ID: 3N9Y) and B) SIX1 (PDB ID: 4EGC). The phosphosite and the basic residues within the basic cluster are shown as balls-and-sticks. The acidic residues in the vicinity of the phosphosite and those within the basic cluster are colored in red and blue, respectively. The sequence that contains the loop and the basic cluster is shown in yellow. The distances between the phosphosite and the first amino acid of the basic cluster for each of the substrates are represented in dashed lines.

(146 substrates out of 327) (S3 File). Then, we mapped the predicted phosphopeptides to the sequences identifying 29 substrates (48 phosphopeptides) with at least one hit (in a total of 187 structures) (S3 File). Further sequence visualization and analysis discarded 15 of the substrates since they had important missing residues inside regions that matched the phosphopeptides (S3 File). From the 14 remaining substrates, two matched the HLH motif (S2 Fig.), two the extended region (S2 Fig.) and the remaining ones showed other motifs (S3 File). Correspondingly, this analysis pointed that in the 40% of the studied substrates; the class-III phosphopeptides locate in a region of the protein where the basic cluster may be closer to the phosphorylation site compared with what is expected in primary sequence observation.

3.4. Intrinsic complexity of CK2 substrate classification

CK2 substrate classification is a simplistic view of a complex regulatory process. Here, we provide two relevant examples on calmodulin (CALM) and B23 CK2 substrates that illustrate the complexity of class-III site prediction and that how a cautious interpretation is needed when analyzing the prediction results.

CALM is a class-II substrate [46] for which phosphorylation is enhanced by polybasic peptides such as polylysines and protamines [15,47]. CK2 phosphorylates CALM *in vitro* with the catalytic subunit modifying the Thr-79, Ser-81, Ser-101, and Thr-117 residues [15]. In the present study, these sites were used for class-III prediction and no matches were expected. Conversely, we identified the Ser-101 residue, located in the Ca-binding loop III of the protein [48], as a putative class-III site. To explain this result we searched the literature and found a work by Arrigoni et al. 2004 that explored the CK2-dependent phosphorylation of these sites using large synthetic peptides of the protein [15]. They concluded that CK2 phosphorylation of different CALM peptides lacking the C-terminal domain (essential for conferring class-II substrate behavior and polylysine dependency) occurs in the presence of either the catalytic subunit or the holoenzyme. Noticeably, two of these peptides, residues 93-106 and 54–106, included the Ser-101 phosphosite [15]. In this example, is made evident that the full-length protein dictates the behavior of the different phosphosites and that the classification of the protein based on *in vitro* experiments using peptides or *in silico* prediction could be misleading.

Other example that showcases classification complexity is the CK2-mediated phosphorylation of B23. This protein has been previously regarded as a class-I CK2 substrate [13]. However, in a recent work by Zanin et al. the authors tested the effect of the beta-dependent phosphorylation inhibitor CK2-MCP peptide on B23. Conversely, they observed a certain inhibitory effect indicating a possible dependence on the regulatory subunit. In agreement, our analysis predicted the Ser-125 residue of B23 as a putative class-III site (Table 2). Although this finding supports the use of the designed patterns for capturing beta-dependent regulatory effect further experiments are mandatory to comprehensively describe B23 substrate behavior *in vivo*.

3.5. Class-III substrates functional classification

3.5.1. Role of CK2 holoenzyme phosphorylation in viral infection

The relevance of CK2 in viral infection is accepted with the enzyme interacting and/or phosphorylating both viral and infectivity-related host proteins. CK2β subunit binds EBNA1 [49] and IE63 [50] viral proteins while CK2α associates to NSI [51], pUL84 [52] and RIG-I (cellular viral infection defense) [53]. An example of functional association between this kinase and viral proteins is the induction of parvovirus capsid phosphorylation by NS1/CK2α complex [51].

The Table 4 summarizes the identified class-III proteins/sites relative to the four viruses selected for prediction and experimental information of the kinase assay. It includes the Ser-5 and Ser-8 phosphoacceptorsites of HIV-1 Rev protein used in pattern design. Literature search shows that the identification of CK2 phosphoacceptor sites among these proteins was carried out using the holoenzyme and that no analysis of subunit contribution was performed as it is the case for Rev protein (Table 4, S4 File). Thus, in the absence of complementary information, pattern-matching becomes a tool of choice guiding the detection of potential class-III sites. For example, in our analysis Vpu, BZLF1, LT-AG, Nef, LT-Ag and EBNA2 matched the designed patterns and were classified as beta-dependent substrates. However, for Nef, LT-Ag and EBNA2 substrates, in particular, literature analysis does suggest that the relevance of the basic cluster in the phosphorylation reaction might be only evident in the full-length protein since derived peptides lacking the basic determinant are phosphorylated by the holoenzyme. This probably resembles the class-I behavior observed in Ser-5 and Ser-8 phosphoacceptor sites in the context of the Rev peptide [16].

Overall, our results for Vpu and BZLF1 proteins might serve to speculate a functional relationship between these viral proteins and CK2 beta-dependent phosphorylation. Hence, based on this assumption, we searched the literature and found interesting evidences connecting Vpu with CK2 beta-dependent phosphorylation. For BZLF1 protein, literature information was insufficient for a further analysis (S4 File).

Vpu is a HIV1 viral protein involved in CD4 proteasomal degradation and in the inhibition of protein transport from ER-Golgi complex to the plasma membrane; functions regulated by CK2-dependent phosphorylation of the Ser-52 and Ser-56 residues [62]. These phosphosites were characterized *in vitro* using CK2 holoenzyme and Vpu recombinant protein and synthetic peptides (Table 4) [55]. The studied peptides constitute variants of the hydrophilic C-terminal domain (residues 32–81) which besides the

Table 4
CK2 phosphorylated viral proteins and their tentative class-III sites.

Virus	Protein Name	CK2 PhosphoSitePlus (***Class-III prediction***)	Kinase assay substrate type	CK2 kinase assay	References
HIV1	Rev	Ser-5, Ser-8	protein, Rev 1-26 peptide	Recombinant α and β subunits of human CK2.	[16,17]
	Vpu	Ser-52, Ser-56	protein, Vpu 32-81 peptide	Recombinant human CK2.	[36,37]
HIV2	Nef	Ser-92	Nef87-96 (MDDVDSDDDD) peptide	Purified CK2 (bovine liver, lung, or brain)	[56]
Epstein-barr virus	EBNA2	Ser-469, Ser-470	protein, EBNA2 462-474 (FETTESPSSDEDY) peptide	Recombinant CK2.	[57]
	BZLF1	Ser-167, Ser-173	protein	Recombinant CK2.	[58–60]
SV40	LT-AG	Ser-106, Ser-111, Ser-112	LT-AG 106-119 (SEEMPSSDDEATAD) peptide	Purified CK2 (bovine liver, lung, or brain)	[56]
Hepatitis delta virus	HDAg	Ser-2	protein	No kinase assay. In vivo labeling and kinase inhibitor experiments	[61]

CK2 consensus sequence contains a basic cluster ($R^{34}QRK^{37}$) at an appropriate distance up-stream the phosphosites [54]. In correspondence both sites were predicted as class-III.

The E^{47}RAEDSGNESEG^{58} dodecapeptide is considered the major determinant of Vpu CK2-mediated phosphorylation [63]. This is a highly conserved sequence found in Vpu protein of different HIV1 strains [63]. To investigate the significance of the basic cluster we ran a BLAST search against UniProt database and identified 10 Vpu proteins (reviewed status) sharing 100% of sequence identity to the dodecapeptide (Fig. S3). The alignment of these 10 sequences shows that the basic cluster is also well-conserved (Fig. S3). This finding suggests that the acidic cluster might be a relevant regulatory element of CK2-mediated phosphorylation in Vpu. The validation of this viral protein as a class-III substrate might provide a strategy in which targeting of holoenzyme integrity will impair protein function in HIV viral infection.

3.5.2. *Functional classification of human class-III substrates*

Bian et al. performed a global screening of human CK2 substrates where they identified 575 substrates of the kinase (988 sites) (Table 2, S1 File), a list enriched in spliceosomal proteins. This experiment combined *in vitro* CK2 holoenzyme reaction and immobilized proteome along with phosphoproteomics. The results were refined by filtering the data based on *in vivo* phosphosites information extracted from phosphorylation databases [29]. Importantly, the authors used CK2 holoenzyme for kinase reaction most likely marginalizing the phosphorylation of class-II sites [29].

Class-III prediction of Bian et al. substrates pointed out that 219 out of the 575 proteins might correspond to this category (Table 2). The functional classification of these putative class-III substrates might serve to speculate, considering the predictive nature of this approach, which biological processes and pathways linked to CK2 signaling might be targeted by class-III phosphorylation inhibitors. As a result, the functional analysis relative to the background of identified CK2 substrates (S5 File) indicated a significant enrichment of RNA processing biological process (P value$=0.003$) and of nuclear lumen subcellular localization (P value$=0.01$) with proteins from both the nucleolus (P value$=0.02$) and the nucleoplasm part (P value$=0.03$). This suggests that the holoenzyme might exert a key role regulating the function of nuclear located CK2 substrates that function on RNA processing events like RNA splicing and mRNA processing. Likewise, selecting the human genome as the background list (S5 File) indicated a marked enrichment of processes related to RNA processing (P value$=6.31e-10$) and of nuclear localization (P value$=8.48e-63$) (Fig. 4). Other enriched biological processes and cellular components are regulation of transcription (P value$=3.73e-4$), cell cycle (P value$=2.24e-2$), apoptosis (P value$=0.007$), cytoplasm (P value$=2.22e-17$), and cytoskeleton (P value$=0.003$) (Fig. 4).

A previous study by Filhol et al. analyzed the intracellular dynamic of CK2 subunits distribution using live-cell imaging [11]. The authors found that the holoenzyme is targeted to the cytoplasm and that binding to certain proteins such as FGF-2 enhances its nuclear accumulation. In addition CK2 independent subunits are imported to nucleus where they may associate to form the holoenzyme or interact with nuclear proteins and mediate independent functions [11]. The subcellular location analysis (S5 File) shows an enrichmentof nuclear (137 proteins) and cytoplasmic (79 proteins) class-III predicted substrates with an overlap of 62 proteins that localize to both compartments (Fig. 4). In correspondence, we also studied the subcellular location of human class-III substrates predicted from PhosphoSitePlus dataset. Similarly, the substrate list was enriched in the nuclear (42 proteins) and cytoplasm (33 proteins) locations with an overlap of 26 proteins. Thus, the results suggest that beta-dependent phosphorylation could modulate primarily the function of nuclear-located

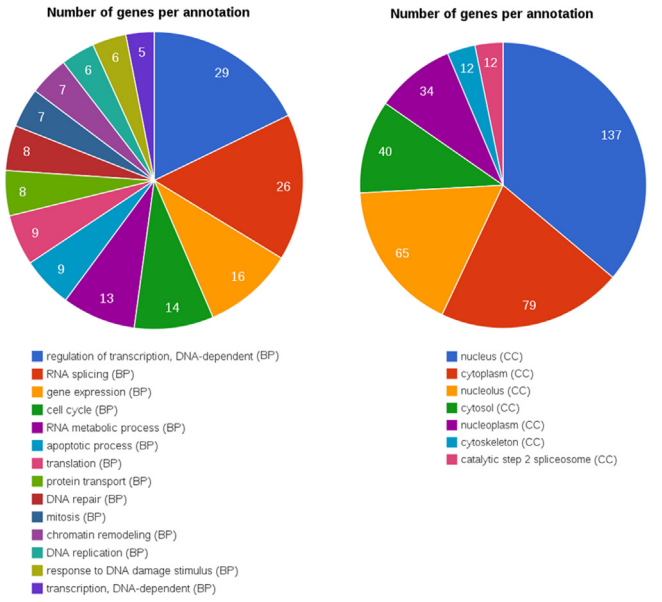

Fig. 4. Biological processes and cellular components enriched in CK2 class-III substrates predicted from Bian et al. dataset. All annotations are significantly enriched (P value < 0.05).

CK2 substrates. In addition, protein overlap points to a possible link between cytoplasmic class-III substrates phosphorylation and their redistribution to the nucleus.

The involvement of CK2 in DNA repair and its regulation has been well-documented with the identification for instance of BRCA1, APC and P53 as binding partners and/or substrates of the enzyme [64–66]. The biological process enrichment analysis (S5 File) supports a possible functional relationship between beta-dependent phosphorylation and the response to DNA damage stimulus and DNA repair. For example, the protein MDC1 (Mediator of DNA damage checkpoint protein 1), here predicted as a class-III substrate, is a scaffold for the recruitment of DNA repair and signal transduction proteins to damaged sites required for cell cycle arrest. Its phosphorylation by CK2 promotes the assembly and retention of the MRN complex to DSBs for the initiation of HR repair [67].

RNA interference and down-regulation of CK2 abundance level result in potent induction of apoptosis [68]. Here a total of 9 proteins associated to apoptosis were predicted as class-III substrates (DIDO1, CDK11A, PDCD4, CLSPN, FXR1, ZC3H8, DPF2, DDX41, and PSMD2) (S5 File) Likewise, some of the predicted class-III substrates were classified as cancer genes based on Futreal et al. 2004 (DEK, NUP98, PSIP1, MYH9, and HSP90AB1) [69] and Gene database (CDC5L, DEK, MTDH, CLSPN, SLC4A1AP, RSF1, and PDCD4) information (S5 File). For example, DEK is an oncogene with chromosomal aberration in AML and the encoded protein is involved in chromatin organization and in splice site selection during mRNA processing. CK2-mediated phosphorylation of the C-terminal domain modifies DNA binding properties of DEK [70].

Furthermore, the mapping of predicted class-III substrates in the human protein–protein interaction network (S5 File) situates them as member of functional modules that correspond to intracellular signaling pathways such as: cell cycle, RNA transport, Wnt signaling pathway, and P53 signaling pathway (Fig. 5). We also mapped the predicted substrates to modules associated to pathways in cancer including prostate, pancreatic and lung cancer and chronic myeloid leukemia. Finally, the network analysis also pointed to the possible modulation of hub proteins including HDAC1, NCL and ESR1 and of proteins essential for connectivity

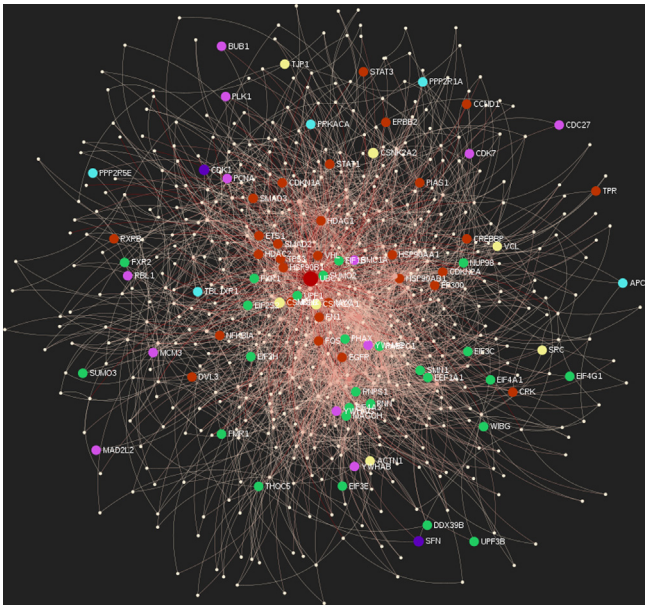

Fig. 5. Protein–protein interaction network depicting the functional heterogeneity of class-III substrates predicted from Bian et. al dataset (193 seed nodes) and of their first neighbors (414 nodes). Enriched pathways: cell cycle (pink, *P* value = 1.64e − 11), RNA transport (green, *P* value = 2.41e − 11), Pathways in cancer (red, *P* value = 9.98e − 05), Adherens junction (yellow, P value = 2.39e − 04), Wnt signaling pathway (blue, *P* value = 0.005), and P53 signaling pathway (violet, *P* value = 0.03). Node size is proportional to node degree.

between modules like ATRX, CPSF2 and CHD1 (S5 File).

4. Conclusions

The four designed linear patterns aimed to assist CK2 beta-dependent substrates prediction. The discrimination power of these patterns relies on the recognition of a basic cluster at a suitable distance from the phosphoacceptor site in the substrate. Since the patterns utilized the CK2 consensus sequence for matching substrates we performed the prediction of class-III substrates on experimentally determined CK2 substrates to reduce noise. As a result we obtained a list of 327 predicted class-III substrates, 467 sites. The functional classification of these substrates indicated a role of beta-dependent regulation in viral infection and biological processes and pathways such as apoptosis, DNA repair and RNA metabolism. It also suggested that the human substrates are primarily nuclear located with a number of them also found in cytoplasm. A cautious interpretation of these results is needed since this analysis derives from an *in silico* predictive approach. Thus, future experiments are required to validate the results of the prediction and the functional analysis.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at 10.1016/j.bbrep.2015.08.011

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