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Review

Roles of CDK/Cyclin complexes in transcription and pre-mRNA splicing: Cyclins L and CDK11 at the cross-roads of cell cycle and regulation of gene expression

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Abstract

Cyclin Dependent Kinases (CDKs) represent a large family of serine/threonine protein kinases that become active upon binding to a Cyclin regulatory partner. CDK/cyclin complexes recently identified, as well as “canonical” CDK/Cyclin complexes regulating cell cycle, are implicated in the regulation of gene expression via the phosphorylation of key components of the transcription and pre-mRNA processing machineries. In this review, we summarize the role of CDK/cyclin-dependent phosphorylation in the regulation of transcription and RNA splicing and highlight recent findings that indicate the involvement of CDK11/cyclin L complexes at the cross-roads of cell cycle, transcription and RNA splicing. Finally, we discuss the potential of CDK11 and Cyclins L as therapeutic targets in cancer.

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1. Cyclin and Cyclin Dependent Kinase families – a brief introduction.

The Cyclin Dependent Kinases (CDKs) represent a large family of serine/threonine protein kinases that become active upon binding to a Cyclin regulatory partner that controls the kinase activity and substrate specificity. Since their discovery in the 1980's as key regulators of cell cycle progression and due to sequencing of the human genome, this family of proteins has grown to include more than 20 CDKs and CDK-Like Kinases (CLKs) [1]. Based on the presence of PSTAIRE-like cyclin-binding elements and high similarities with the original canonical CDKs, Malumbres and co-authors proposed CDK nomenclature [1] from CDK1 to CDK20, and CDK-like kinase nomenclature from CDKL1 to CDKL5. The reader is referred to reviews that cover the timeline and milestones describing the functional characterization of CDKs and CDK-like protein kinases [1-4].

Similarly to CDKs, the diversity of Cyclin family members has increased during evolution [5], and the mammalian and human genome encodes more than 30 Cyclins and Cyclin-like proteins [6]. Cyclins were initially classified into sub-families represented by capital letters (A to L, O, P, Q, T and Y) as well as numbers for orthologs within a family. However, in the past few years several proteins showing structural similarities with Cyclins have been identified introducing a greater diversity and, sometimes, a confusing nomenclature with attempts to harmonize the classification of this extended family [6]. In addition, some of the Cyclin genes are subject to alternative splicing producing several isoforms, such as Cyclins L1 and L2 [7]. An extensive analysis of the literature and RNA and protein databases remains to be conducted to establish an exhaustive list of all Cyclin isoforms expressed in human cells.

1.1 Breaking down the cyclin family – similarities and differences

The Cyclin proteins show a remarkable diversity of structure, regulation, and function. The first discovered members of this family were named mitotic or cell cycle Cyclins because their expression levels markedly fluctuate during the cell cycle. This group of canonical Cyclins includes the sub-families A, B, D and E [8], which bind to specific CDKs to regulate the cell cycle [2, 3]. A second group of Cyclins, referred to as transcriptional or non-canonical Cyclins (Cyclins C, H, K, L, Q and T), bind to CDKs to form complexes involved in the regulation of transcription and pre-RNA processing in addition to other processes [3, 6, 9]. Then, a third group includes the more recently identified atypical Cyclins G, I, J, O, P and Y and other proteins not named after the conventional Cyclin nomenclature, such as CABLES 1 and 2, for which the CDK partners are less documented [6]. Both canonical and non-canonical Cyclins share low sequence homology, but are structurally defined by the presence of one or two relatively well conserved Cyclin Box Domains (CBD) of about 100 amino acids [10], which is formed of closely packed helical modules [4].

Several proteins sharing low identity with Cyclins also bear a CBD, such as retinoblastoma pocket protein (Rb) and general transcription factor TFIIB [10, 11]. While these proteins do not serve as CDK activators, the similarity between Cyclins, TFIIB and Rb encompassing the CBD suggest common ancestry [10].

Based on similarities in the CBD sequences, empirical experimental evidence, and crystal structures, authors have defined motifs and amino acids that are critical for Cyclin binding to CDKs [10]. Quandt et al. identified the conservation of a Lysine-Glutamate pair (KxEE), located at residues K257 and E259/260 in Cyclin B1, combined with a second highly conserved ExxxLxxL sequence (E286/L290/L293 in Cyclin B1) found in nearly all Cyclins [6]. They concluded that Cyclin F may not be a “real” Cyclin since it does not have the highly conserved Lysine-Glutamine pair and the surrounding conserved residues required for CDK interaction. We performed a similar alignment of human Cyclin box family members to highlight the conserved amino acids contained in Cyclins L1 and L2. Our alignment confirmed the presence of the highly conserved FLA(S/A)KxEE and ExxxLxxL motifs (**Figure 1**). Cyclins L1 and L2 contain the ExxxLxxL motif at positions E185/180, L189/184, L192/187, respectively; however, they lack the Phenylalanine present in most Cyclins (F253 in Cyclin B1), which is replaced by Asparagine-N134 and Histidine-H129. The extended alignment of the amino acid sequences on both amino- and carboxyl-terminal ends of these two “Cyclin defining motifs” also indicated that Arginine-R113 and Phenylalanine-F114 in Cyclin L1 (R232/F233 in Cyclin B1), initially reported in canonical Cyclins [10], as well as Tyrosine-Y208 and Glutamine-Q210 are highly conserved in most Cyclin CBDs. The CBD of Cyclin L1 and L2 share >90% similarity but show much lower conservation with other Cyclins, with Cyclins Q, T and Y the most highly related at ~35% similarity over the CBD. Our alignment also showed low similarity between Cyclin F and other Cyclins (**Figure 1**) without conservation of the KxEE and ExxxLxxL motifs.

1.2 Roles of various CDK/Cyclin complexes in transcription and pre-mRNA splicing

Numerous data demonstrate that accurate transition from G1 phase to DNA replication is controlled by a specific transcriptional program regulated by canonical CDK/Cyclin complexes. It is also now established that both canonical and non-canonical CDK/cyclin complexes show functional evolution as they are implicated in the control of diverse processes including differentiation, metabolism regulation, DNA damage repair and caspase-dependent apoptosis [3, 12]. However, in this section we focus on the well-studied roles of the transcriptional CDK/Cyclin complexes in regulation of gene expression via phosphorylation of the transcriptional [3, 11, 13-15] and pre-mRNA processing machineries [15, 16]. We will briefly introduce some of the key protein complexes involved in mRNA production in order to emphasize the crucial roles of CDK/Cyclin complexes in the regulation of gene expression.

The general transcription machinery performs complex multi-step processes responsible for the production of pre-mRNAs [17] that include pre-initiation, initiation, promoter clearance, elongation and termination (**Figure 2**). Several other catalytic activities, which include capping, splicing, polyadenylation and cleavage, occur co-transcriptionally to ensure maturation of pre-mRNAs into mRNAs [17-19]. All of these processes are controlled by a large group of proteins which form very dynamic complexes interacting with DNA and pre-mRNAs to coordinate these activities [20]. Eukaryotic transcription starts with the sequence-specific binding of transcription factors (TFs) to enhancer DNA elements, promoting recruitment of the three multimeric subunits of the transcription machinery: the Mediator complex [13], the general transcription factors (GTFs), and RNA Polymerase II (RNAPII) [13, 19]. While TFs drive the transcription of specific sets of genes, they do not interact directly with RNAPII but with the Mediator complex (**Figure 2**), a large complex of at least 30 subunits organized into four modules: head, middle, tail and kinase [13]. The tail module binds to TFs while head and middle modules interact with RNAPII and GTFs including TFIIB, -E, -F, -H and -S [11] at the core promoters to stabilize the pre-initiation complex (**Figure 2**). The kinase module contains several protein kinases and is reversibly associated to the Mediator to regulate recruitment of subunits and transcription activity [13]. RNAPII transcribes protein-coding genes by catalyzing the synthesis of pre-mRNA, which requires the GTFs as auxiliary factors for accurate initiation at the core promoter. In addition to these co-factors, the large subunit Rpb1 of RNAPII contains multiple hepta-peptide repeats with the consensus amino acid sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (YSPTSPS), which has been highly conserved throughout evolution, and has fifty-two repeats in mammals [21, 22]. The CTD is a key regulator of pre-mRNA synthesis and coordinates different activities involved in mRNA maturation, including alternative splicing [17, 21], polyadenylation [18] and RNA quality control [19] by acting as a platform or docking site. Pre-mRNA splicing takes place in a supramolecular ribonucleoprotein (RNP) complex called the spliceosome [23, 24] that contains two main types of splicing factors: the small nuclear ribonucleoproteins (snRNPs) and non-snRNPs including the superfamily of arginine/serine di-peptide rich proteins called SR proteins [23, 24]. Splicing of pre-mRNA occurs through four steps (assembly, activation, catalysis and disassembly) characterized by sequential recruitment of subunits and structural rearrangements of this multi-megadalton machinery that catalyzes the intron excision and exon ligation during both constitutive and alternative splicing [23, 24].

A wealth of evidence has accumulated during the last 20 years establishing that more than 30 protein kinases are capable of phosphorylating components of the transcription and pre-mRNA machineries. One of the best examples is the CTD of RNAPII for which changes in phosphorylation level not only affect the polymerase activity but also coordinate the recruitment of various co-regulators of

transcription and RNA processing [3, 11, 13-15, 22, 25-27]. Similarly, SR proteins, snRNP components, splicing regulators and co-activators, many containing RS domains with multiple putative sites, undergo changes in their phosphorylation status with direct effect on their protein-protein interactions, subcellular localization and activity [15, 16, 28]. Protein kinases involved in these phospho-regulatory modifications include several CDK/cyclin complexes: CDK7/Cyclin H, CDK8/Cyclin C, CDK9/Cyclin T, CDK11^{p110}/Cyclins L, CDK12/Cyclin K, and CDK13/Cyclin K.

CDK7 is the catalytic subunit of the trimeric CDK7/Cyclin H/MAT1 complex, originally purified from cell extracts based on its ability to phosphorylate and activate CDKs from which its name, CDK Activating Kinase (CAK) was derived [29]. The CAK complex consists of equal stoichiometric association between CDK7, Cyclin H and the RING finger protein MAT1 [29]. The CDK7/Cyclin H/MAT1 complex was also found to be a component of the general transcription factor IIH (TFIIH) [11]. TFIIH plays a crucial role during the first steps of RNAPII-dependent transcription as well as nucleotide excision repair [11, 30]. When CAK is part of TFIIH, its substrate specificity is increased towards the CTD [11, 30]. Following assembly of the pre-initiation complex, RNAPII is released from the core promoter in a process known as promoter escape [13]. This mechanism involves TFIIH-dependent phosphorylation of the CTD on S2 and S5 positions via CDK7 activity (**Figure 2**) together with other CTD kinases such as CDK9/Cyclin T, CDK12/Cyclin K, ERK1/2 and DYRK1A. These CTD phosphorylation events release interaction of RNAPII with the Mediator complex to facilitate progression from initiation into elongation during transcription and trigger the recruitment of factors involved in co-transcriptional events [21], creating a CDK7-dependent "CTD code" regulating chromatin marks [31]. CDK7 also participates in transcription initiation and RNAPII pausing via activation of the CDK9/Cyclin T complex, and phosphorylates other transcription regulators such as TFIIE [32] and the p53 tumor suppressor [33].

Cyclin C is the sole partner of CDK8, as CDK8 contains a "specific" Cyclin C α -helix recognition domain limiting binding to other Cyclins [34]. Neither CDK8 and Cyclin C protein levels nor kinase activity vary significantly during cell cycle [34]. The CDK8/Cyclin C complex and accessory proteins MED12 and MED13 form the CDK8-dependent kinase module (CKM) of the Mediator complex [13, 34, 35]. The CKM also contains a paralog of CDK8, called CDK19, as well as paralogs of MED12 and MED13, named MED12L and MED13L [13]. CDK8 and CDK19 regulate transcription through different mechanisms, one of which is phosphorylation of RNAPII CTD (**Figure 2**). Through phospho-regulation of RNAPII CTD, CDK8 and CDK19 function in several specific transcriptional programs, including NF κ B and the glycolytic cascade, and are proposed as potential oncogenes [36, 37]. In addition, the CDK8/Cyclin C complex also phosphorylates S5

and S304 within the cyclin H subunit of TFIIH, negatively regulating TFIIH kinase activity toward the CTD and transcription [11]. It is of interest to note that CDK8 lacks the conserved CAK phosphorylation Threonine residue in its T-loop, indicating that CDK8 activation is independent of CAK activity.

Two CDK9 isoforms, short 42 kDa CDK9-S and long 55 kDa CDK9-L proteins, are encoded by the same gene resulting from transcription using two distinct promoters [14]. While CDK9-L is involved in apoptosis and DNA repair, CDK9-S is the kinase subunit of positive-transcription elongation factor b (P-TEFb) as well as the HIV Tat-associated kinase (TAK), thus providing the first indications of CDK9-S involvement in transcriptional regulation [38]. Several Cyclin partner proteins are capable of activating CDK9 kinase activity, including Cyclins T1, T2a, T2b, and K [39]. The biological relevance of CDK9/Cyclin K remains unclear, while it is well established that Cyclins T are the predominant partner proteins for CDK9 to form P-TEFb [14, 35]. Cyclin T binding not only regulates CDK9 kinase activity but is required for CDK9 binding to its substrates at active sites of transcription [40]. CDK9 is required for the global regulation of gene transcription in both basal and stimulated conditions as indicated by its wide distribution at sites of active transcription [14, 40]. Transcription by RNAP II is stalled shortly after initiation of transcription, and this pause is facilitated by the negative transcription elongation factors NELF (Negative Elongation Factor) and DSIF (DRB-Inducing Sensitivity Factor) [13, 14, 35]. CDK9 T-loop phosphorylation by CDK7/TFIIH activates P-TEFb-mediated kinase activity required to allow the RNAP II complex to escape from transcriptional arrest and enter productive elongation (**Figure 2**). CDK9 phosphorylates the CTD of RNAP II predominantly on S2 and S5, but can also phosphorylate S7 [13, 14, 35]. CDK9-mediated phosphorylation of two other targets facilitates release of paused RNAP II into productive elongation: the Spt5 (p160) subunit of human DSIF and the NELF protein, resulting in NELF ejection from the transcription complex [13, 14, 35]. Phosphorylation of these three targets alleviates the negative effects of DSIF and NELF on transcription, and tips the balance to positive regulation of transcriptional elongation. CDK9 also contributes to recruitment of chromatin remodeling enzymes [14], RNA processing regulation [17, 26, 41], and transcription promoted by active distal enhancers using a chromatin looping mechanism [14].

CDK12 and CDK13 are atypical high molecular weight CDKs (~180 kDa) with a central CDK-related kinase domain, large amino- and carboxyl-terminal extensions, and RS domains [42, 43]. Using co-expression of tagged proteins, it was initially reported that Cyclins L were binding partners of both CDK12 and CDK13, to form CDK/Cyclin complexes co-localizing and/or interacting with splicing factors such as SC35 (SFRS2) and ASF/SF2 (SFRS1) [42, 44], and regulating alternative splicing of TNF β and E1a reporter minigenes [43, 44]. In addition, CDK12 immunoprecipitate complexes exhibited a CTD kinase activity [42].

These data strongly suggested the involvement of CDK12 and CDK13 in the regulation of transcription and pre-mRNA processing. Using different immunoprecipitation approaches of endogenous CDK12, CDK13 and Cyclin K, it was demonstrated that Cyclin K was the principal binding partner of these CDKs to form two distinct CDK/Cyclin complexes co-existing in cells [42, 43]. Multiple in vitro and cellular evidence indicated that CDK12/Cyclin K and CDK13/Cyclin K complexes phosphorylated the CTD of RNAPII predominantly on S2 and S5 and with higher activities when S7 is pre-phosphorylated [45, 46]. Knocking-down CDK12 and CDK13 expression by siRNA or pharmacological inhibition of their kinase activity led to global gene repression and cell death, suggesting that they are major CTD kinases during RNAPII-dependent transcription [46].

Mitotic CDK/Cyclin complexes also phosphorylate key components of the transcription and polyadenylation machineries to induce gene silencing during M phase. Transcription is inhibited in mitosis coinciding with hyperphosphorylation of RNAPII CTD as well as phosphorylation and release of TFIID and TFIIF from core gene promoters [9]. CDK1/Cyclin A/B complexes phosphorylate the CTD [47] and S164 within the CDK7 T-loop, which induces inhibition of CDK7 kinase activity [9, 11]. In addition, TFIIF-associated CDK7 kinase activity fluctuates throughout the cell cycle adding to the idea that transcription and cell cycle are tightly connected [25]. Similarly, the polyadenylation machinery is inhibited during mitosis through hyperphosphorylation and inhibition of the poly(A)polymerase (PAP) by CDK1/cyclin B [48]. Together, these data demonstrated the involvement of multiple CDK/cyclin complexes, including those with crucial cell cycle functions, in the regulation of transcription and RNA processing during the cell cycle.

2. CDK11/cyclin L complexes: Gene cloning, protein structures and pairings of CDK11 and Cyclin L isoforms

The first CDK11 protein (formerly named PITSLRE kinase) was identified by co-purification with a mammalian glycosyltransferase [49]. The cloning of the corresponding CDK11 cDNA indicated that it encoded a 58-kDa protein kinase highly related to CDK1 [50]. From this first isoform, it was next demonstrated that CDK11 is well conserved throughout evolution with two almost identical CDK11 genes in humans (CDK11A and CDK11B) but only one gene in other organisms; CDK11 genes encode numerous protein isoforms through complex alternative splicing [49]. Two predominant CDK11 protein isoforms of 110 and 58 kDa are produced from mammalian CDK11 gene(s), designated CDK11^{p110} and CDK11^{p58} [49] (**Figure 3A, B**). The larger CDK11^{p110} proteins, comprising many highly related isoforms [49], bear the kinase domain within their carboxyl-terminal half and have a large amino-terminal extension containing

important functional domains: nuclear localization signals, an arginine (R) and glutamic acid (E)-rich (RE) domain, a putative 14-3-3 consensus-binding site and caspase cleavage sites, which are functionally important (see transcription and pre-mRNA splicing section).

In a unique manner among CDKs, the shorter CDK11^{p58} proteins are produced from the same mRNA used to translate the CDK11^{p110} isoforms through the use of an internal ribosome entry site (IRES) located within the open reading frame of the transcripts (**Figure 3A, B**). This IRES-mediated translation occurs specifically during the G2/M phases of the cell cycle [51]. A third CDK11 isoform is generated by caspase-dependent proteolytic cleavage of both CDK11^{p110} and CDK11^{p58} proteins during apoptosis to produce CDK11^{p46}, retaining an intact catalytic domain [52, 53]. Transient expression of catalytically active CDK11^{p46} in CHO cells triggers apoptosis [52]. Cleavage of CDK11^{p110} also generates a p60 N-terminal protein, CDK11^{p60}, that re-locates to mitochondria, causing cytochrome *C* release and apoptosis [53-55]. A large body of evidence indicates that CDK11^{p46} is involved in apoptosis through phosphorylation of proteins regulating mRNA translation and apoptotic signaling pathways [49-56].

In order to identify binding or regulatory partners of CDK11^{p110}, we and others performed yeast two-hybrid screens and biochemical purifications, leading to the identification of transcription and splicing regulators; however, no Cyclin partner was found using these approaches. Independently, Berke and colleagues [57] identified Cyclin L1 (formerly named Ania-6a) as an immediate-early gene strongly induced in rat striatum by cocaine and direct dopamine stimulation and in neuron-like PC12 cells in response to growth factors. They also identified a closely related but distinct gene they called Ania-6b, which was later renamed Cyclin L2 [58, 59]. The authors showed that Cyclin L1 co-immunoprecipitated with CDK11^{p110} as well as the splicing factor SRSF2 (SC35) and the hyperphosphorylated form of RNAPII, providing early evidence that these proteins could be part of a nuclear complex regulating transcription and/or pre-mRNA processing. Two other groups described the human Cyclin L2 (Ania-6b) gene encoding two proteins, a long isoform with a ~65 kDa apparent electrophoretic mobility [58, 59] and a shorter isoform at ~25 kDa generated by alternative splicing [59]. In these reports, the authors showed that the long Cyclin L2 protein, like Cyclin L1 [57], contains a N-terminal CBD and a C-terminal RS domain (**Figure 3C**), co-immunoprecipitates with hyperphosphorylated RNAPII, CDK11^{p110} and splicing factors SRSF2 and SRSF7 (9G8), and localizes to nuclear speckles [58, 59].

We extended these data by describing that Cyclin L1 and L2 genes produce multiple transcripts encoding six distinct proteins [7], and proposed the following nomenclature for the L-type Cyclins: Cyclins L1 α , L1 β , L1 γ and Cyclins L2 α , L2 β A/B (**Figure 3C, D**). Cyclins L α and L β contain a full CBD with the consensus peptide domains [6], and we demonstrated that both α and β isoforms interacted with

CDK11^{p110}, mitosis-specific CDK11^{p58}, and apoptosis-specific CDK11^{p46} [7]. In contrast, Cyclin L1 γ has a truncated version of the CBD containing the highly conserved Lysine-Glutamate pair but lacking the ExxxLxxL motif [6]; as expected, L1 γ did not interact with any CDK11 proteins [7]. Both Cyclins L1 α and L2 α co-localize with CDK11^{p110} within nuclear speckles (**Figure 3E**) and have a long carboxyl-terminal extension that contains a RS domain very similar to that characterizing the SR splicing factors (**Figure 3F**). The Cyclin L's CBDs share nearly 90% identity, while the RS domains are much more divergent with 45% identity (**Figure 3C**). The ~80 amino acids from residues 300 to 380, located between the CBD and RS domains in the α isoforms, share low identity among all Cyclins (**Figures 1, 3F**) and were used to develop L1 α - and L2 α -specific antibodies. We demonstrated that both CDK11^{p110} and CDK11^{p58} co-immunoprecipitate with endogenous Cyclin L1 α and L2 α [7]. These data collectively identified the Cyclins L as the main partners of CDK11 proteins. Cyclin D3 was pulled out from a yeast two-hybrid screen using CDK11^{p58} as bait, and the biological relevance of this interaction continues to be explored [60].

3. Functional roles of CDK11/Cyclin L complexes

3.1 CDK11^{p110}/Cyclin L complexes in transcription and pre-mRNA splicing

The demonstration that CDK11^{p110} proteins localized within nuclear speckles and co-immunoprecipitated with splicing factors and RNAPII [57-59] led us to postulate that CDK11^{p110} was involved in transcription and RNA processing. Using chromatographic purification, we demonstrated that CDK11^{p110} is present in nucleoplasmic protein complexes of human cells eluting in two major molecular mass ranges, corresponding to 1 to 2 MDa and 500 to 800 kDa [7, 61]. Using mass spectrometry, co-immunoprecipitation and yeast two-hybrid screens, we showed that CDK11^{p110} associated with the largest subunit of RNAP II (Rpb1), numerous transcription elongation factors including ELL2, TFIIS, and the SSRP1 and SPT16 subunits of FACT, as well as the Rap30 and Rap74 subunits of TFIIF, casein kinase 2 (CK2) [61, 62], Cyclins L1 α /L2 α and several pre-mRNA splicing factors [7]. While the CDK11^{p110} protein was found associated with both hypo-(IIA) and hyperphosphorylated (IIO) forms of Rpb1, it preferentially associated with the hyperphosphorylated (IIO) form [61]. Importantly, antibody mediated inhibition of CDK11^{p110} protein suppressed RNAPII-dependent in vitro transcription suggesting that this protein kinase regulates some aspect of transcription and pre-mRNA processing. In addition to evidence that CDK11^{p110} protein directly phosphorylates the CTD of RNAPII, CK2 present in CDK11^{p110} immunoprecipitates also phosphorylates the CTD [62-65] as well as CDK11^{p110} at serine 227 (**Figure 2**) [62, 63]. Deletion of the arginine and glutamic acid-rich (RE) domain of CDK11^{p110} (**Figure 3**) abolishes interaction with CK2 and

nuclear speckle localization, demonstrating the importance of the RE domain in protein–protein interactions [62].

Independently, CDK11^{p110} has been shown to have an important role in Human Immunodeficiency Virus (HIV1) gene expression. Following integration of viral DNA sequences into the human genome, RNAPII is recruited to viral promoters by TFs binding to 5′ long terminal repeat sequences of the HIV genome. RNAPII-mediated transcriptional elongation of HIV DNA sequences is dependent on the viral Tat protein that binds the nascent viral transcript. The Tat protein recruits pTEFb, which phosphorylates multiple substrates in the transcriptional complex to facilitate transcription of the HIV genome [15]. In a genetic screen designed to identify critical components for virus replication, an amino terminal fragment of the eukaryotic translational initiation factor eIF3 was isolated as a potent inhibitor of HIV-1 RNA 3′ end processing [66]. As eIF3 was known to interact with CDK11, it was next demonstrated that the CDK11^{p110}/Cyclin L2 complex is recruited to viral DNA by the transcription/export complex TREX/THOC and phosphorylates RNAPII CTD at S2 near the 3′ end of HIV genes, promoting recruitment of cleavage and polyadenylation (CPA) factors [65]. Longer polyA tails enhance mRNA stability and, ultimately, viral gene expression levels [65, 67]. Interestingly, Cyclin L2 also affects HIV latency in resting CD4⁺ T cells and macrophages in a CDK11-independent manner. Cyclin L2 binds to and induces proteasome-mediated degradation of SAMHD1, a key factor in the HIV1 cycle [68]. Knowing that Cyclin L2 was phosphorylated by DYRK1A [57], Kisaka and colleagues [69] have recently shown that Cyclin L2 interacts with DYRK1A in cycling and resting cells and that depletion of DYRK1A increases both Cyclin L2 levels and HIV replication, providing evidence that DYRK1A modulates the levels of Cyclin L2 to control HIV replication.

Using yeast two-hybrid screening, we identified splicing-associated partner proteins of CDK11^{p110}, providing the first indication that it was connected to RNA processing. RNPS1 and 9G8 were identified as *in vitro* and *in vivo* partners of CDK11^{p110} and colocalized in nuclear speckles [70, 71]. RNPS1 is an SR protein-related polypeptide which activates pre-mRNA splicing [72]. RNPS1 is also a component of the exon–exon junction complex, linking pre-mRNA splicing to nuclear export of mature mRNA, nonsense-mediated mRNA decay and translation. Interestingly, RNPS1 also interacts with and is phosphorylated by CK2 *in vivo* [72]. 9G8 is another general SR splicing factor which shuttles between the nucleus and the cytoplasm and also participates in exporting mature mRNA to the cytoplasm. The CDK11^{p110}/Cyclins L complex not only interacts with 9G8, but also phosphorylates 9G8 *in vitro* and *in vivo* to promote pre-mRNA splicing [71]. The discovery of Cyclin L1 α and L2 α as CDK11^{p110} binding partners further strengthened the link between CDK11^{p110} and pre-mRNA splicing. These two Cyclins have a remarkable structure combining the conventional CBD (**Figure 1**) and carboxyl-terminal extension containing an RS

domain typically found in SR proteins such as SFRS1 (SF2/ASF) and SFRS7 (9G8), although the RS domains of Cyclin L1 α and L2 α have shorter Arginine and Serine-rich stretches than SR proteins (**Figure 3F**). Notably, the divergence in the RS domains of these two Cyclins could explain their differential localization within nuclear speckles [73], with possible resultant functional distinctions.

In vitro and in vivo splicing assays demonstrated that CDK11^{p110}/Cyclin L complexes regulate pre-mRNA splicing. Using a β -globin pre-mRNA substrate and nuclear extracts in standard in vitro splicing assays, we and others showed that depletion of CDK11^{p110} [71] and Cyclins L [7, 58] from nuclear extract greatly reduced splicing activity, while addition of bacterially produced Cyclin L1 α [74] and L2 α [58] stimulated splicing. However, there is no evidence that Cyclins L trigger splicing of a β -globin pre-mRNA substrate in S100 extract, a cytosolic fraction lacking SR proteins, which is a criteria to identify SR proteins. In addition, the recombinant amino-terminal segment of CDK11^{p110} involved in protein–protein interactions also substantially reduced splicing activity when added to in vitro splicing assays in nuclear extracts [71]. In cell-based splicing assays, using synthetic minigenes, we showed that ectopic expression of Cyclins L or active CDK11^{p110} individually enhanced intron splicing activity, whereas, expression of CDK11^{p58/p46} or kinase-dead CDK11^{p110} repressed splicing activity [7]. Moreover, co-expression of Cyclins L α /L β and CDK11^{p110} strongly and differentially affected alternative splicing *in vivo*. Together, these data established that CDK11^{p110} interacts physically and functionally with Cyclins L α and splicing factors to regulate pre-mRNA splicing events in cells.

3.2 Cell cycle regulation

While the CDK11^{p110} protein isoforms are ubiquitously expressed in quiescent mammalian tissues and throughout the cell cycle of synchronized cell lines [7], the expression of the Cyclins L is strongly induced in early G1 phase of the cell cycle [57] (Loyer and Trembley, unpublished data) with CDK11^{p110} as the putative binding partner, suggesting a possible role in the regulation of gene expression required for cell proliferation.

The expression of CDK11^{p58} is restricted to mitosis [51] and the high homology with CDK1 strongly suggested that CDK11^{p58} was a cell cycle regulator. This was first confirmed with ectopically expressed CDK11^{p58} in CHO cells that led to longer cell-doubling time and telophase delay [50]. In addition, cells stably expressing CDK11^{p58} exhibited increased micronucleation and aneuploidy [50, 52]. Considering these data, it was not surprising that disruption of the single murine CDK11 gene resulted in early embryonic lethality at the blastocyst stage of development due to cell proliferation failure and mitotic arrest [75].

CDK11^{p58}, but not CDK11^{p110}, associated with mitotic centrosomes, and knock-down of CDK11^{p58} activated a mitotic checkpoint, resulting in mitotic arrest and micronucleation defects [76]. In addition, a moderate depletion of CDK11^{p58} induced misaligned and lagging chromosomes without mitotic arrest, while a strong depletion caused defective chromosome congression, premature sister chromatid separation and mitotic failure [77]. It was also demonstrated that CDK11^{p58} was responsible for Ran-GTP-dependent microtubule stabilization required for normal mitotic spindle assembly [78], centriole duplication [79], and regulation of abscission site assembly [80].

In mitosis, translation of most mRNA species is strongly repressed. It was recently reported that CDK11^{p58} repressed cap-dependent mRNA translation by phosphorylating subunit eIF3F of the translation factor eIF3 complex [81], confirming a previous study showing that CDK11^{p46} also phosphorylated eIF3F [55]. Cross-regulation between cap-dependent and IRES-mediated translation in M phase has been shown, involving the 14-3-3 σ protein to control mitotic translation [82]. Cells lacking 14-3-3 σ cannot suppress cap-dependent translation in M phase and do not induce IRES-mediated translation, resulting in reduced expression of CDK11^{p58} and impaired cytokinesis [82]. Similarly, in *E μ -Myc/+* transgenic mice, increased cap-dependent protein synthesis associated with impairment of IRES-mediated protein translation at mitosis [83]. This resulted in reduced production of CDK11^{p58}, cytokinesis defects, and genome instability in B lymphocytes.

4 Cyclin L1, Cyclin L2, CDK11 and Cancer

While the informational base concerning CDK11 expression and its potential as a therapeutic target in cancer has expanded in the last decade, information on expression and potential roles for Cyclin L1 and Cyclin L2 in malignant cells has lagged in the same period. Here, we summarize what is known about Cyclin L1, Cyclin L2 and CDK11 in relation to cancer.

4.1 Cyclin L1

Several publications over the past 2 decades have implicated *CCNL1* genomic amplification and/or expression levels as contributing factors in cancer. *CCNL1* was first identified in 2002 as a candidate oncogene on chromosome 3q25 in head and neck cancer [84]. In this study, amplification of *CCNL1* in the tongue cancer cell line CAL 27 corresponded with 10.7-fold increased RNA expression relative to a non-transformed control tongue cell line. In head and neck squamous cell carcinoma (HNSCC) primary tumors, 8 of 9 well-differentiated tumors showed high Cyclin L1 RNA expression relative to matched normal tissue. This group subsequently published analysis of 96 HNSCC tumors in which elevated Cyclin L1 RNA was

detected in 57% of cases, and *CCNL1* gene amplification was determined in 26% of tumors analyzed [85]. Cyclin L1 protein was detected by immunohistochemistry, with normal epithelium displaying weak nuclear staining of the parabasal cells and HNSCC tumors displaying strong nuclear signal in epithelial cells. A separate laboratory found *CCNL1* copy number gain in 32% of HNSCC tumors associated with lymph node metastasis, and high level amplification of *CCNL1* correlated with shorter overall patient survival [5]. Gain or amplification of *CCNL1* also correlated with worse disease-free survival in nasopharyngeal carcinoma positive for Epstein-Barr virus [87]. Finally, *CCNL1* amplification in uterine cervical carcinoma is concordant with increased RNA and protein expression, and is a determinant of poor patient outcome [88]. Together, these publications demonstrate a likely oncogenic role for increased Cyclin L1 gene expression in different head and neck as well as cervical cancers.

4.2 Cyclin L2

In contrast to an oncogenic role for Cyclin L1 in cancer, several reports suggest that high levels of Cyclin L2 can inhibit tumor cell growth. In an original functional characterization report, overexpression of Cyclin L2 protein in one human hepatocellular carcinoma cell line (SMMC 7721) slowed growth and induced apoptosis in both cultured cells and xenograft tumors [58]. Another laboratory showed that forced expression of Cyclin L2 in the lung adenocarcinoma cell line A549 inhibited growth, increased the proportion of G0/G1 cells, and slightly induced apoptosis [89]. This same group later published results in a human gastric cancer cell line (BCG823) demonstrating similar effects of Cyclin L2 overexpression on cell growth, altered cell cycle, and apoptosis [90]. They also showed that increased Cyclin L2 levels enhanced the cytotoxic effects of fluorouracil, docetaxel and cisplatin in these cells. Overall, although data on Cyclin L2 is relatively scarce, the current knowledge suggests anti-oncogenic function in several cancer types.

4.3 CDK11 – survival gene and promising target in cancer

The identification of CDK11 as a survival gene in numerous large scale cancer-related screens published between 2005 and 2012 introduced the concept of cancer cell dependency on CDK11 expression. In a RNAi-based screen of the human kinase genome, loss of expression of 73 kinases, including CDK11, caused apoptosis of HeLa cervical cancer cells [91]. A genome-wide siRNA library screen for targets whose loss eliminates tumor-initiating cells in breast cancer identified CDK11 [92]. *CDK11A* and *CDK11B* also emerged as survival genes in multiple myeloma [93]. Finally, an shRNA library screen in osteosarcoma revealed CDK11 expression as essential for cell survival [94]. This publication represented the first in a series of investigations from multiple laboratories into the utility of targeting CDK11 in several types of cancer.

In osteosarcoma, CDK11 knockdown by shRNA and siRNA transfection resulted in cell growth inhibition, loss of anti-apoptotic proteins such as MCL-1, Bcl-xL, and survivin, and induction of markers of apoptosis [94]. Examination of CDK11 protein levels by western blot revealed extremely low expression in normal osteoblast cell lines, and variable expression levels in primary osteosarcoma specimens. By immunohistochemical staining in osteosarcoma tissue microarrays, it was determined that patients with high CDK11-scoring tumors had significantly worse survival outcome than patients with low CDK11 scores. Finally, intra-tumoral injection of CDK11 siRNA into KHOS osteosarcoma xenografts significantly suppressed tumor growth [94]. These authors subsequently published reduced migration and invasion activities and loss of integrin β 3, VEGF, and MT1-MMP expression after CRISPR/Cas9-mediated interruption of CDK11 expression in osteosarcoma cell lines [95]. This group next reported that CDK11 expression is involved in androgen receptor (AR) signaling [96]. Finally, they identified core-binding factor subunit β (CBF β), also known as Polyomavirus Enhancer-Binding Protein 2 Beta Subunit (PEBP2B), as a direct positive transcriptional target gene of CDK11^{p110}, but not CDK11^{p58}, in osteosarcoma [97]. High CDK11 and CBF β protein levels in patient tissues correlated with worse disease-free survival, and CDK11 protein expression levels were higher in tissues from metastases compared to primary tumor tissues in the same patients.

The role and targeting of CDK11 in breast cancer has been investigated by multiple research groups. The first publication in 2014 focused on CDK11^{p58}, and data is reported on CDK11^{p58} expression by immunohistochemical staining in patient tissues [98]. This data needs to be interpreted with the understanding that the p110 and p58 isoforms of CDK11 cannot be distinguished by antibody detection in tissue sections. The majority of signal detected in a tumor section is most likely the p110 isoform, as the number of cells undergoing mitosis at any given point in time is relatively low [99]. In this study, disease-free survival was worse in breast cancer patients with low CDK11 expression [98]. This same group showed that stable overexpression of CDK11^{p58} in two breast cancer cell lines slowed the growth of xenograft tumors [100]. These results from constitutive expression of the mitotic form of CDK11 throughout the cell cycle, which could also partially mimic the apoptotic CDK11^{p46} cleavage product, are similar to published data on slowed growth, suppression of translation, and induction of apoptosis after ectopic expression of CDK11^{p58} and CDK11^{p46} [52, 101, 102]. The next publication on CDK11 focused on triple negative breast cancer and demonstrated that immunohistochemical detection of CDK11 protein was significantly higher in triple negative breast cancer than in normal breast tissue [103]. Downregulation of CDK11 expression in breast cancer cells caused significant loss of cell viability and clonal survival and induced cell death. Further, tumor cell-specific targeting of CDK11 using a tenfibgen nanocapsule

containing an anti-CDK11 siRNA significantly shrank MDA-MB-231 xenograft tumors [103]. Another group also demonstrated that CDK11 expression is crucial for breast cancer cell proliferation and growth [104]. They detected significantly higher CDK11^{p110} protein expression in breast tumor tissue relative to adjacent non-cancer tissue and reported that elevated CDK11 expression correlated significantly with poor differentiation of tumor. Finally, their data showed that clinical outcome for patients with high CDK11 staining levels was worse than for those with low CDK11 staining, however, the difference was not statistically significant [104].

Examination of CDK11 expression in liposarcoma established that this kinase is highly expressed in malignant tissue relative to non-malignant lipoma tissue [105]. Blocking CDK11 expression via RNAi methods greatly reduced cell viability, induced apoptosis, and enhanced the cytotoxic effects of doxorubicin in 2 liposarcoma cell lines. In ovarian cancer, this same laboratory found linearly increased CDK11 protein staining levels from primary tumor and metastasis to recurrent metastasis [106]. They utilized a microarray containing primary tumor, a synchronous metastasis, and a metachronous metastasis (collected at the time of disease recurrence following treatment) from the same patient. Similar to other cancers studied, knockdown of CDK11 levels decreased cell growth, caused apoptosis, and enhanced the anti-proliferative effects of paclitaxel in 2 ovarian cancer cell lines. Intra-tumoral injection of siRNAs targeting CDK11 shrank SCOV-3 xenograft ovarian tumors over a 29 day study.

Investigation in BRAF- and NRAS-mutant melanoma cell lines showed elevated CDK11^{p110}, Cyclin L1 α , and Cyclin L2 α proteins compared to non-transformed primary melanocytes [107]. Down-regulation of CDK11 significantly reduced cell viability, clonal survival, and tumorsphere formation. A pan-cancer gene expression analysis performed after siRNA-mediated CDK11 loss indicated impact on cell cycle and DNA repair pathways in A375 cells. Further investigation in A375 and WM1366 cell lines confirmed changes in cell cycle and DNA repair gene expression levels, and demonstrated altered cell cycle distribution with G1-phase accumulation and G2/M-phase diminution due to CDK11 down-regulation. Patient survival analysis using the Cancer Genome Atlas (TCGA) RNA-seq data suggested that high level expression of CDK11 mRNA is a marker of poor prognosis in melanoma [107].

A recent body of work identified CDK11 as the unexpected target of the anticancer drug OTS964, which was designed to target PDZ binding kinase (PBK, also known as TOPK) [108]. In this work, the authors made OTS964-resistant HCT116 colorectal cancer cell line clones. All resistant clones were found to contain a missense mutation in 1 copy of *CDK11B* which changed the glycine residue just upstream of the DFG magnesium chelating motif to a serine. Molecular manipulation of multiple cancer cell types to contain this mutation in 1 copy of *CDK11B* also rendered the cell lines resistant to OTS964. When cancer

cells were transduced with pan-CDK11 guide RNAs using the CRISPR-Cas9 system, the cells accumulated in G2/M. When A375 cancer cells were synchronized into G1/S by double thymidine block and then released into medium containing OTS964, these cells arrested in G2 with 4C DNA content. These results open up exciting possibilities for targeting CDK11 function *via* small molecular inhibitors.

5 Concluding remarks

CDK/Cyclin complexes were first identified as key regulators of cell cycle progression; more recently, discovery of new Cyclin family members and CDK/Cyclin complexes together with continuing functional studies expanded the roles of these proteins to many other cellular processes. Here we have described CDK/Cyclin regulation of gene expression via the phosphorylation of the transcriptional machinery and other substrates regulating transcription and mRNA processing. The CDK11 and Cyclin L protein isoforms represent a unique microcosm spanning both cell cycle and transcriptional regulatory activities, and we summarized more than 30 years of investigation into the functions of these proteins. CDK11^{p110} isoforms in association with Cyclin L1 and L2 proteins have the potential to orchestrate different gene expression programs through impact on RNA elongation, splicing and processing. CDK11^{p58} plays crucial roles in the completion of the cell cycle and cellular division. Together, these wide-ranging roles place Cyclins L and CDK11 at the cross-roads of cell cycle and gene expression.

Even with focus on transcriptional functions, description of the roles for CDKs and Cyclins in different disease states and physiological processes is beyond the scope of this review. Here, we specifically summarized what is known about Cyclins L1 and L2 and CDK11 in different cancers. The biochemical and biological data thus far indicate that reducing CDK11 protein levels (via RNA interference) or blocking CDK11 function (via small molecule inhibitor) is effective in multiple cancer types to slow growth, interfere with cell cycle function, and promote cell death. It is likely that anti-CDK11 treatment impact will differ depending on the growth rate of the cancer cells, the oncogenic factors contributing to malignancy, and on the tissue and cell type involved. Regardless, through blocking of transcription and splicing activities as well as disruption of the cell cycle, CDK11-targeted drugs have important potential for efficacy in numerous cancers.

6 References

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7 Figure legends

Figure 1: Alignment of several human cyclin box family members performed using Cobalt at NCBI (NIH, USA) and refined manually. Green squares show conserved amino acids between Cyclin L1 and/or L2 versus other indicated Cyclins. Yellow squares show conserved amino acids between either Cyclin L1 or L2 versus other indicated cyclins. Grey squares represent conservative substitution. *last amino acid. Gaps are represented by “-“. Blue [X] symbol with X for the number of amino acids, indicated partial amino acid sequences not presented. The highly conserved Arginine-Phenylalanine di-peptide (R232/F233, R113/F114 in Cyclins B1 and L1, respectively), the Phenylalanine F253, the Lysine-Glutamate pair (KxEE) located at residues K257 and E259/260 in Cyclin B1, and the ExxxLxxL sequence (E286/L290/L293 in Cyclin B1) are shown in bold font on the lane above the alignment.

Figure 2: Network of CDK/Cyclin complexes regulating transcription and mRNA processing via cross-talk between multiple complexes. Arrows and bars indicate phosphorylation events resulting in activation and inhibition of protein targets, respectively, during the transcription cycle and pre-mRNA splicing. Details of transcript splicing and 3'-end processing are not depicted

Figure 3: A) Schematic representation of the CDK11 family members. B) Western blot of ectopically expressed FLAG-tagged CDK11 isoforms in 293T cells. CDK11 proteins are detected with anti-FLAG antibody. C) Schematic representation of the Cyclin L family members and percentages of identity within the Cyclin Box and the RS Domains. D) Western blot of overexpressed HA-tagged cyclin L isoforms in 293T cells. Cyclin L proteins are detected with anti-HA antibody. E) Immunodetection of CDK11^{p110}, Cyclin L1 α and Cyclin L2 α in nuclei of 293T cells. F) Alignment of the last ~370 amino acids of human Cyclins L1 α , L2 α , SFRS1 and SFRS7. Green squares show conserved amino acids between cyclins L1 α , L2 α and underlined amino-acids indicate the arginine/serine di-peptide rich stretches. Grey squares represent conservative substitution. *last amino acid. The panels C and D were originally published in the Journal of Biological Chemistry: P. Loyer, J.H. Trembley, J.A. Grenet, A. Busson, A. Corlu, W. Zhao, M. Kocak, V.J. Kidd, J.M. Lahti. Characterization of cyclin L1 and L2 interactions with CDK11 and splicing factors: influence of cyclin L isoforms on splice site selection, J Biol Chem, 2008, 283(12), 7721-7732 [7]. © the American Society for Biochemistry and Molecular Biology.

A1 219 **L**R**A**E**I**R**H**R**F**K**A**H**Y**M**K**K**Q**P**D**I**E**G**M**R**T**I**V**D**W**V**E**V**G**E**E**Y**K**L**R**A**E**T**L**Y**L**A--**N**F**L**D**R**F**L**S**C**M**V**L**R**G**K**L**-**Q**L****G**T**A**-**A**M**L**-**L**A**S**K**-**Y**E**E**I**Y**P**P**E**V**E****F**V**Y** 313
 B1 110 **V**K**E**E**K**L**S**P**E**I**L**V**T**A**S**P**S**[72]**M**R**A**-**I**L**I**D**W**V**O**V**Q**M**K**F**R**L**Q**E**T**M**Y**M--**S**I**I**D**R**F**M**Q**N**N**C**V**P**K**M**L**-**Q**L****G**V**T**-**A**M**F**-**I**A**S**K**-**Y**E**E**M**Y**P**P**E**I**G**D**F**A**F** 271
 C 17 **D**K**Q**D**L**K**E**R**Q**K**D**L**K**F**I**S**E**E**E**Y**W**K**Q**--**I**F**F**T**N**V**I**G**A**L**G**E**H**K**R**Q**V**I**A**T**A**T**Y**E**K**-**R**E**Y**A**R**Y**S**L**K**-**S**I**D**P**V**L**M**A**P**T**-**C**V**F**-**L**A**S**K**-**V**E**E**F**G**V**V**S**N**T**R**L**-**I 109
 D1 31 **M**L**K**A**E**E**T**C**A**P**S**V**S**Y**F**K**C**V**Q**K**V**L**P**S**M**R**K**I**V**A**T**W**M**L**E**V**C**E**Q**C**E**E**E**V**F**L**A**M**N**Y**-**L**D**R**F**-**L**S**L**E**P**V**K**K**S**R**L**Q**L**L**G**A**T**-**C**M**F**-**V**A**S**K**-**M**K**E**T**I**P**L**T**A**E**K**L**C**I** 126
 D3 31 **L**L**R**L**E**E**R**Y**V**F**A**S**Y**F**Q**C**V**Q**R**E**I**K**P**H**M**R**K**M**L**A**Y**W**M**L**E**V**C**E**Q**C**E**E**E**V**F**L**A**M**N**Y**-**L**D**R**F**-**L**S**C**V**P**T**R**A**Q**L**Q**L**L**G**A**V**-**C**M**L**-**L**A**S**K**-**L**R**E**T**T**P**L**T**I**E**K**L**C**I 126
 E1 121 **L**N**K**E**K**T**Y**L**R**D**Q**H**F**L**E**Q**H**P**L**L**Q**P**K**M**R**A--**I**L**L**D**W**V**E**V**C**E**V**Y**K**L**H**R**E**T**F**Y**L**A--**C**D**F**F**-**D**R**Y**M**A**T**Q**E**N**V**V**K**T**L**L**Q**L**I**G**I**S**-**S**L**F**-**I**A**A**K**-**L**E**E**I**Y**P**P**K**L**H**Q**A**-**Y 215
 F 24 **R**R**P**R**N**I**T**I**L**S**L**P**E**V**V**F**H**I**L**K**W**L**S**V**E**D**L**A**V**R**A**V**H**S**O****K**D**V**D**N**H--**S**V**W**A**C**A**S**E**Q**E**L**W**P**-**S**P**G**N**L**-**K**L**-**F**E**R**A**E**K**G**-**N**F**E**A**I**V**K**-**L**G**I**-**N**Y**L**Y**N**E**G**-**S**V**S 119
 G1 28 **Q**F**K**V**C**G**L**R**L**I**E**S**A**H**D**N**G****R**M**T**A**R**L**R**--**D**F**E**V**K**D**L**L**S**L**T**Q**F**F**G**D**T**E**T**F**S**L**A**V**N**--**L**D**R**F**-**L**S**K**M**K**V**-**Q**P**K**H**L**G**C**V**G**L**S**C**-**F**Y**L**A**V**K**S**I**E**E**E**-**R**N**V**L**A**T**D**L** 121
 G2 29 **E**R**F**Q**P**R**E**K**G**L**S**L**I**E**A**T**P**E**N**D**T**L**C**P**L**R**N**A**K**V**E**D**L**R**S**L**A**N**F**F**G**S**C**T**E**T**F**V**L**A**V**N**I**L**D**R**F**-**L**A**L**M**K**V**-**K**P**K**H**L**S**C**I**G**V**-**C**S**F**L**L**A**A**R**I**V**E**D**-**C**N**T**I**P**S**T**H**D**V** 125
 H 35 **V**A**N**G**K**V**L****N**D**P**V**F**L**E**P**H**E**E**M**T**L**C**K**Y**--**Y**E**K**R**L****E**F**C**S**V**F**K**P**A**M**R**S**V**V**G****I**A**C**M**Y**F**K**-**R**F**Y**L**N**N**V**M**-**E**Y**H**P**R**I****I**M**L**T**-**C**A**F**-**L**A**S**K**-**V**D**E**F**N**V**S**S**Q**P**F**V**G**- 127
 I 21 **T**R**E**A**Q**M**K**V**N**V**R**K**M**P**S**N**Q**N**V**S**P**S**Q**--**D**E**V**I**Q**W**L**A**K**L**K**Y**Q**F**N****Y**P**E**T**F**A**L**A**S**S**-**L**D**R**E**L**A**T**V**K**A**H**P****K**Y**L**S**C**H**A**I**S**--**C**F**F**-**L**A**A**K**T**V**E**D**-**E**R**I**P**V**L**K**V**L 114
 J 14 **A**D**I**H**Q**A**L**R**Y**K**E**L**K**L**P**S**Y**K**G**Q**S**P**O****S**L**R**R**Y**F**A**D**L**I**A**I**V**S**N**R**E**T**L**C**P**S**R**H**L**A--**V**Y**L**L**D**L**F**M**D**R**Y**D**I**S**-**I**Q**Q**L**H**L**V**A**L**S**-**C**L**L**-**L**A**S**K**-**F**E**E**K**E**D**S**V**P**K**L**E**Q**L** 109
 K 26 **W**D**K**K**D**L**A**H**T**P**S**Q**L**E**G**L**P**A**T**H**A**R**Y**--**R**E**A**R**F****F**D**V**T**R**I**G**L**H**Y**D**T**L**A**T**G**I**I**Y**F**H**-**R**F**Y**M**F**H**S**E**K**-**Q**F**P**R**Y**-**T**G**A**C**C**L**F**-**L**A**K**-**V**E**E**T**K**H**C**K**I**I**K**T 119
 L1 59 **I**P**E**R**L**S**P**T**P**S**M**Q**D**G**L**D**L**P**S**E**T**D**L**R--**I**L**G**C**E**L**I**Q**A**A**G**I**L**L**R**L**P**Q**V**A**M**A**T**Q**V**L**F**H--**R**F**F**Y**S**K**S**F**V**-**K**H**S**F**E**I**V**A**M**A--**C**I**N**-**L**A**S**K**-**I**E**E**A**P**R**I**R**I**D**L**I**N**V** 152
 L2 54 **L**P**D**D**K**L**R**F**T**P**S**M**S**S**G**L**D**T**D**T**E**T**D**L**R**V**V**--**G**C**E**L**I**Q**A**A**G**I**L**L**R**L**P**Q**V**A**M**A**T**Q**V**L**F**Q--**R**F**F**Y**T**K**S**F**V**-**K**H**S**M**E**H**V**S**M**A--**C**V**H**-**L**A**S**K**-**I**E**E**A**P**R**I**R**I**D**L**I**N**V** 147
 O 109 **R**K**A**Q**E**S**H**F**H****R**E**A**L**A**R**Q**P**V**T**A**E**S**R**C**-**K**L**L**S**W**L**P**V**H**R**Q**F**L**S**F**E**S**C**L**V**N**T**L**--**D**R**E**L**T**T**P**-**V**A**A**D**C**H**Q**L**L**G**V**T**-**S**L**L**-**I**A**C**K**Q**V**E**V**H**P**P**V**K**O**L**L**A**L** 203
 Q 3 **A**P**E**G**G**G**G**P**A**A**R**G**P**E**G**O**P**A**P**-**A**R**V**H--**F**R**V**A**R**F**I**M**E**A**G**V**K**I**G**M**R**S**I**P**I****A**C**T**I**Y**H**K**F**C**E**T**N**L**D**A**--**Y**D**P**Y**L**I**A**M**S**-**S**I**Y**-**L**A**G**K**-**V**E**E**Q**H**L**T**R**I**I**N**V** 96
 T1 14 **F**T**R**E**Q**L**E**N**S**P**S**R**R**F**G**V**P**P**K**L**S**Y**Q**Q--**A**A**N**L**I**Q**D**M**Q**R**I**N**V**S**L**I**N**I**A**I**V**Y**M**H--**R**E**Y**M**I**Q**S**E**T**-**Q**F**P**G**N**S**V**A**P**I**-**A**L**E**-**L**A**A**K**-**V**E**E**Q**E**K**L**E**H**V**I**K**Y** 107
 Y 144 **I**F**D**E**N**I**H**P**L**-**S**K**C**E**-**V**P**E**D**Y**D**K**H**N**P**E**Q**K**Q**M**Y**R**F**V**R**T**L**F**S**A**Q****L**T**A**E**C**-**A**I**V**T**L**V**Y**L**E****L**L**T**Y**A**E**I**D**I**C**P**A**N**W**K**R**I**A**L**G**A**L**L**-**L**A**S**K**-**V**W**D**D**Q**A**V**W**N**V**D**Y**Q**Q** 239

A1 314 I-----**T****D****T****Y**--**T**K**R**O**L**L**K**M**E**H**L**L**L**V**L**-**A**F**D**L**T**V**P**T**N**Q**F**L**L**Q**Y**T----**R**R**Q**V**C**V**R**T**E**N**L**A**K**Y**A**E**L**S**L**E**A**D**P**F**L**K**Y**L**P**S**L**I**A**A 389
 B1 272 V-----**T****I****N**T**Y**--**T**K**H**O**I**R**O**M**E**M**K**L**L**R**A**L**-**N**E**G**L**G**R**P[22]-**A**K**-**Y**L**[16]----**I**A**A**G**A**F**L**C**A**L**K**I**L**D**N**G**E**W**T**P**T**L**Q**H**Y**L**S**Y**E**E**S**L**L** 377
 C 110 **A**A**A**T**S**V**L**--**K**T**R**F**Y**F**E**K**--**E**F**F**N**--**R**M**N**H**L**E**C**E**F**Y**L**L**E**-**L**M**D**C**L**I**V**E**F**Y**R**P**L**L**Q**V**--**---**C**D**M**G**E**D**M**---**L**L**P**L**A**M**R**I**V**---**N**D**Y**T**R**I**L**C**L**L 191
 D1 127 Y-----**T****N**S**I**--**R**P**E**L**L**Q**M**E**L**L**V**N**K**L**K**W**N**L**A**A**M**T**P**H**D**E**H**-**F**[8]-**N**K**I**I**R**--**K**H**A**-**Q**T**F****A**L**C**A**T**D**V**K**F**I**S**N**P**P**S**M**V**A**G**S 207
 D3 127 Y-----**T**H**A**V--**S**P**R****L**R**D**W**E**V**L**V**I**G**K**L**K**W**D**L**A**A**-**I**A**H**D**F**L**A--**F**I[8]-**D**R**A**I**V**-**K**H**A**-**Q**T**F****A**L**C**A**T**D**Y**T**F**A**M**Y**P**P**S**M**I**A**T**G**S** 207
 E1 216 V-----**T****G**A**C**--**S**G**D**E**L**T**M**E**L**M**I**M**A**L**K**W**R**L**S**P**L**T**I**V**S**W**L**N**-**I**M**-----**Q**V**A**Y**L**N**D**L**H**E**V**L**P****Y**Q**Q**I**F**I**Q**I**A**E**L****-**D**L**C**L****V**D**V** 289
 F 120 **D**E**A**R**A**E**V**N**G**L**K**A**R**F**F**S**L**A**E**R**L**N**V**G**A**A**P**F**I**W**L**F**I**R**P**P**S**V**S**--**S**C**C**K**A**V**V**H**E**S**L**R**A**E**C**-----**L**Q**R**T**H**K**A**S**I**L**H**C**L**G**R**V**L**S**L**F**E**D**E**E**K**Q**Q**A**H**D**L**F**E** 211
 G1 122 I**R**-----**I**S**C**--**R**F**T**V**S**D**L**M**R**M**K**I**V**I**E**K**V**C**K**V**K**A**T**A**F**Q**L**Q**-**Y**Y**[20]**S**L**Q**L**K**A**H**C**R**I**I**F**S**K**A**K**P**S**V**L**A**L**S**I**A**L**E**I**Q**A**O**K**C****E** 219
 G2 126 I**R**-----**I**S**O**C**-**C**A**S**D****K**R**M**E**K**I**I**S**E**K**L**H**Y**E**L**A**T**T**A**L**N**F**L**H**L**-**L**H[21]--**Q**L**K**A**N**C**R**L**I**F**S**K**A**K**P**S**V**L**A**L**C**L**L****-**L**E**V**E**L**K**S**-**E 222
 H 128 --**N****L****R**E-----**S**--**P**L**-**C**O**E**K**--**A**L**E**O**L**E**Y**E**L**L**I**Q**Q**L**-**N**E**H**L**I**V**H**N****E**Y**R**P**F**E**G**F**--**---**I**D**L**K**T**R**Y**P**I**L**L**E**N**P**E**L**R**T**A**D**D**F**L****R**I**A**L**T****A**Y**L**L 206
 I 115 A-----**R****S**F**G**C**S**S**E**L**L**R**M**E**I**I**L**D**K****N**W**D**L**H**T**A**T**P**L**D**F**L**H**I**-**F**H[8]--**L**L**F**S**L**P**K**L**S**P**S**Q**H**L**A**V**L**T**K**Q**L**L**H**C**M**A**C**N**Q**L**L**Q**F** 198
 J 110 **N**S**G**C**M**T**N**M**N**L--**V****T**K**O**N**L**L-----**H**M**E**L**L**L**L**E**T**F**Q**W**N**L**C**L**P**T**A**A**F**L**E**Y**-**Y**L**-----**S**E**A**V**H**E**T**D**L**H--**D**G**W**P**M**I**C**L**E**K**T**K**L**Y**M**A**Y**A**D**Y**F**L**E**V 190
 K 120 **A**R**S****L**L-----**N**D**V**--**Q**F**G**F**G**D**D**P**E**E**V**M**V**L**E**R**I**L**Q**T**I**-**K**F**D**L**Q**V**E**H**F**Y**Q**F**L**L**K**Y**A**K-----**Q**L**K**G**D**-**K**N**K**I**K**L**V**Q**M**A**V**T**F**--**N**D**S**I**C**T**L**S**L**Q 200
 L1 153 **F**H**L**R**Q**L**R**G**K**T**P**S--**P**L**I**L**D**O**N**Y**I**N**T**K**N**Q**V**I**K**A**E**R**R**V**L**K**E**L**-**G**F**C**V**H**V**K**H**P**H**K**I**I**V**M**Y**L-----**Q**V**L**E**C**E**R**N--**Q**T**L**V**Q**T**A**N**N**Y**M**---**N**D**S**L**R**T**N**V**F**V**H** 239
 L2 148 **F**H**R**L**R**Q**L**R**D**K**K**K**F**V--**P**L**L**L**D**Q**D**V**N**L**K**N**O**I**K**A**E**R**R**V**L**K**E**L**-**G**F**C**V**H**V**K**H**P**H**K**I**I**V**M**Y**L-----**Q**V**L**E**C**E**R**N--**Q**H**L**V**Q**T**S**W**N**Y**M**---**N**D**S**L**R**T**D**V**F**V**R** 234
 O 204 -----**C**C**G**A**-**F--**S**R**Q**L**C**N**L**E**C**I**V**L**H**K**L**-**H**F**T**L**G**A**P**T**I**S**F**L**E**H**F**T[5]-**A**G**A**E**A**S**E**A**L**E**A****A**L**A**R**G**V**A**E**L**S**L**A**D**Y**A**F**T**S**Y**S**P**S**L**L 283
 Q 97 **S**N**R**Y**F**N-----**P**S**G**E**P**L**E**L**I**S**R**E**W**E**L**R**D**S**I**V**Q**C**E**L**L**M**L**R**V**L**-**R**F**O**N**S**F**O**H**P**H**Y**L**L**H**Y**V**---**S**L**N**W**L**---**N**--**R**H**S**W**R**T**P**V**A**V**T**A**-**W**A**L**L**R**D**S**Y**H**G**A 181
 T1 108 **A**T**C**L**H**P**Q**E-----**S**-**L**D**T**R**S**E**A**M**L**Q**O**V**Q**D**L**V**I**L**S**I**L**Q**T**L**-**S**F**E**L**T**I**D**H**P**H**T**H**V**K**C**T**-----**Q**L**V**R**A**S**K**D---**L**A**C**T**S**--**F**M**A**T**N**-**S**L**H**L**T**T**S**I 189
 Y 240 I-----**L**K**I**I--**V**E**D**M**N**E**L**E**R**Q**F**L**L**-**C**F**N**I**N**V**P**F**S**V**-**Y**A**K**-**Y**F**D**L**R**S**L**A**E**A**N**N**L**S**F**P**L**E**F**L**S**R**E**R**A**H**K**L**E**A**I**S**R**L**C**E**D**K**Y**K**D**L** 316

Figure 1

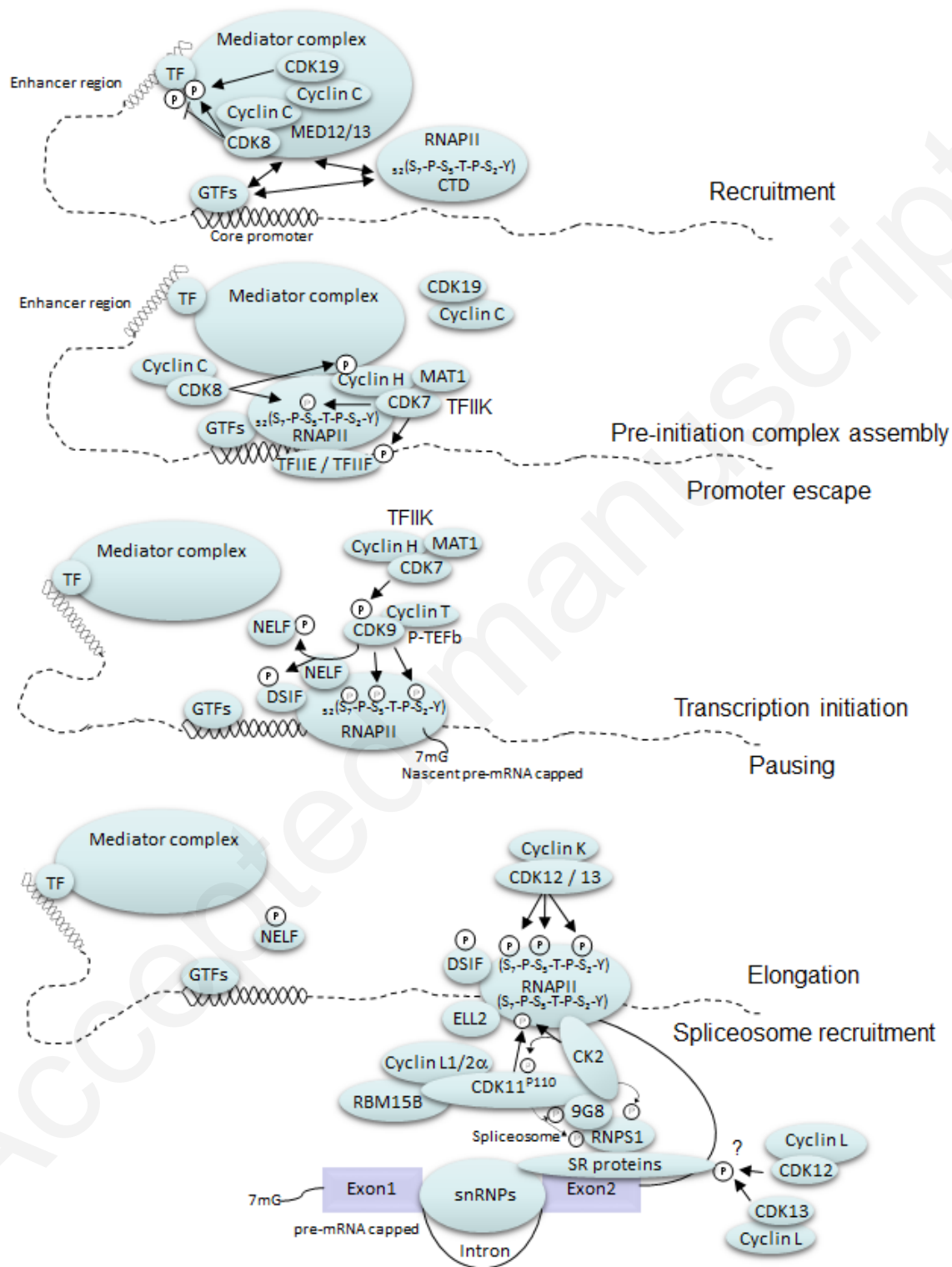


Figure 2

