A novel role of Aurora-A in pre-mRNA splicing

Arun Prasath Damodaran

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Par

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A novel role of Aurora-A in pre-mRNA splicing

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Abstract

Aurora-A is a serine/threonine kinase known to regulate mitotic events in cells. Its over-expression in tumours promote cell survival by modulating cell proliferation and overcoming apoptosis. Previously, it has been shown that Aurora-A is important for the stability of a serine/arginine (SR)-rich protein, ASF/SF2, thereby altering the alternative splicing of apoptosis-related genes. In consistence with this role in splicing, we have discovered that the interactome of Aurora-A contains a large number of spliceosome components - both core and non-core spliceosomal proteins.

My PhD work aimed to characterize the function of Aurora-A in pre-mRNA splicing and to investigate its function at the molecular level. I show that Aurora-A interacts directly with splicing factors such as SR proteins and hnRNP proteins and also phosphorylates few of them *in vitro*. I demonstrate that Aurora-A is important for the stability of ASF/SF2 and many other SR proteins. Using immunofluorescence microscopy, I further demonstrate that Aurora-A localizes to nuclear speckles, the sites where splicing proteins are stored, as well as to the periphery of nuclear speckles where a majority of pre-mRNA splicing is known to take place. Notably, addition of recombinant Aurora-A enhances the splicing efficiency of beta-globin pre-mRNA *in vitro*. Through RNA-seq analysis using the VAST tool, I identified 414 alternative splicing events that are altered upon inhibition of Aurora-A. Surprisingly the important SR protein kinases, CLK1 and CLK4 are also identified to be alternatively spliced in an Aurora-A dependent manner.

Altogether my work reveals a broad and novel role of Aurora-A in pre-mRNA splicing and suggests molecular means through which Aurora-A could modulate the spliceosome and splicing factors.

Résumé

Aurora-A est une sérine/thréonine kinase connue pour réguler les événements mitotiques dans les cellules. Sa surexpression dans les tumeurs favorise la survie cellulaire en modulant la prolifération cellulaire et en inhibant l'apoptose. Auparavant, il avait été montré qu'Aurora-A était important pour la stabilité d'une protéine riche en sérine / arginine (SR), ASF/SF2, modifiant ainsi l'épissage alternatif des gènes liés à l'apoptose. En cohérence avec ce rôle dans l'épissage, nous avons découvert que l'interactome d'Aurora-A contient un grand nombre de composants du spliceosome - des protéines spliceosomales centrales et non essentielles.

Mon travail de thèse a consisté à caractériser la fonction de Aurora-A dans l'épissage pré-ARNm et à étudier sa fonction au niveau moléculaire. J'ai montré qu'Aurora-A interagissait directement avec des facteurs d'épissage, tels que les protéines SR et les protéines hnRNP, et phosphorylait également quelques-unes d'entre elles in vitro. J'ai démontré qu'Aurora-A est important pour la stabilité de ASF/SF2 et de nombreuses autres protéines SR. En utilisant la microscopie à immunofluorescence, j'ai démontré en outre qu'Aurora-A localise vers les taches nucléaires, les sites où les protéines d'épissage sont stockées, ainsi qu'à la périphérie des taches nucléaires où une majorité d'épissage de pré-ARNm est connue. L'ajout d'Aurora-A recombinant améliore notamment l'efficacité d'épissage du pré-ARNm de la bêta-globine in vitro. En analysant la méthode RNA-seq à l'aide de l'outil VAST, j'ai identifié 414 événements d'épissage alternatifs qui sont modifiés lors de l'inhibition de Aurora-A. De manière surprenante, les protéines kinases SR importantes, CLK1 et CLK4, sont également identifiées comme étant épissées de manière alternative d'une manière dépendante de Aurora-A.

Dans l'ensemble, mes travaux révèlent le rôle important et novateur d'Aurora-A dans l'épissage des ARNm et suggèrent des voies moléculaires permettant à Aurora-A de moduler les facteurs d'épissage et d'épissage.

Abbreviations

3’ - 3 prime

3’SS - 3’ splice site

5’- 5 prime

5’SS - 5’ splice site

A Box - Aurora box

AIK-1/2 - Human Aurora-A Kinase 1/ 2

APC/C - anaphase promoting complex/cyclin

AS - alternative splicing

ATP - Adenosine 5'-triphosphate

BP - branch point

BTAK - Breast tumour activated kinase

Cdk - Cyclin Dependent Kinases

cDNA - Complementary DNA

cryo-EM - cryo-electron microscopy

CTD - C-terminal repeat domain

DMEM - Dulbecco's modified Eagle's medium

DMSO - Dimethyl Sulfoxide

DNA - Deoxyribo Nucleic Acid

dNTPs - deoxynucleotides

EDTA Ethylene Diamine Tetraacetic Acid

ESE - exonic splicing enhancer

ESS - exonic splicing silencer

G0 - Gap zero

G1/G2 - Gap1/Gap2

GE - gene expression

GFP - Green Fluorescent Protein

GO - gene ontology

GST - Glutathione S-Transferase

h - hour

HeLa - Henrietta Lacks

ISE - intronic splicing enhancer

ISS - intronic splicing silencer

kDa - kilodalton

M-phase - Mitotic phase

mRNA - messenger RNA

MS - mass spectrometry

MYC - myelocytomatosis

NEK-2 - NimA-related kinase

NMD - nonsense mediated decay

NTP - nucleoside triphosphate

PAGE - polyacrylamide gel electrophoresis

PBS - Phosphate Buffered Saline

PCR - polymerase chain reaction

pre-mRNA - premature mRNA

PSI - Percent Splicing Index or Percent Spliced In

RBP - RNA binding protein

RNA - ribonucleic acid

RNA - Ribo Nucleic Acid

RNA-Seq - RNA sequencing

RRM - RNA recognition motif

RT-PCR - reverse-transcription polymerase chain reaction

SDS - Sodium dodecyl sulfate

Ser - Serine

SF1 - splicing factor 1

SF2/ASF - splicing factor 2/alternative splicing factor

siRNA - small interfering RNA

snRNA - small nuclear RNA

snRNP - small nuclear ribonuclear protein

SR protein - serine/arginine-rich protein

SRPK - SR protein kinase

Thr - Threonine

TPX2 - Targeting protein for Xklp2

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1. Introduction

1.1. The splicing of RNA

1.1.1. The central dogma of life and RNA processing

DNA acts as a source of genetic information in most of the living organisms with the exception of RNA viruses, which can reverse transcribe their genetic information from RNA to DNA. The highly stable DNA is transcribed into highly reactive and unstable RNA. This intermediary RNA is then translated into a final functional product called proteins. The whole process is described as “central dogma of life”. In the late 1970s, it was assumed that the eukaryotic genetic information is organized and regulated in the same way as that of the bacterial genome. However, the descriptive understanding of the eukaryotic genetic material and its appearance inside the cells with a nucleus shattered the initial beliefs regarding the eukaryotic genome (Sharp, 1994).

On the other hand, the RNA is not directly converted to protein. The transcribed pre-mRNA undergoes three main processes before it is recognized by the translational machinery to be decoded into proteins. These processes include 5’ capping, RNA splicing and 3' polyadenylation. All these processes are predominantly co-transcriptional and contribute to both the efficiency and fidelity of gene expression (Moore and Proudfoot, 2009). The 5’ capping is the first RNA processing on the nascent transcript by the action of three enzymes namely triphosphatase, a guanylyltransferase, and a methyltransferase, where the pppA in the 5’ terminus is converted to 7meGpppA (Shuman, 2000). The RNA splicing is a process of removal of introns and joining of exons from the pre-mRNA transcript. 3' polyadenylation is the process of adding poly(A) tail to a messenger RNA and is important for the nuclear export, translation, and stability of mRNA (Proudfoot et al., 2002).

1.1.2. Pre-mRNA splicing

The intervening nonsense DNA sequences are present in-between the coding DNA sequences and are then as such transcribed to the RNA molecule. These intervening sequences are removed from the newly formed RNA molecule and this process is called

RNA splicing (Crick, 1979; Sharp, 1994) (**Figure 1**). The sense coding region is called exon and the intervening non-sense sequence is called intron. In the 1970s, another puzzling observation was the finding of long RNA in the nucleus of vertebrate cells when compared to the shorter mRNA that appeared in the cytoplasm. The comparison of complementarity between the mRNA sequence in the cytoplasm to that of the adenoviral DNA sequence from which it was transcribed led to the discovery of "split genes" and "RNA splicing" (Berget et al., 1977; Chow et al., 1977). Later these split genes and RNA splicing have also been identified in higher organisms. For example, the globin gene of Rabbit contains the intervening or intronic sequences (Jeffreys and Flavell, 1977).

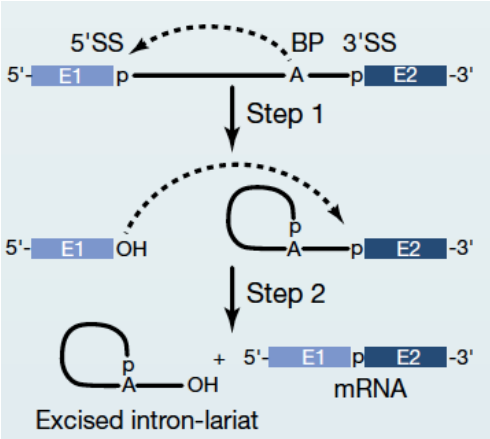

Figure 1 | The RNA splicing reaction happens in two consecutive transesterification steps. During the first step, the 2'-hydroxyl of an adenosine on the branch point sequence (BPS) in the intron attack the phosphodiester bond at the 5' splice site (SS). This generates a free 5' exon and an intron lariat-3' exon (Step1). Subsequently, the phosphodiester bond at the 3'SS is attacked by the 3'-hydroxyl of the 5' exon, leading to ligation of exons and excision of the lariat intron (step 2). Figure adapted from (Wahl and Lührmann, 2015a)

The most important questions till date are "Why are introns present" and "Where do introns come from?". Though the presence of intron can be answered by relating it to the protein diversity as a result of alternative splicing, the question on the origin of introns still remains a mystery leading to controversial discussions. Based on the structure and functional features, the introns are classified into 4 types namely, the group I self-splicing introns, group II self-splicing introns, tRNA introns and spliceosomal intron. Group I introns catalyze their own splicing reaction and are present either in nuclear ribosomal RNA genes or in mitochondrial and/or plastid genes in most eukaryotic supergroups as well as in some bacteria and viruses. tRNA introns are very short introns and their splicing

is carried out by protein enzymes and not by ribozymes. Spliceosomal introns are present in all eukaryotic nuclear genomes and their splicing is carried out by the spliceosome. Group II self-splicing introns are found in bacterial genomes and in chloroplast and mitochondrial genomes of widely diverged eukaryotes. They are also believed to be responsible for the origin of eukaryotes. It is also proposed that the eukaryotic introns are evolved from the integration of early mitochondrion containing group-II introns to the eukaryotic cells. The spliceosomal intron is believed to be originated from the group-II self-splicing introns due to their similarity in the splicing catalysis (Irimia and Roy, 2014).

Pre-mRNA splicing gives rise to protein diversity and thus acts as an important factor for deciding the complexity of an organism. It is worth noting that *C. elegans* and humans have almost the same number of genes. However, humans remain more complex than *C. elegans* and this complexity is believed to be determined by the increase in protein diversity as a result of the complex splicing mechanism in humans **(Figure 2)**. RNA splicing is also known to be important for the development of an organism, tissue diversity, circadian cycle, health and disease.

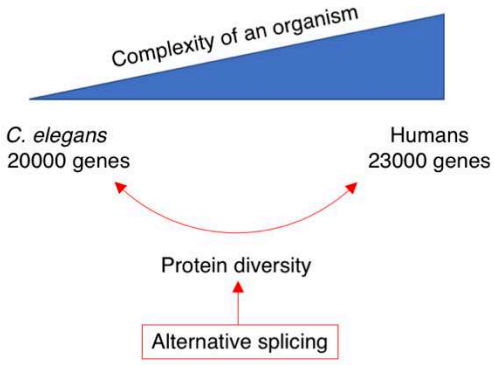

Figure 2 | The complexity of an organism decided by alternative splicing.

1.1.3. Cis-acting elements in pre-mRNA

This section is focussed on the elements present in pre-mRNA that defines the introns to be recognized and removed by the spliceosome during the splicing reaction. The pre-mRNA contains elements that are short and conserved sequences such as 5′ splice site (SS), 3′SS and branch site (BS) (**Figure 3**). During the initial stages of splicing reaction, the 5'SS is recognized by the U1 snRNP and later U2 snRNP is recruited to the branch site/point (Will and Lührmann, 2011).

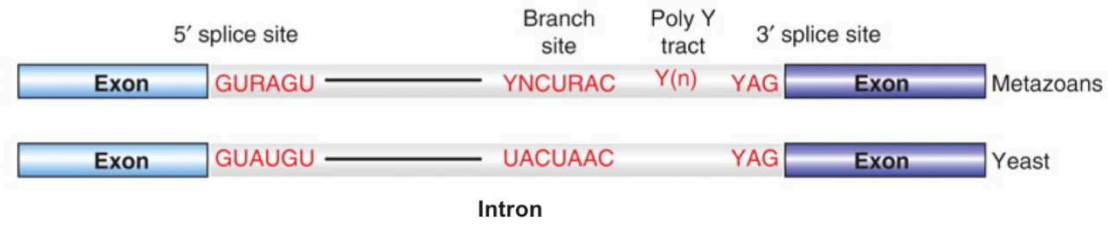

Figure 3 | The cis-acting elements of a metazoan and yeast pre-mRNA. Conserved sequences found at the 5′ and 3′ splice sites and branch site of a pre-mRNA introns in metazoans and budding yeast (*S. cerevisiae*) are shown in red letter. Y = pyrimidine and R = purine. The polypyrimidine tract is indicated as (Yn). The figure is adapted from (Will and Lührmann, 2011)

1.1.4. Spliceosome – the splicing machinery

Every cellular process is carried out by a specific machinery. For instance, replication is carried out by a DNA polymerase whereas transcription and translation are carried out by RNA polymerase and ribosomes respectively. Similarly, pre-mRNA splicing is carried out by a complex and dynamic machinery called spliceosome.

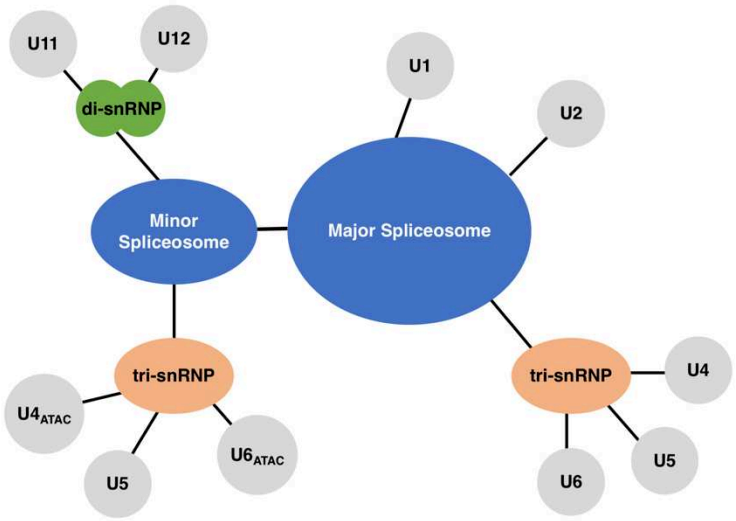

Figure 4 | The components of the major and minor spliceosome. U1, U2, U4 and U6 snRNPs are specific to major spliceosome. The U11, U12, $U4_{ATAC}$ and $U6_{ATAC}$ snRNPs are specific to minor spliceosome. U5 snRNP is common to both spliceosomes. The figure is adapted from (Vazquez-Arango and O'Reilly, 2017)

The spliceosome is of two types, major and minor spliceosome. The major spliceosome (containing U1, U2, U4, U5 and U6 snRNP) is responsible for the splicing of a vast majority of pre-mRNA introns (called U2 type introns). The minor spliceosome made of U11, U12, U5, U4atac and U6atac is responsible for splicing of a rare subset of

pre-mRNA introns called U12 type intron (**Figure 4**). These U snRNPs are responsible for carrying out a number of catalytic functions during splicing through their interaction with the RNA and protein components of the spliceosome. Altogether, these snRNPs bring about a 3D structure containing two active sites that catalyze the two steps of splicing reactions (shown in **Figure 1)** (Will and Lührmann, 2001).

1.1.4.1. U snRNPs

Both the spliceosomes are made of five different snRNP proteins and their associated non-snRNP proteins (Wahl and Lührmann, 2015a). Each snRNP protein is again made of different components such as snRNA and proteins. All the five snRNPs (except U6 snRNP) contains the same set of seven Sm proteins (B/B′, D3, D2, D1, E, F, and G). The U6 snRNP contains LSm proteins (Lsm2-8) in place of Sm proteins. All the RNA and protein components of the six snRNPs are shown in **Figure 5**.

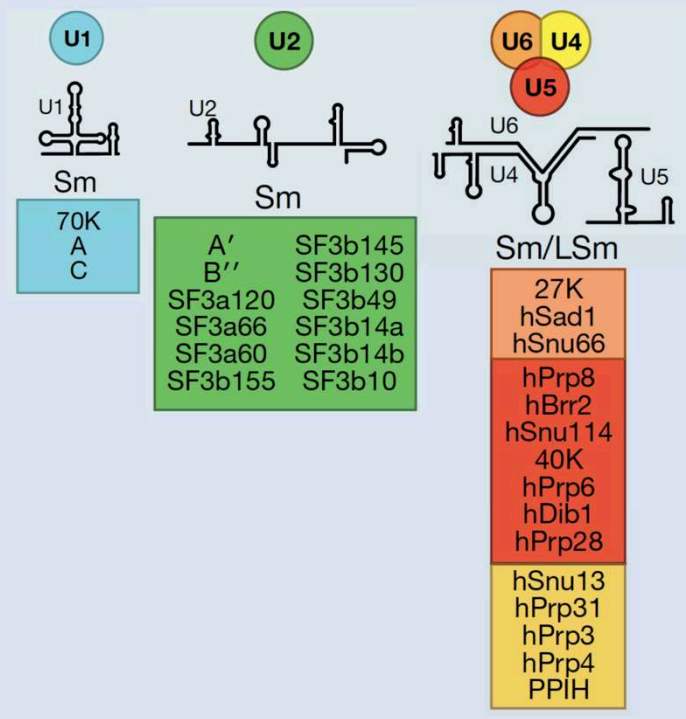

Figure 5 | The protein and RNA components of human spliceosomal snRNPs. All seven Sm proteins (B/B', D3, D2, D1, E, F, and G) or LSm proteins (Lsm2-8) are indicated by "Sm" or "LSm" at the top of the boxes showing the proteins associated with each snRNP. The U4/U6.U5 tri-snRNP contains two sets of Sm proteins and one set of LSm proteins. The figure is adapted from (Wahl and Lührmann, 2015a).

1.1.4.2. Multi-protein subunits of the spliceosome

Apart from the core spliceosomal snRNPs, spliceosome also contains many pre-organized multi-protein subunits such as the Prp19 complex (Prp19), the Intron-Binding Complex (IBC), the pre-mRNA REtention and Splicing complex (RES) and the Exon Junction Complex (EJC) (**Figure 6**).

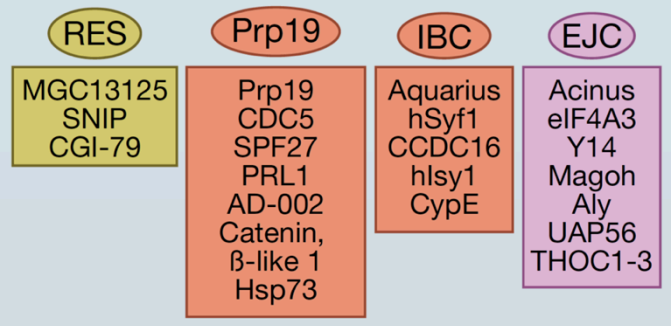

Figure 6 | The multi-protein subunits of the spliceosome. The different proteins that make each of the multi-protein complexes are shown. The figure is adapted from (Wahl and Lührmann, 2015a).

The RES complex is an evolutionarily conserved trimeric complex that associates with spliceosome before the first step of splicing reaction. It enhances the splicing of a subset of transcripts and promotes the nuclear retention of unspliced pre-mRNAs in yeast (Dziembowski et al., 2004). It is required for the efficient transformation of the precatalytic B spliceosome into an activated B^{act} complex (Bao et al., 2017). The Prp19 or NineTeen complex is important for the stable association of U5 and U6 with the spliceosome during spliceosome activation (Chan and Cheng, 2005). This complex is necessary for the first step of splicing (Makarova et al., 2004). This complex is also shown to be important for the formation of stable tri-snRNP (U4/U6.U5 snRNP) by K63 mediated ubiquitination of Prp3 (a component of U4 snRNP), which increases the affinity of Prp3 towards Prp8 (a component of U5 snRNP) (Song et al., 2010). IBC is a pentameric complex and it plays a significant role in Exon Junction Complex (EJC) assembly (De et al., 2015). The exon junction complex (EJC) is a dynamic multi-protein complex loaded onto nuclear-spliced mRNAs upstream of exon-exon junctions. It plays an important role in splicing, translational yield, NMD (nonsense-mediated mRNA decay), subcellular mRNA localization and mRNA export (Tange et al., 2004; Wang et al., 2014b). All these multi-protein subunits help in the different steps of spliceosome assembly enabling the faithful splicing reaction.

The other components of the spliceosome such as the RNA helicases will be discussed along with the spliceosome assembly.

1.1.5. Spliceosome assembly

The spliceosome is known to contain U1, U2, U4, U6, and U5 snRNPs and non-snRNP proteins. Unlike other types of machinery, the spliceosome is not a pre-assembled complex. Instead, it assembles on a pre-mRNA in a stepwise manner (Frendewey and Keller, 1985; Konarska and Sharp, 1986). Yeast and humans are widely used as a model system to study the assembly of the spliceosome.

The spliceosome assembly starts with the U1 snRNP where 5'end of the U1 snRNA forms base-pairing with the 5'SS of the intron in an ATP independent manner. In higher eukaryotes, this interaction is stabilized by the serine-arginine rich (SR) proteins and the protein components of U1 snRNP (Cho et al., 2011; Staknis and Reed, 1994) **(Figure 7)**. In the next step, SF1/BBP protein bind to BS (branch site) and the U2 auxiliary factor (U2AF) subunits, U2AF65 and U2AF35, bind to the polypyrimidine tract and 3'SS respectively (**Figure 7**). At this stage, the spliceosome complex is called E complex.

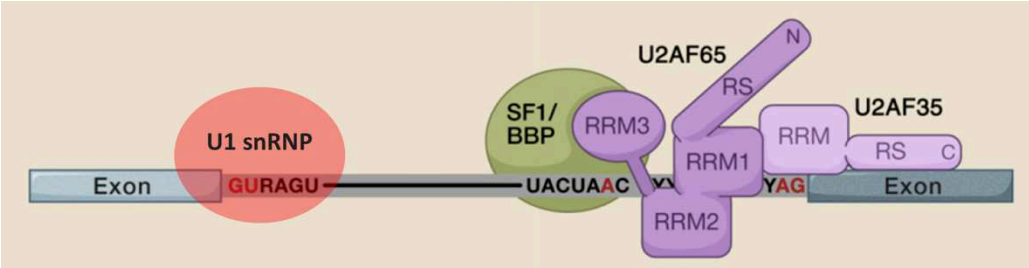

Figure 7 | The spliceosomal E complex. The pre-mRNA is shown with exons in blue and introns in grey. The 5'SS is recognized by the U1 snRNP (in orange). The branch site is bound by SF1/BBP, whereas the polypyrimidine tract and 3' splice site (SS) are bound by the U2 auxiliary factor (U2AF) subunits U2AF65 and U2AF35, respectively. U2AF65 binds both SF1/BBP and U2AF35. Figure adapted from (Wahl et al., 2009).

The next step is an ATP dependent process catalyzed by Prp5 and Sub2. During this step, the U2 snRNA base-pairs with the branch site (BS) and this weak interaction is stabilized by heteromeric protein complexes of U2snRNP (SF3a and SF3b) and SR domain of U2AF65 (Gozani et al., 1996; Valcárcel et al., 1996). Then it is followed by replacement of SF1 from BS with the p14. At this stage, SF3b155 interacts with the C

terminal RRM (RNA recognition motif) of U2AF65 (Gozani et al., 1998). This complex is called pre-spliceosomal complex or A complex (**Figure 8**).

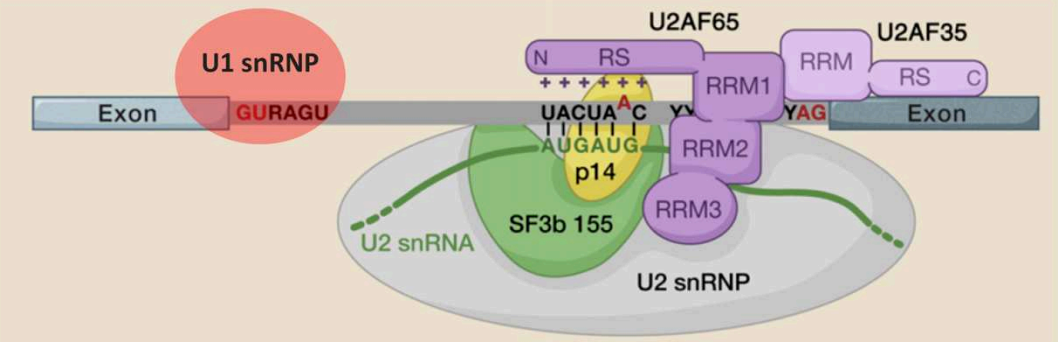

Figure 8 | The spliceosomal A complex. During the formation of A complex, the SF1/BBP is replaced with the U2-associated protein p14 which interacts with the BS while U2AF65 interacts with SF3b155. The base-pairing between U2 snRNA and BS is stabilized by components of the U2 snRNP and by the arginine-serine rich (RS) domain of U2AF65. Figure adapted from (Wahl et al., 2009)

Subsequently, the pre-assembled tri-snRNP (U4/U6.U5 snRNP) is recruited to the A complex. This leads to the formation of the B complex or pre-catalytic spliceosome. Though this complex contains all the proteins that are necessary for carrying out splicing reaction, it is still catalytically inactive (**Figure 9**). This B complex undergoes structural and component re-arrangement before it transforms into an active spliceosomal complex. At this stage, the U1 and U4 snRNP are eliminated from the B complex. It is further remodeled by Brr2 and stabilized by a Prp19 complex (not shown in Figure 9) to form the B^{act} complex. The B^{act} complex is subsequently transformed into a catalytically active B* complex by the ATPase, Prp2. The activated B* complex carries out the first step of splicing and thus leads to the formation of C complex. Later the C complex undergoes additional rearrangement by the ATPase, Prp16 in order to perform the second step of splicing. Subsequently, the post-splicing complex is released from mRNA by the ATPase, Prp22. Finally, all the snRNPs are individualized by ATPases Prp43 and Brr2 and recycled for the next cycle of spliceosome assembly (Wahl and Lührmann, 2015a; Wahl et al., 2009) (**Figure 9**).

Though the initial steps of spliceosome are well studied, the late complexes are still not well characterized. With the evolution of cryo-EM techniques and discovery of new splicing inhibitors the intermediate and less transient complex are being more and more discovered. The "resolution revolution" (Kühlbrandt, 2014) inspired the groups of Luhrmann, Nagai and Shi to apply state-of-the-art electron cryo-microscopy techniques

to the spliceosome. They identified and resolved the structures of few other intermediate complexes such as pre-B complex (Boesler et al., 2016), C* complex (Bertram et al., 2017; Fica et al., 2017; Yan et al., 2017; Zhang et al., 2017), ILS (intron-lariat spliceosome) (Yan et al., 2015). The high resolution of these cryo-EM structures of spliceosome provided the mechanistic insights and revealed the molecular mechanism of the pre-mRNA splicing (Scheres and Nagai, 2017). The ordered assembly of spliceosome containing these newly identified complexes is shown in **Figure 10**.

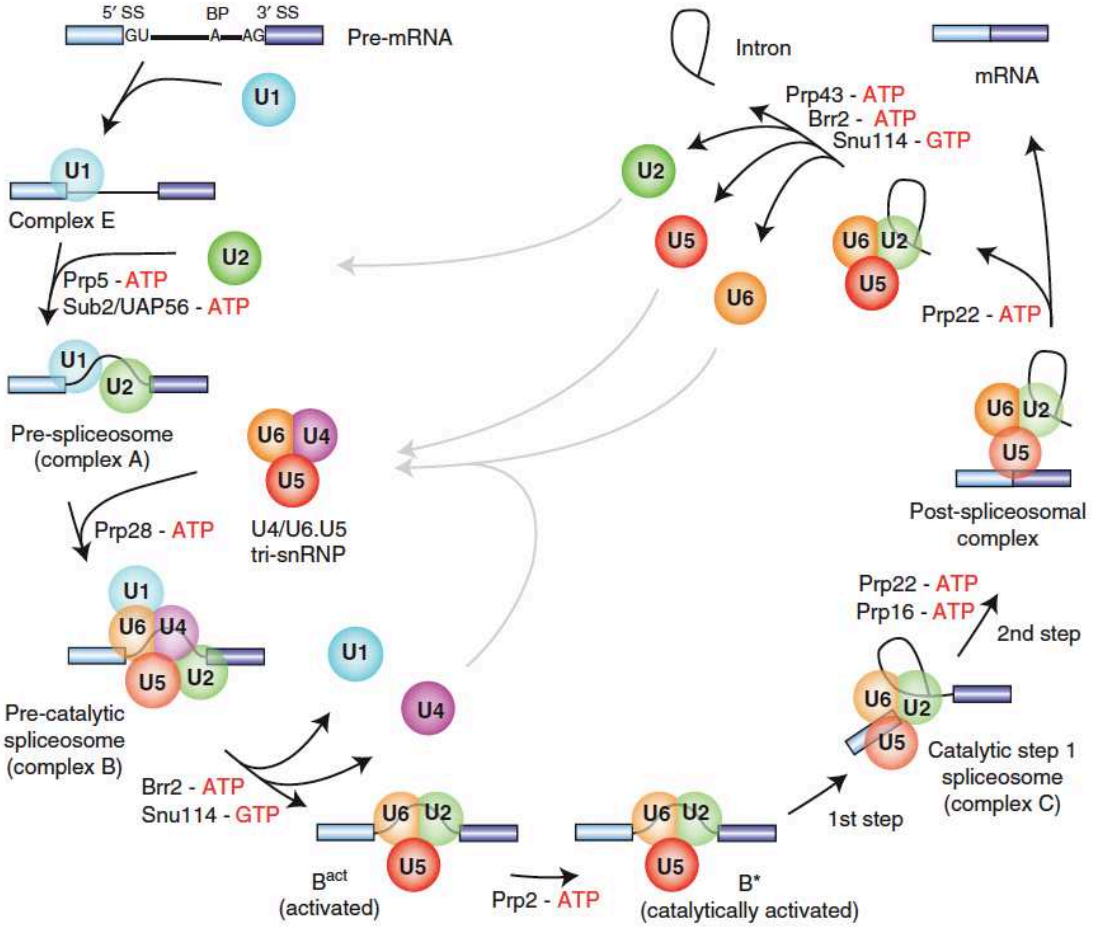

Figure 9 | Canonical assembly and disassembly pathway of the major spliceosome. The pre-mRNA containing exon and intron sequences are indicated by boxes and lines, respectively. The step-wise interactions of the snRNPs (indicated by circles) are shown. For simplicity, the non-snRNP proteins (like Prp19) are not included in the figure. The RNA helicases and ATPases which facilitate structural and conformational remodeling at each stage are indicated (Prp5, Sub2/UAP56, Prp28, Brr2, Prp2, Prp16, Prp22 and Prp43, or the GTPase Snu114). At the end of the splicing reaction, the spliceosome is released from mRNA and its components are recycled. Figure adapted from (Will and Lührmann, 2011).

Throughout the spliceosome assembly, a lot of structural and compositional re-arrangements enable the formation of different spliceosomal complexes. This remodeling is carried out by a class of proteins containing DEAD- and DEAH-box ATPases called RNA helicases (**Figure 9**). These helicases are responsible for the fidelity of the spliceosome assembly and hence ensures the faithful splicing reaction (Wahl et al., 2009).

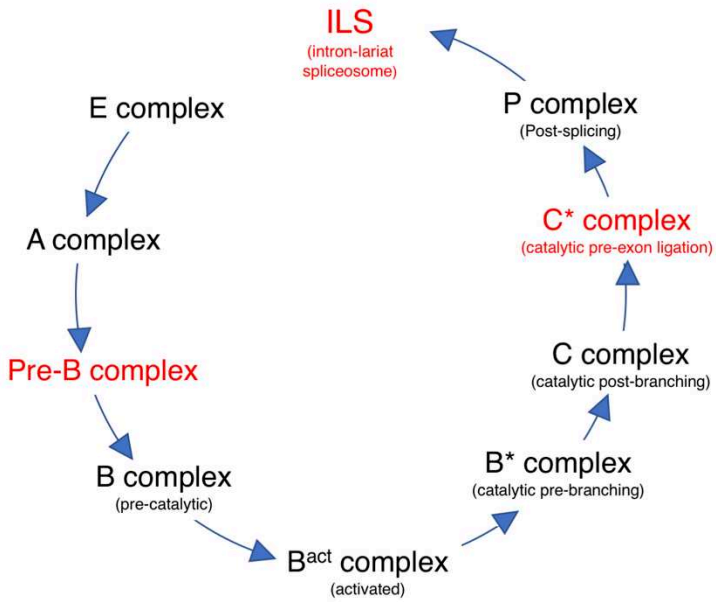

Figure 10 | Spliceosome assembly cycle. The schematic representation showing the order of spliceosome complexes. The newly identified complexes are shown in red. Figure modified from (Fica and Nagai, 2017).

1.1.6. Alternative pre-mRNA splicing

Alternative pre-mRNA splicing is a mode of gene regulation and results in the synthesis of different mRNA from a single primary transcript and thus is responsible for protein diversity in eukaryotes. The protein isoforms originating from alternative splicing can have different activities, interactions and differential localization and thus can serve different functions (Kelemen et al., 2013). Alternative splicing can also cause mRNA downregulation by nonsense-mediated decay (NMD) by introducing premature termination codons (PTCs). In certain cases, alternative splicing can impact regulatory regions that are important for mRNA stability, localization or translation efficiency (Kalsotra and Cooper, 2011). It is also important for all biological processes and determines the cell- and tissue-specificity and normal functioning of cells (Kalsotra and Cooper, 2011). Almost 95% of human multi-exon genes undergo alternative splicing (Pan et al., 2008). In the 1980s, the splicing of endogenous mammalian genes such as

immunoglobin and calcitonin was reported for the first time (Liu et al., 1980; Rosenfeld et al., 1981, 1982).

Depending on the physiological conditions, alternative splicing patterns of genes constantly change, allowing an organism to respond to changes in the environment. The real-life scenario of such alternative splicing is shown by Kurokawa et al., where the alternative splicing of phosphatidylinositol 3-kinase-related protein kinase (SMG-1) is altered as a result of stress in medical students due to exams. This might have later effects on nonsense-mediated RNA decay and the p53 pathway (Kurokawa et al., 2010). It would be very interesting to carry out similar studies with the Indian employees working in the field of "information technology" and "business-process-outsourcing" since they are diagnosed with serious health issues such as musculoskeletal symptoms, hypertension, diabetes, dyslipidemia, depression, anxiety, insomnia and obesity (Padma et al., 2015).

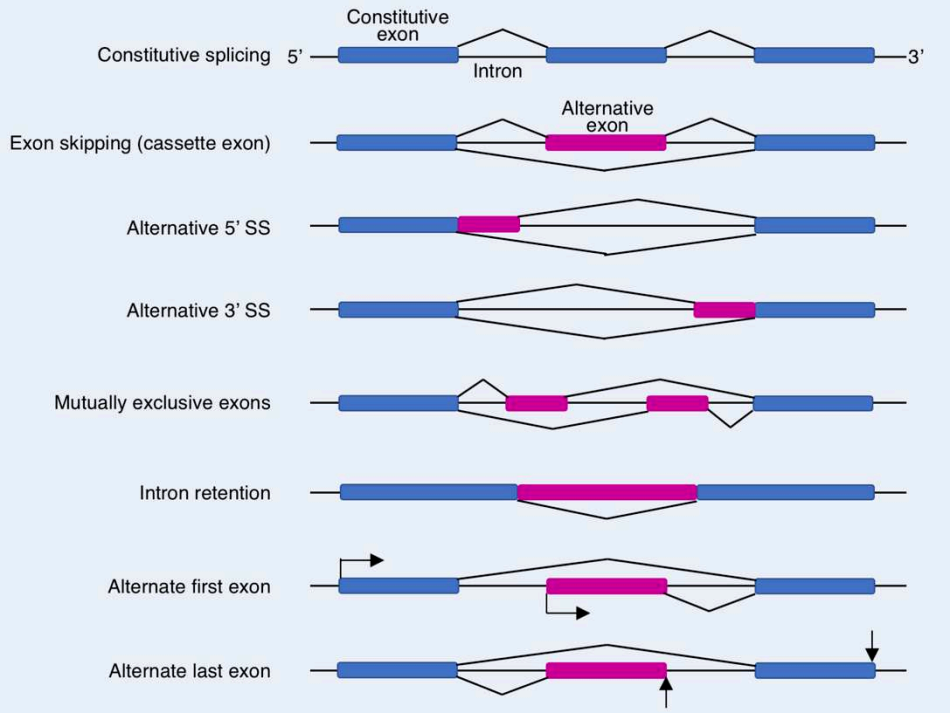

Figure 11 | Alternative splicing patterns. The different patterns of alternative splicing events that lead to inclusion or skipping of regions in mRNAs. Exons and introns that are not spliced are called constitutive. The main types of alternative splicing events are alternative 3' and 5' splice sites, cassette exons, retained introns and mutually exclusive exons. Figure adapted from (Wagner and Berglund, 2014; Wahl and Lührmann, 2015b).

In a mRNA that contains multiple exons, the splicing pattern can be of different types (**Figure 11**). Most of the exons are always spliced or included in the mRNA and this pattern of splicing is called constitutive splicing. An exon that is regulated in order to be either included or excluded from the mRNA is called a cassette exon. In some cases, the multiple cassette exons are mutually exclusive and thus produce mRNAs that always include exactly one of the several possible exon choices. The exon sizes can be altered by the use of splice sites (5' or 3 SS) at different position and the resulting splicing patterns are called Alternative 5'SS or Alternative 3'SS. Alternative promoters that alter the transcription start sites result in mutually exclusive alternate first exons. Similarly, regulation of alternative polyadenylation sites results in alternate last exons that are mutually exclusive. Finally, in some transcripts, the failure to remove an intron results in the splicing pattern called intron retention (Wagner and Berglund, 2014).

1.1.7. Regulation of alternative splicing

Alternative splicing can be regulated by the "additional cis-acting elements" and the trans-acting splicing factors. Apart from the cis-acting elements (5'SS, 3'SS and branch site), pre-mRNA also contains cis-regulatory elements (also called additional cis-acting elements) which are divided into four groups according to their position in the pre-mRNA (exon/intron) and their function (enhancers/silencers). These four elements are exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs) (Goren et al., 2006). These cis-regulatory elements are targeted by RNA-binding proteins (RBPs) called trans-acting splicing regulators. These trans-acting splicing factors include heterogeneous nuclear ribonucleoprotein (hnRNPs) (Gallinaro et al., 1981) and serine-arginine-rich proteins (SR proteins). These RBPs are non-snRNP (non-core) proteins that are not strictly required for spliceosome assembly or splicing catalysis. Instead, these RBPs promote or interfere with the recruitment of spliceosome components (U1 and U2 snRNPs) to particular splice sites (**Figure 12**). For instance, hnRNPs bind to silencer sequences on introns and exons (ESS and ISS) and prevent the recruitment of spliceosome components and thus prevent a particular exon from being included or spliced. On the other hand, SR proteins bind to the enhancer sequences on introns and exons (ESE and ISE) to promote the recruitment of spliceosome components and thus favours the inclusion or splicing of particular exons (Coelho and Smith, 2014). Overall these two factors (cis-regulatory elements and trans-acting splicing factors) together majorly regulate the alternative splicing.

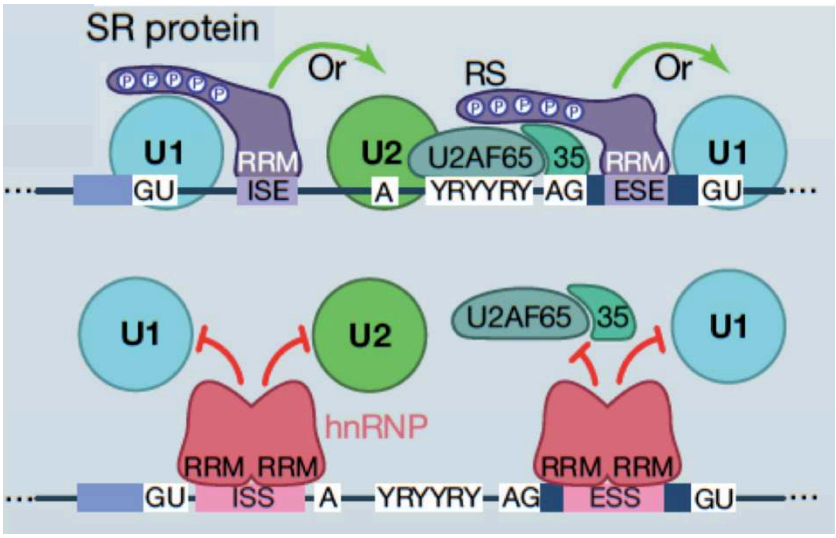

Figure 12 | The regulators of alternative splicing. The pre-mRNA containing the cis-regulatory elements (ESS, ESE, ISS, ISE) are shown in pink and purple colour. The trans-acting splicing factors that bind to these cis-regulatory elements are shown in violet colour (called SR protein) (in top panel) and maroon colour (called hnRNPs) (in bottom panel). These trans-acting factors either promote or prevent the recruitment of the spliceosomal components U1 and U2 snRNPs to particular splice sites. The figure is adapted from (Wahl and Lührmann, 2015b).

Another mode of regulating alternative splicing is through the combinatorial effect (cooperation or competition between splicing factors) of different RBPs (**Figure 13a and 13b**). For instance, different cell types express different amounts of RBPs and this can influence alternative splicing. These kinds of combined effects of RBPs are attributed to cell and tissue-type specific alternative splicing patterns (Fu and Ares, 2014). The expression and localization of these splicing factors can also vary in certain physiological conditions based on the environmental cues and in turn, can also regulate the alternative splicing patterns. An increasing amount of evidence suggests that the speed of the transcription elongation may also influence the splice site selection thus modulating the alternative splicing (**Figure 13c**) (Fu and Ares, 2014; Kornblihtt, 2007).

In addition to these regulatory elements, the secondary structure of the pre-mRNAs can sequester the cis-regulatory elements from being recognized by the splicing factors. The splicing factors usually recognize only the single-stranded form of cis-regulatory elements (Coelho and Smith, 2014). Also, the epigenetic changes such as DNA and RNA methylation, chromatin structure and histone modifications also regulate alternative splicing (Coelho and Smith, 2014; Luco et al., 2010).

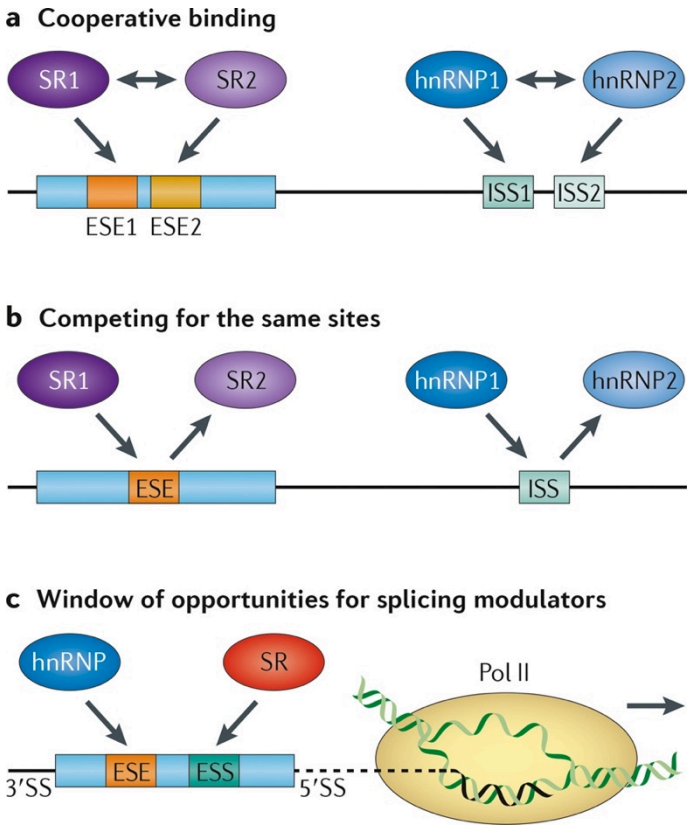

Figure 13 | Cooperation and competition between splicing factors in regulating alternative splicing. (a) Distinct splicing factors may cooperatively bind to adjacent cis-regulatory elements, which may be mediated, at least partly, by their protein–protein interactions (indicated by two-headed arrows). (b)The same cis-regulatory elements, regardless of whether they are located in exons or introns, may be recognized by related RNA-binding splicing regulators. This results in competition between related splicing regulators. (c) The speed of transcription elongation may influence splice site selection, which creates opportunities for the splicing factors to recognize their binding sites co-transcriptionally, thus modulating alternative splicing during these integrated events in gene expression. 3′SS, 3′ splice site; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; hnRNP, heterogeneous nuclear ribonucleoprotein; ISS, intronic splicing silencer; Pol II, RNA polymerase II. The figure is adapted from (Fu and Ares, 2014)

Generally, SR and hnRNP proteins are considered as trans-acting splicing factors. However, a subset of the core spliceosomal proteins apart from its general role in spliceosome assembly is also regulating alternative splicing (Papasaikas et al., 2015). For instance, the depletion of core snRNP protein SmB/B9 resulted in a reduction in the inclusion of multiple alternative exons including its own pre-mRNA in HeLa cells (Saltzman et al., 2011). Depletion of an another spliceosome component, PRPF8 is reported to be preferentially altering the splicing of a subset of genes that contain weak 5’ splice site and these genes are enriched to mitotic progression (Wickramasinghe et al., 2015). The effect of depletion of core spliceosomal proteins on alternative splicing could

result from a decrease in the ratio of spliceosomal components to splice sites and thus creates a competitive environment. When the core spliceosomal proteins are abundantly present, their recruitment to splice sites would not be rate limiting and under such a situation, the usual factors regulate alternative splicing. But when the core spliceosomal proteins are less abundant, the spliceosomal components are recruited efficiently only to certain splice site pairs and thus would result in altered splicing pattern (Nilsen and Graveley, 2010). In most of the cases, depletion of spliceosome components alter the dynamics of spliceosome complex assembly and the minimum availability of core spliceosome components creates a tug-of-war between strong and weak splice sites and in turn leads to alteration of splicing pattern.

1.1.7.1. Heterogeneous nuclear ribonucleoproteins (hnRNPs)

The hnRNPs belongs to a large family of RNA binding proteins (RBPs) that are involved in multiple nucleic acid related metabolism, including alternative splicing, mRNA stabilization, and transcriptional and translational regulation. The hnRNP proteins were initially described as a major group of chromatin-associated RBPs (Dreyfuss et al., 1993; Swanson, 1995). They are bound to nascent RNA as soon as they are transcribed and form a hnRNP complex which is essential for their function in RNA metabolism. Approximately 30 hnRNP proteins were identified from the immunoprecipitation of the hnRNP complex proteins (Dreyfuss et al., 1993; Swanson, 1995). Many experimental pieces of evidence indicate shuttling of many hnRNPs between nucleus and cytoplasm (Dreyfuss et al., 1993; Nakielny and Dreyfuss, 1997; Swanson, 1995). The hnRNP proteins play diverse roles both in nucleus and in cytoplasm and regulate the gene expression at various levels.

The hnRNP proteins possess different types of RNA binding domains, which directly interact with RNA, most likely in a sequence-specific manner. One such prevalent domain is the RRM (RNA recognition motif) that mediates specific interactions with the pre-mRNA. All hnRNPs contain this RRM except for hnRNPs E/K, which interact with RNA via the hnRNP KH (K homology) domain. Most hnRNPs also harbour RGG boxes (repeats of Arg-Gly-Gly tripeptides), and additional glycine-rich, acidic or, proline-rich domains (Dreyfuss et al., 1993) (**Figure 14**). Typically, the RRM is characterized by a β_1-α_1-β_2-β_3'-α_2'-β_4' structure and the presence of two highly degenerate RNP consensus sequences, RNP-1 and RNP-2. Some hnRNPs also contain

atypical RRM (aRRM) that differ from the usual RRM. For instance, hnRNP-H/F contains an extra β_3' loop compared with classical RRMs and lack the RNP consensus sequences. Similarly, hnRNP-I contain five strands in the β-sheet of its second and third RRM domains and also lacks the consensus RNP sequences (Geuens et al., 2016; Han et al., 2010). These domain variabilities in hnRNPs contribute to their structural and functional diversity allowing them to have different RNA-binding efficiency.

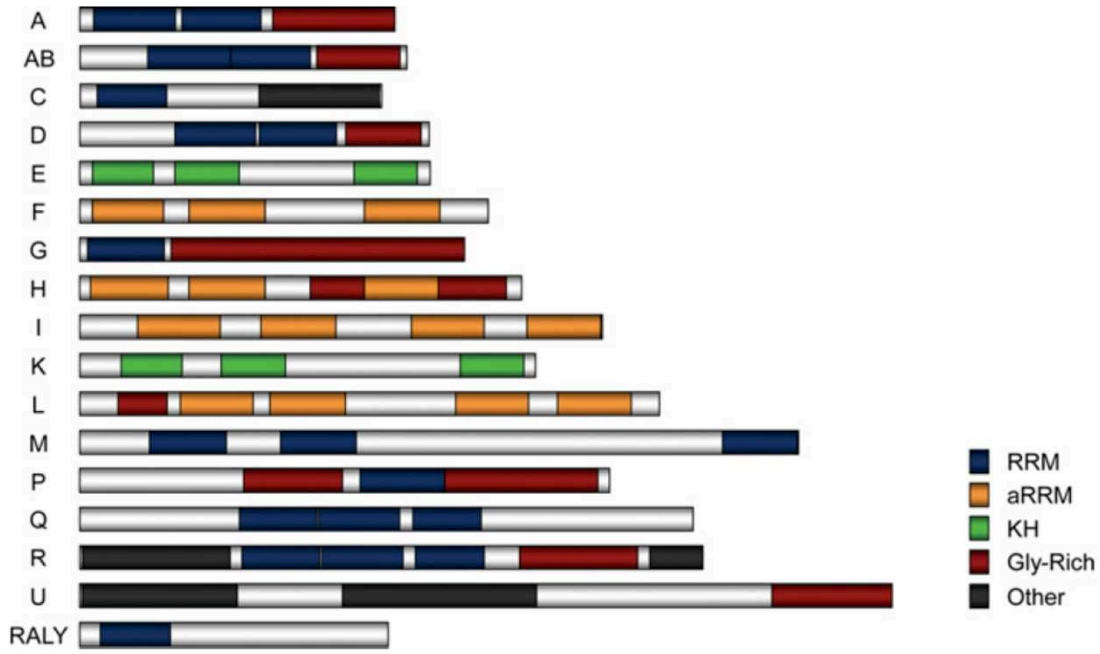

Figure 14 | Domain structure of hnRNP proteins. The alphabets on the left side of the figure represent different hnRNP proteins. 'A' represents the hnRNP A/B proteins (A0, A1, A2/B1 and A3) that are structurally similar. Gly-Rich – glycine-rich; RRM – RNA recognition motif; aRRM – atypical RNA recognition motif; KH – K homology. The figure is adapted from (Han et al., 2010).

1.1.7.2. Serine- and arginine-rich proteins (SR proteins)

The SR proteins are named after their typical RS domain that is rich in serine (Ser, S) and arginine (Arg, R). They are conserved in evolution and are present in all metazoan organisms and plants. They function in both constitutive and alternative splicing. SRSF1 (ASF/SF2) is the first member of SR proteins that was identified as a splicing factor that is required for the splicing of a β-globin pre-mRNA *in vitro* (Krainer and Maniatis, 1985). SRSF2 (SC35) is the second member of SR proteins that was identified to be important for the first step of splicing reaction (Fu and Maniatis, 1992). These two SR proteins are characterized with one or two conserved RRM in the N-terminal and an RS domain in the

C-terminal. Soon it was identified that there are many other proteins that contain similar domains. There were many proteins that share some but not all the features, such as the presence of RRM and RS domains and the function in splicing, and yet they are not grouped into the same family. In order to avoid this confusion, Manley and Krainer defined that SR proteins are the proteins with the following minimal attributes "one or two N-terminal RRMs, followed by a downstream RS domain of at least 50 amino acids with >40% RS content, characterized by consecutive RS or SR repeats". Finally, they came up with 12 proteins following those features and grouped them as canonical or classical SR proteins. They named these 12 proteins as SRSF 1-12 (**Figure 15**) (Manley and Krainer, 2010). The remaining proteins that follow only a few of these defined features are termed as SR like or SR related proteins (Long and Caceres, 2009). For instance, RNPS1 protein which contains both SR and RRM domains are yet considered as SR like proteins since its SR domain is in N-terminal and RRM domain is in the C-terminal.

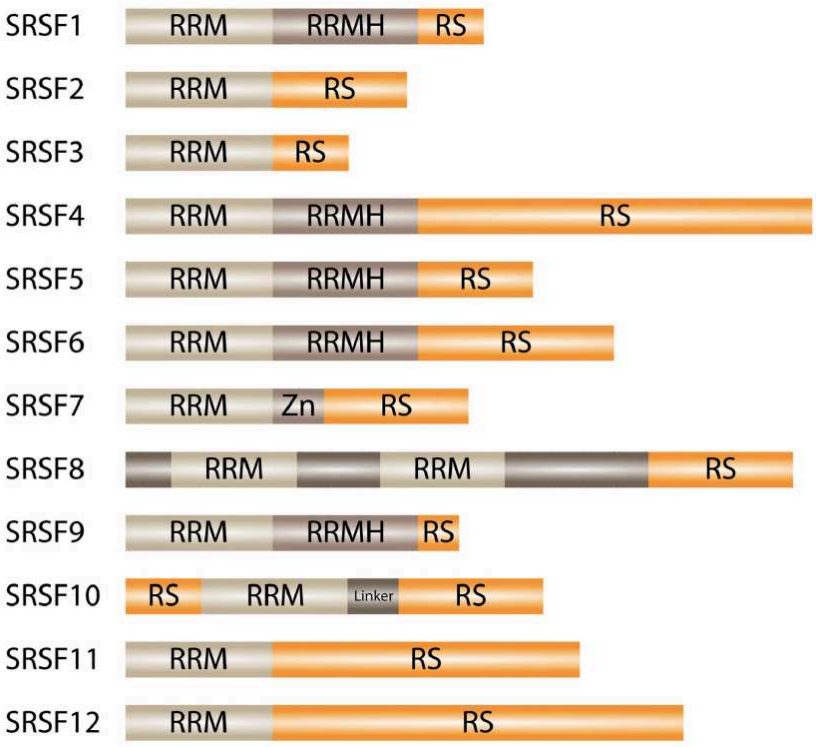

Figure 15 | Domain structure of human serine/arginine (SR) proteins. Serine/arginine-rich splicing factor (SRSF) 1–12 are members of the canonical SR protein splicing family that is defined by the N-terminal RNA recognition motifs (RRMs) followed by a downstream arginine/serine (RS) domain. The RRM is responsible for RNA binding, while the RS domain mediates protein/protein interactions.

1.1.7.2.1. Regulation of SR proteins

The RRM domain is generally known for its ability to bind to RNA whereas RS domain is known for its ability to promote protein-protein or protein-RNA interaction. Additionally, the RS domain acts as an NLS (nuclear localization signal), affecting the subcellular localization of SR proteins by mediating the interaction with the SR protein nuclear import receptor, transportin-SR (Kataoka et al., 1999; Lai et al., 2000). Both the RRM and RS domains are required for the recruitment of SR proteins to the site of transcription from the nuclear speckles (Misteli et al., 1998).

Many SR proteins are exclusively present in the nucleus whereas few SR proteins (SRSF1, SRSF3, SRSF4, SRSF6, SRSF7 and SRSF10) are known to shuttle between the nucleus and cytoplasm. The differential expression, localization and regulatory pattern suggest non-redundant and distinct roles in pre-mRNA splicing. The shuttling of SR proteins is also implicated in protein translation (Sanford et al., 2004; Swartz et al., 2007). Botti et al. showed the shuttling of two SR proteins (SRSF2 and SRSF5), which was previously thought to be nuclear. However they observed this effect only in pluripotent P19 mouse embryonic carcinoma cells but not in P19 differentiated neural cells and HeLa cells and this effect is dependent on the post-translational status of the SR proteins (Botti et al., 2017).

Post-translational modifications (PTMs) of SR proteins is an important signal that modulates various aspects of SR proteins namely localization, activity, interaction and stability. All SR proteins are phosphorylated predominantly in their SR domains. Phosphorylation is the most studied PTMs of SR proteins. It is known that phosphorylation on SR domains is responsible for their recruitment to the site of transcription from the nuclear speckles (Misteli et al., 1998). Gui et al., purified the first member of the SR-specific kinase called SRPK1 (SR protein specific kinase 1), that is responsible for the redistribution of SR proteins from highly localized nuclear speckled domains in interphase cells to the nucleoplasm when cells enter mitosis. SRPK1 is specific for the phosphorylation of the SR domain present in all SR proteins (Gui et al., 1994a, 1994b). Moreover, SRPK1 shows the highest activity during mitosis which is responsible for the re-distribution of SR proteins from nuclear speckles.

The phosphorylation-dephosphorylation cycle of SR proteins is important for the spliceosome assembly and splicing catalysis. For instance, phosphorylation of the RS domain in SF2/ASF is responsible for enhancing the protein–protein interactions with

other RS domain containing splicing factors, such as U1-70K (Xiao and Manley, 1997). On the other hand, dephosphorylation of SR and SR-related proteins is also required for splicing catalysis to proceed (Cao et al., 1997; Tazi et al., 1993). Inhibition of any of these two ordered events (phosphorylation and dephosphorylation) does not favour the completion of the splicing catalysis.

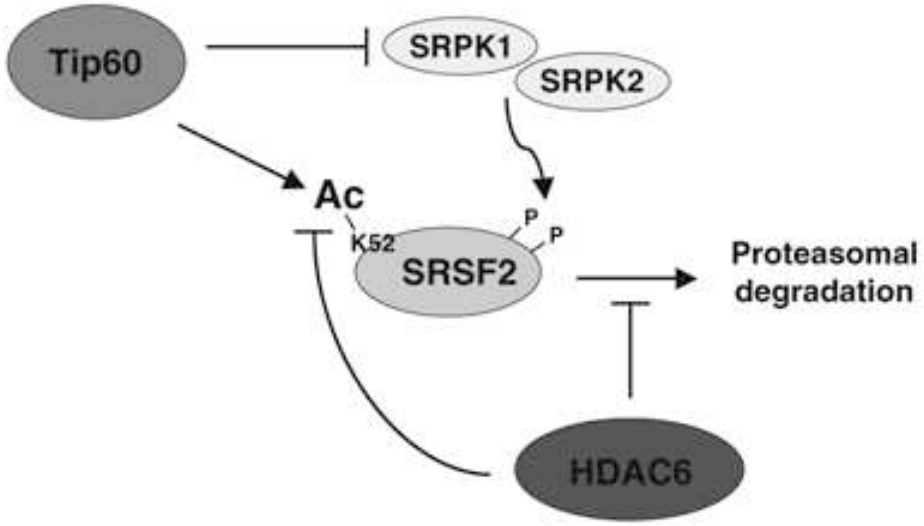

Figure 16 | Destabilization of SC35 by TIP60. TIP60 prevents the translocation of SRPK1 and SRPK2 to the nucleus and acetylates SC35. The prevention of the nuclear translocation of SRPK1 and SRPK2 maintains SC35 in hypo-phosphorylated form. This hyperacetylated/hypo-phosphorylated form of SC35 is degraded by the proteasome. On the other hand, HDAC6 counteracts this effect of TIP60 on SC35. The figure adapted from (Edmond et al., 2011).

A hand full of studies shows that the phosphorylation and other PTMs of SR proteins are known to impact on the stability of the SR proteins. SRSF2 or SC35 is the best example to explain the role of PTMs in regulating the stability of SR proteins. Edmond et al., described that SC35 undergoes acetylation at lysine 52 at RRM by the acetyltransferase Tip60 which leads to degradation of SC35 by the proteasome. TIP60 also prevents the phosphorylation of SC35 by inhibiting the nuclear translocation of SRPK1 and SRPK2 and thus leading to degradation of SC35 proteins. This effect of TIP60 can be counteracted by HDAC6 (Edmond et al., 2011) (**Figure 16**). From this study, it can also be assumed that nuclear SRPK could act as an important factor for the stability of SR proteins such as SC35. Gonçalves et al., showed that the inhibition of GSK3β leads to decrease in SRPK1 protein levels, which in turn leads to cytoplasmic accumulation and degradation of ASF/SF2 (SRSF1) (**Figure 17**) (Gonçalves et al., 2014).

Similarly, Moore et al and Jones et al., reported the destabilization of ASF/SF2 protein levels upon inhibition of Aurora-A (Jones et al., 2017; Moore et al., 2010).

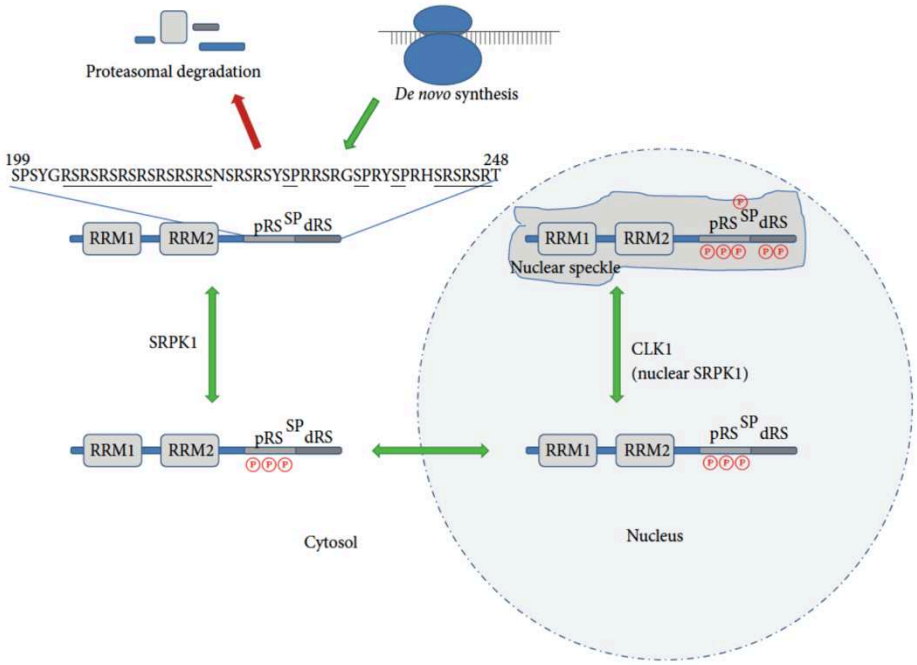

Figure 17 | Posttranslational modifications regulating SRSF1 (ASF/SF2) protein. Cytoplasmic SRPK1 constitutively phosphorylate the just synthesized cytoplasmic SRSF1 protein at serine residues in the proximal SR domain (pRS). This allows the nuclear import of SRSF1, followed by a second phosphorylation step at the distal SR domain (dRS) that includes several serine-proline (SP) motifs by CLK1 or nuclear SRPK1. SRSF1 phosphorylated at both proximal and distal SR allows its accumulation in nuclear speckles from where it is recruited to the spliceosome. SRSF1 dephosphorylation induces its nuclear to cytoplasmic translocation and lack of phosphorylation by SRPK1 in the cytosol leads to its proteolytic degradation. The figure is adapted from (Gonçalves and Jordan, 2015).

Another study shows the increased ubiquitylation of SRSF1 in activated T cells and its degradation by proteasome. Interestingly, T cells from patients with SLE (systemic lupus erythematosus) showed increased ubiquitylation of SRSF1 when compared to those from healthy individuals (Moulton et al., 2014). A similar proteasome mediated degradation was observed in other SR proteins such as SRSF5 during late erythroid differentiation (Breig and Baklouti, 2013). In contrast to SC35 (SRSF2), acetylation of SRSF5 by TIP60 on K125 antagonizes the Smurf1 mediated ubiquitylation and degradation of SRSF5 upon glucose intake. Upon glucose starvation, the HDAC1 deacetylates SRSF5 and mediates its degradation by proteasome (Chen et al., 2018). Overall all these reports suggest that each SR protein follows a different mode of degradation. The stabilization/degradation of the same SR proteins can vary depending

on the cell and tissue type since the degradation of each SR protein relies on more than one regulatory proteins and the expression and localization of these regulatory proteins might vary between different cell and tissue types.

1.1.8. Alternative splicing and disease

The defects in the regulation of splicing have been implicated in many different diseases. It is known that almost 15% of the mutation that causes genetic diseases alter the pre-mRNA splicing (Srebrow and Kornblihtt, 2006). The mutation in cis-acting elements or cis-regulatory elements or trans-acting splicing factors or spliceosomal proteins can be responsible for human pathogenesis such as cancer. In addition, the mis-regulation of the expression of these trans-acting splicing factors and spliceosomal proteins can also cause human diseases. The defects in these elements give rise to the selection of a wrong splice site and, which in turn, alter the alternative splicing pattern. The emerging knowledge in understanding the normal and pathological splicing regulation would help in developing treatment options.

The mutation in the core spliceosomal protein, PRPF31 and PRP8 is responsible for the autosomal dominant forms of retinitis pigmentosa (Boon et al., 2007; Vithana et al., 2001; Wilkie et al., 2008). A point mutation generates an alternative 3´SS in HBB (the gene that encodes β-globin) results in β^{+}-thalassaemia, a condition that is characterized by reduced β-globin protein levels and anaemia (Busslinger et al., 1981).

Spinal muscular atrophy (SMA) is a neurodegenerative disease caused due to deletion or loss-of-function mutations in the gene SMN1 which results in a decrease in SMN protein levels. Interestingly there is a homologous gene, SMN2. But then a single nucleotide difference in exon 7 causes decreased inclusion of exon 7 that in turn results in truncated protein isoform. It was hypothesized that the exon 7 of SMN2 could be more included by using an antisense oligonucleotide (Hua et al., 2011). This antisense oligonucleotide is now been approved by the FDA under the name of nusinersen/Spinraza (Biogen) and it is being used to treat SMA patients (Corey, 2017).

Cancer cells acquire 8 main traits for its development. These include sustaining proliferative signaling, reprogramming of energy metabolism, resistance to cell death, enabling replicative immortality, evasion of growth suppressors, acquisition of the ability to invade normal tissues and metastasize, induction of angiogenesis and avoidance of

immune destruction (Hanahan and Weinberg, 2011). Each of these fundamental traits could be linked to aberrant splicing regulations (Liu and Cheng, 2013) (**Figure 18**).

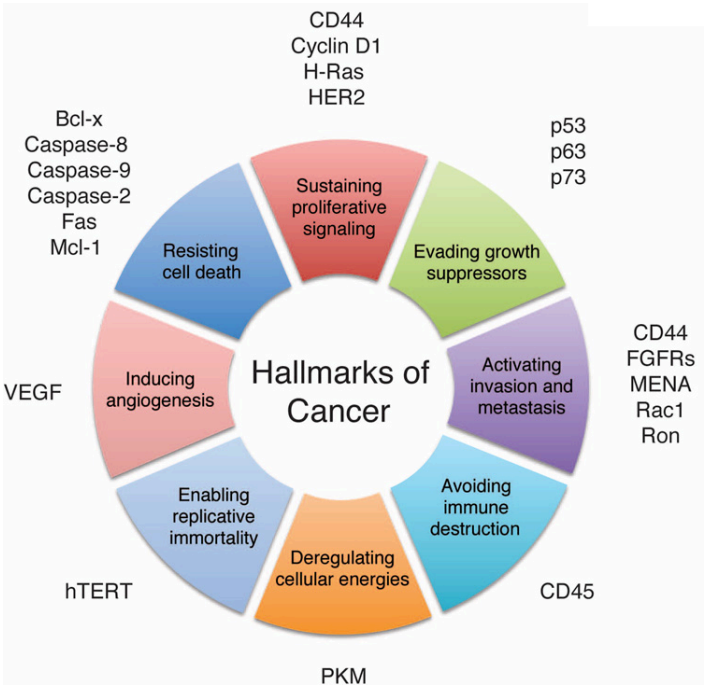

Figure 18 | Mis-regulated alternative splicing of genes responsible for cancer. The 8 traits that are obtained by cancer cells are shown. The splicing of genes that are mis-regulated is shown next to each trait. The figure is adapted from (Liu and Cheng, 2013).

Genome analysis of multiple tumours has revealed the frequent mutations in the core and auxiliary components of spliceosomes such as *SF3b1*, *U2AF35, SRSF2* and *ZRSR2* in haematological malignancies (Dvinge et al., 2016). Similarly, in solid tumours, the splicing factors are mutated but are less frequent. For instance, *SF3B1* in breast cancer and melanoma (Darman et al., 2015; Furney et al., 2013), *U2AF35* and *RBM10* in non-small cell lung tumours (Brooks et al., 2014) and *HNRNPL* in colon tumours (Sebestyén et al., 2016) are mutated. A large scale analysis of known and putative RBPs coding genes has shown that mutations in known and putative RBPs are mostly limited to these cases in solid tumours (Sebestyén et al., 2016). Furthermore, change in the expression of splicing factors greatly impacted on the alternative splicing when compared to the mutation in splicing regulators or factors (Jung et al., 2015; Sebestyén et al., 2015). Also, the mutation and differential expression of splicing factors seem to produce different splicing outcomes. For instance, modulating the expression of *SF3B1* in cells does not produce the same splicing outcome when *SF3B1* is mutated (Alsafadi et al., 2016). The

complete understanding of the relation between the splicing outcome and the mis-regulated splicing factors would enable developing a therapy against cancer. Many small molecule inhibitors are being identified and their anti-tumour properties are also being observed. For instance, pladienolides, spliceostatin A and its analogues sudemycins that target SF3b1 are known to have anti-tumour activity (Bonnal et al., 2012).

Alternatively, the mis-regulation of the splicing kinases such as SRPKs, CLKs and PRP4K is also responsible for the tumorigenesis (Corkery et al., 2015; Dominguez et al., 2016; Wang et al., 2014a). Targeting such kinases for cancer treatment does not represent a solid therapeutic option since increased or decreased activity or expression of these kinases are known to promote tumorigenesis (Wang et al., 2014a). Additionally, many recent studies report that many kinases previously known for their cell signalling activity are also involved in splicing regulation (Moore et al., 2010; Naro and Sette, 2013; Naro et al., 2014). These kinases include Nek2, Aurora-A, GSK3β, Akt, PKA, MAPKs, which are also known to be mis-regulated in cancer. Finally, few studies report the functional interaction between these signalling kinases and splicing specific kinases in cancer. For instance, GSK3β is known to regulate SRPK1 level (Gonçalves et al., 2014; Naro and Sette, 2013). Hence disruption of the functional interactions between these signalling and splicing kinases may represent a better strategy in cancer treatment, leaving the normal functions of these kinases untouched.

1.1.9. Nuclear speckles

Nuclear speckles or interchromatin granule clusters are dynamic structures that are enriched with proteins in pre-mRNA splicing and other RNA processing. They are 20 to 50 irregular shaped structures and are present throughout the nucleoplasm in regions where there are no or less DNA (**Figure 19**) (Lamond and Spector, 2003). Santiago Ramo´n y Cajal described a nuclear domain in 1910, which is now called as nuclear speckles (Lafarga et al., 2009). These structures act as storage, assembly and modification sites and supply splicing proteins to transcription sites called perichromatin fibrils (Lamond and Spector, 2003; Misteli et al., 1998; Spector and Lamond, 2011). Nuclear speckles contain many factors such as SR specific kinases that phosphorylate the splicing proteins allowing their re-localization to active transcription sites (Misteli et al., 1997).

Nuclear speckles are dynamic structures whose shape and size vary between different cell types depending on the transcription state of the cells. Many studies show

that the treatment of cells with drugs that inhibit the transcription directly or indirectly leads to accumulation of splicing factors in nuclear speckles. This in turn leads to enlarged and rounded nuclear speckles (Spector et al., 1984, 1991). Conversely, during viral infection where the transcription levels are very high, the splicing proteins are recruited to transcription sites and hence lead to reduced accumulation of splicing proteins in speckles (Spector and Lamond, 2011). This shows the importance of this structure in pre-mRNA splicing and gene regulation.

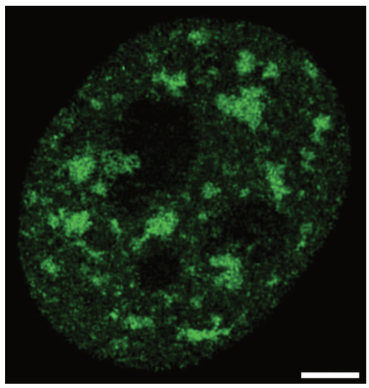

Figure 19 | Microscopy image of nuclear speckles. Hela cells expressing SC35-EYFP showing speckled pattern in the nucleus called nuclear speckles. The scale bar is 2 μm. The figure is adapted from (Spector and Lamond, 2011).

1.2. The Cell Cycle

Cell division is a fundamental mechanism conserved in all cell types that allow one cell to divide and give rise to two distinct and genetically identical daughter cells. The cell division cycle (cell cycle) describes the ordered sequence of events that lead to cell division. The cell cycle and its different phases were first described by Howard and Pele in 1951(Blanchet et al., 2009; Golias et al., 2004). Prokaryotes undergo cell division leading to the birth of a new organism through a process called binary fission. Many eukaryotes, on the other hand, undergo cell division for their growth and differentiation to form specialized organs. In the case of eukaryotes, cell division also helps in the renewal of lost cells. The cell cycle allows a number of phases that ensure very precisely defined functions of phases that follow one another but are also very closely interdependent. The two main phases are the interphase and mitosis (**Figure 20**).

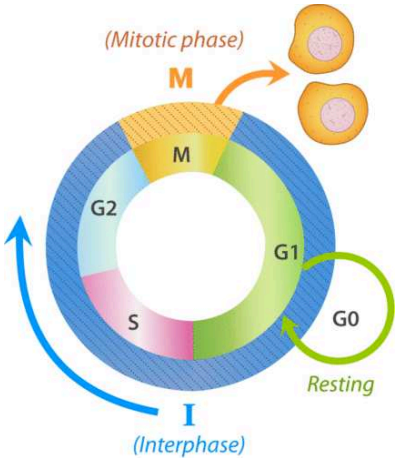

Figure 20 | Overview of cell cycle. In eukaryotic cells, the cell cycle is characterized by four distinct phases (G1, S, G2 and M phase). In G0 phase, cells enter a state of resting or quiescence. Figure Adapted from CK-12 Foundation (https://www.ck12.org/biology/cell-cycle/lesson/Cell-Cycle-MS-LS/)

1.2.1. Interphase

It is the phase that separates two successive mitosis phases. During interphase, the cell grows, duplicates its genetic material and prepares for its entry into the mitosis phase. This is the longest phase of the cell cycle. It consists of three phases, namely G1, S and G2.

1.2.1.1 G0 Phase

The G0 or quiescence phase corresponds to an exit from the cell cycle that occurs either normally after mitosis or during G1 in case of insufficient cell growth or damaged DNA. The two main reasons for a cell to enter the G0 phase are lack of nutrients and differentiation into a non-dividing cell type (eg muscle cell, neuron etc). On the other hand, senescence is a stage where the cell is not capable of further cell division (**Figure 20**) (Ekholm and Reed, 2000; Nurse, 2000).

1.2.1.2. G1 Phase

The cell cycle typically begins with the G1 phase. This phase is characterized by cell growth regulated by growth factors in the extracellular environment. The cell prepares itself for the duplication of its equipment. This phase is intervened by a check point and in case of insufficient nutrients or DNA damage; it exits the G1 to enter G0

(**Figure 20**). When the conditions for growth are ideal, the cells pass the check point and enter a new cycle.

1.2.1.3. S Phase

The S phase gets its name from DNA synthesis. During this phase, the cell duplicates not only its genetic material, but also the centrosomes. These duplications occur only once per cell cycle and allow each daughter cell to receive equivalent content. The intra-S-checkpoint detects faulty DNA replication and delay mitosis until replication is complete (Bartek et al., 2004) (**Figure 20**).

1.2.1.4. G2 Phase

The G2 phase is the second phase interval following the S phase and preceding mitosis (**Figure 20**). During this phase, the cell grows rapidly and prepares for entry into mitosis. During this phase a second point of control of the cell cycle intervenes, it is called G2 / M control point or DNA damage control. This control point, in case of DNA damage, allows the cell cycle to stop before entering into mitosis (Muotri and Gage, 2006). This allows the cell to repair its DNA before resuming its cycle. In case of irreparable damage, it triggers cell death. Defects in this checkpoint are associated with cancer.

1.2.2. Mitosis

Mitosis was described for the first time in 1882 by Walter Flemming (Dröscher, 2015). In this stage, the cell separates its content into two identical parts and distributes them among the two daughter cells (Nigg, 2001; Norbury and Nurse, 1992; Nurse, 2000). Mitosis consists of five stages namely, prophase, prometaphase, metaphase, anaphase and telophase. During prophase, the chromosomes are dense, the mitotic spindles begin to form. Prometaphase is characterized by the breakdown of the nuclear envelop, releasing chromosomes and allowing mitotic spindles to capture the chromosomes. In metaphase, the chromosomes are aligned and the kinetochores of each chromosome are attached to the microtubules from opposite spindle poles. Anaphase is characterized by the separation of the chromatids into separate chromosomes. Finally, in the telophase, two new nuclei, for each set of chromosome are formed. (**Figure 21**).

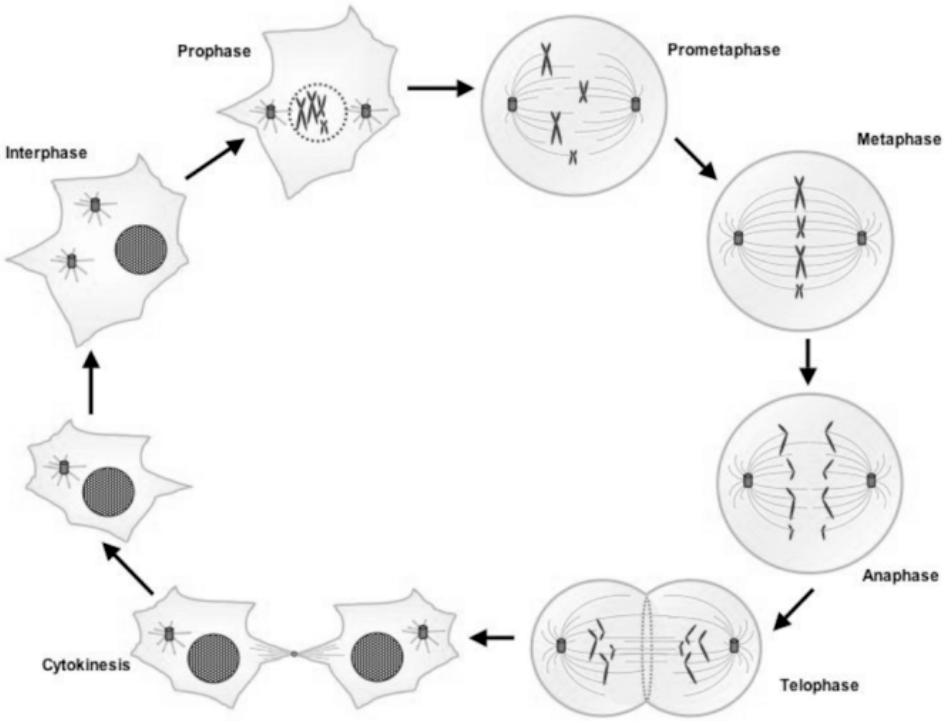

Figure 21 | The different phases of the mitosis. The ordered sequence of interphase and mitotic phases namely, prophase, prometaphase, anaphase, telophase and cytokinesis are shown. Figure adapted from (Salaün et al., 2008)

The physical separation of the two new cells is called cytokinesis. It may either start in the anaphase or telophase, based on the cell and finishes shortly after the telophase giving rise to two new cells, each with a complete set of chromosomes identical to that of the mother cell. These cells further participate in the cell cycle.

1.2.3. Cell cycle and pre-mRNA splicing

The eukaryotic cells are compartmentalized in nucleus and cytoplasm and this spatial separation enables the uncoupling of cellular process such as transcription and translation (Hofmann et al., 2010). Also in most eukaryotes, nucleus is contains many membrane-less structures that characteristically increase the local concentration of essential factors and facilitate the nuclear events such as DNA replication, DNA repair, transcription and pre-mRNA processing (Lamond and Earnshaw, 1998; Lanctôt et al., 2007; Sexton et al., 2007; Takizawa et al., 2008; Zhao et al., 2009). However, most of the eukaryotic cell cycle is characterized by open mitosis where the nuclear envelope breaks down as cells enter mitosis (Güttinger et al., 2009; Kutay and Hetzer, 2008). Apart from the nuclear envelope breakdown, the nuclear compartments are disassembled and their

functions are hampered in open mitosis. For instance, the nuclear speckles, the structure known for its function in gene expression and splicing, is disassembled as cells enter mitosis. All the component in the nuclear speckles become diffusely distributed throughout the mitotic cytoplasm (Ferreira et al., 1994; Lamond and Spector, 2003; Reuter et al., 1985; Spector and Smith, 1986; Thiry, 1995). The mitotic phase also negatively impacts on transcription machinery through two modes, the cyclin-B/cdk1 dependent phosphorylation and inhibition of RNA polymerase-II (Colgan et al., 1996, 1998) and the hyper condensation of mitotic chromosomes (Gottesfeld and Forbes, 1997). Mitotic onset also hampers pre-mRNA splicing and Shin and Manley explain a possible means through which this is attained (Shin and Manley, 2002). They demonstrate that one of the SR proteins, SRp38 (SRSF10) is phosphorylated during interphase and acts as splicing activator whereas at mitotic phase it undergoes dephosphorylation and represses splicing.

For long it was believed that transcription and splicing are completely inhibited at mitosis. Recently, Palozola et al revealed low level of transcription (8074 transcripts corresponding to 3689 genes) during mitosis in HeLa cells by labelling nascent RNA with 5-ethynyluridine (a cell-permeable uridine analogue) that is coupled to biotin and subsequently pulling down the nascent transcript and sequencing (Palozola et al., 2017a, 2017b; Strzyz, 2017). These genes that are transcribed in mitosis does not contribute to cell-specific functions but instead are involved in basic cell growth. They conclude that mitotic phase is not transcriptionally silent as it was believed so far.

Kittler et al reported the function of splicing factors (SmB/B′, U2A′, SART1 and SKIP/SNW1) in mitosis. The depletion of few splicing proteins leads to mitotic spindle defects and mitotic arrest (Kittler et al., 2004). In an another study, the same group identified 18 splicing proteins whose knock-down resulted in delays in cell cycle entry (G1), S-phase or at several stages of M-phase (Kittler et al., 2007a, 2007b). Similarly, Neumann et al also identified 18 splicing proteins whose depletion caused mitotic defects (Neumann et al., 2010). But it remains elusive whether the mitotic defects caused upon the depletion of splicing proteins are the direct function of splicing proteins in mitosis or a secondary effect due to mis-splicing of pre-mRNAs that express mitotic proteins (Lelij et al., 2014; Oka et al., 2014; Sundaramoorthy et al., 2014; Valcárcel and Malumbres, 2014; Watrin et al., 2014).

Though we know that the splicing is hampered in mitosis and SRp38 (SRSF10) contributes to this repression, it is still not known if the disassembly of nuclear speckles

in mitosis also contributes to the repression of splicing during mitosis. The other reasons for splicing repression during mitosis could be because of change in factors that regulate splicing such as chromatin structure, the rate of transcription and localization and levels of splicing proteins. Since it is known that splicing is predominantly co-transcriptional and there is a low level of transcription happening in mitosis, it can be hypothesized that low level is pre-mRNA splicing is also present in mitosis. Moore et al observed the pro-apoptotic splicing of apoptotic genes upon treatment with drugs that induce mitotic arrest (Moore et al., 2010). Since this study uses the mitotic arrested cells, it does not indicate the occurrence of splicing in normal mitosis. The study by Dominguez et al identified periodic alternative splicing of genes throughout the cell cycle including mitosis and they also report that CLK1 (SR protein kinase) is important for cell cycle progression by modulating the periodic alternative splicing across the cell cycle. They show that CLK1 modulated the splicing of genes involved in cell cycle entry, G1/S transition, replication, DNA repair and centriole duplication during interphase and altered splicing of genes involved in kinetochore, metaphase-anaphase and cytokinesis (Dominguez et al., 2016). Overall, the work of Dominguez et al indicated the occurrence of splicing in mitosis. However, it still remains elusive how splicing happens in mitosis, a cell cycle phase where the structures such as nuclear speckles are disassembled and the splicing proteins are dispersed to the cytoplasm. It is to be remembered that splicing proteins are stored in the nuclear speckles and are recruited to the site of splicing during interphase. It therefore remains an open question how all the splicing proteins are brought together in order to perform splicing reaction in mitosis.

1.2.3.1. Cell signalling kinases in pre-mRNA splicing

Apart from the splicing specific kinases such as CLKs and SRPKs, which are known to phosphorylate splicing proteins and regulate their localization and function, there are also many cell signalling and cell cycle kinases that function also in splicing. Such kinases include Nek2, MAPKs, GSK3β, Akt, PKA and Aurora-A (Naro and Sette, 2013). Nek2 is the centrosomal kinase that phosphorylates SR proteins and modulates alternative splicing of *BCL-X, BIN1* and *MKNK2* (Naro et al., 2014). Akt phosphorylates hnRNP-L and induces the anti-apoptotic splicing of Caspase 9 (Vu et al., 2013). PKA modulates alternative splicing of involved in neuronal differentiation by phosphorylating hnRNP-K (Cao et al., 2012). Consistent with these reports and in an effort to identify

genome-wide regulators of alternative splicing, Moore et al identified that many cell cycle and cell signal regulating kinases such as MAPK3K3, PLK1, AKT1, CDK1/CDC2 and Aurora-A regulate the alternative splicing of *BCL-X* and/or *MCL-1*. Interestingly, they found Aurora-A to take the centre of protein interaction in a network analysis of splicing regulators. They also found that inhibition or depletion of Aurora-A leads to post-translational turn-over of ASF/SF2 (SRSF1) and in turn alters the alternative splicing of *BCL-X* and *MCL-1* (Moore et al., 2010). Following Moore et al, Jones et al showed the depletion of Aurora-A leading to decrease in ASF/SF2, which in turn alters the splicing of androgen receptor (AR) in castrate-resistant prostate cancer (CRPC). Inhibition of Aurora-A decreased the inclusion of cryptic exon3 that is responsible for producing AR variant, AR-V7 and in turn this decreased the proliferation of CRPC (Jones et al., 2017). It remains a big question why these signalling kinases are taking part in splicing when there are splicing specific kinases that regulate pre-mRNA splicing. Since all the above mentioned works were carried using cancer cell lines as a model and since many cancers over-express these cell signalling and cell cycle kinases, it can be hypothesized that these kinases that perform their basic function in normal cells might be performing an additional splicing function in cancer cells as result of their over-expression.

1.3. The family of Aurora kinases

Aurora kinases form a conserved family of serine/threonine kinase, the first member of which was identified in 1993 as Ipl1 (increase in ploidy 1) during a screen planned to identify genes involved in chromosome segregation in *Saccharomyces cerevisiae* (Chan and Botstein, 1993). The Ipl1 related kinases, Aurora and Eg2 were also later identified in *Drosophila melanogaster* and *Xenopus laevis* respectively (Glover et al., 1995; Roghi et al., 1998). Subsequent research revealed that yeasts possess a single Aurora kinase gene called *IPL1* in *S. cerevisiae* and *Ark1* in *S. pombe*. Drosophila, *C. elegans* and *X. laevis* possess 2 aurora kinases namely Aurora-A and Aurora-B. In addition to Aurora-A and Aurora-B, mammals have a third Aurora kinase called Aurora-C, which is specifically expressed in testis and helps in spermatogenesis (Nikonova et al., 2013; Yan et al., 2016)

The mammalian Aurora kinases are implicated as mitotic regulators and also play a significant role in tumorigenesis due to their elevated expression profiles detected in many human cancers. Since over-expression of these kinases have frequently been

reported in many cancers, it is important to understand the molecular mechanisms responsible for their malignant transforming property. Also, elucidation of the signaling pathways that by either activating or inhibiting the Aurora kinases is likely to teach us novel concepts of how growth stimulatory or inhibitory signals are transduced to affect the subcellular structures like centrosomes, microtubules, mitotic apparatus and mitochondria and in turn maintain the chromosomal stability of cells (Bertolin et al., 2018; Fu et al., 2007; Giet and Prigent, 1999; Glotzer, 2005; Zhou et al., 1998)

Isolation of Aurora kinase family members from eukaryotes such as yeast, Drosophila, *Caenorhabditis elegans*, *Xenopus laevis*, *Mus musculus*, *Rattus norvegicus* and *Homo sapiens* revealed the conservation of this serine/threonine protein kinase family through evolution.

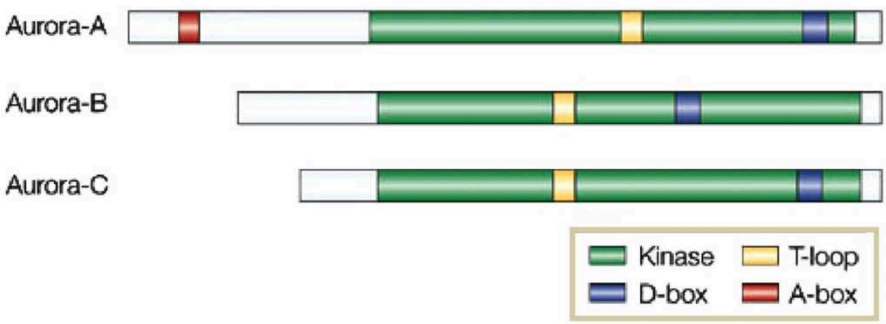

Figure 22 | Domain Organization of Aurora kinases. N terminal domains (in white box) contain regulatory sequences. The C-terminal domain (in green) is the catalytic domain responsible for the kinase activity. D box is a destruction box and A box activates D box. The T loop is the activation loop. Figure adapted from (Umene et al., 2013).

All the three Aurora kinases possess a variable N terminal domain of varying length. They also have a highly conserved C terminal catalytic domain (71% identity) which is responsible for their kinase activity. Additionally, they also possess D box and T loop. Aurora-A alone contains a special domain called A box which activates the D box (**Figure 22**) (Umene et al., 2013). The A box in Aurora-A helps in its degradation at the end of mitosis through APC/C complex mediated proteasomal degradation (Littlepage and Ruderman, 2002). The variable N-terminal region of Aurora kinases is responsible for their differential localization and hence they possess different substrates and interactors (Adams et al., 2001). Aurora-A is predominantly localized to the centrosome at interphase, and at spindles and spindle poles during mitosis (Bischoff et al., 1998). Recently Aurora-A was also reported to be localized to the mitochondria during

interphase (Bertolin et al., 2018). By contrast, Aurora-B localizes to kinetochores, midzone and midbody at different stages of mitosis (Bischoff et al., 1998). Finally, Aurora C is predominantly expressed in testis and localizes to centrosomes from anaphase to cytokinesis (Bernard et al., 1998; Kimura et al., 1999). All three kinases are known to be over-expressed in cancer. Ever since the identification of their role in tumorigenesis, all three Aurora kinases are a priority target for cancer therapy.

1.3.1. Aurora kinase A

Aurora-A is also known as Aurora-2, AIR-1 (*C.elegans*), AIK1 (mouse Aurora-A kinase), AIRK1 (rat), AYK1 (mammalian-murine), BTAK (human-breast tumour activated kinase), Eg2 (xenopus), IAK1 (human) and STK15 (serine/threonine kinase). Its gene maps to chromosome 20q13.2-q13.3, a region frequently amplified in many types of cancer (Kollareddy et al., 2008).

1.3.1.1. Functions of Aurora-A

The different functional aspects of Aurora-A such as bipolar spindle assembly, G2/M transition, actin network and the regulation of Aurora-A by different E3 ubiquitin ligases are discussed in detail in the following article titled “Regulation of Aurora Kinases and Their Activity” (Section 1.3.1.1.1).

Chapter 3

Regulation of Aurora Kinases and Their Activity

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Additional information is available at the end of the chapter

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Abstract

The three mitotic protein kinases Aurora-A, B and C are complementary enzymes that regulate multiple mitotic events. To do so, the different kinases must be locally activated, and the control of their activity is tightly regulated in time and space during mitosis. For instance, Aurora-A is first active at the centrosomes, then on microtubules at the spindle pole, Aurora-B is active in the nucleus, then at chromosome kinetochores and later one at the midbody. Aurora kinase activity is regulated in space and time by locally binding to regulators. Aurora kinases must bind to protein partners to be activated. Aurora-A for instance binds to targeting protein for *Xenopus* kinesin-like protein 2 (TPX2) and is activated at the spindle pole, Aurora-B and Aurora-C to INner CENtromer Protein (INCENP) and is activated on the chromosomes. These activations go through an autophosphorylation of a threonine residue in the T-loop of the kinase. Other protein partners are using different mechanisms to activate Auroras. These allow activation of the kinase at different time and location in the cell. This review is an up-to-date list of regulators of Aurora kinases. The subcellular localization of these regulators explains the presence of an active Aurora kinase. It also explains the changes in the localizations of the Aurora kinases activity observed during cell cycle progression in mitosis. Aurora kinases have been recently reported to be involved in nonmitotic events, and the identity of their activators in these events must be searched.

Keywords: Aurora kinase, regulation, posttranslational modification

1. Introduction

During the process of division, the cell goes through two main phases such as interphase and mitosis that is followed by the physical separation of the two daughter cells. During interphase, the cell duplicates its contents that will be segregated during mitosis to generate two daughter

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cells. The whole process lasts about 24 h in the case of human cells during which mitosis takes only 1 h. This short phase is highly regulated by phosphorylation and dephosphorylation reactions [1]. Among the key protein kinases involved, there are cyclin-dependent kinase 1 (CDK1), polo-like kinase 1 (Plk1), NIMA-related kinase 2 (Nek2) and the Aurora kinases (Aurora-A, B and C) [2]. The three mitotic protein kinases Aurora-A, B and C are complementary enzymes that regulate multiple mitotic events [3].

During mitosis, the cell segregates its two centrosomes that migrate around the nucleus to reach opposite position. The nuclear membrane breaks down, and the chromatin starts to condense to form chromosomes. Microtubules nucleate from both the centrosomes and the chromosomes to form a bipolar spindle [4]. The force exerted by the spindle microtubules contributes to the alignment of chromosomes on the metaphase plate. This event is immediately followed by the separation of each pair of sister chromatids and the beginning of their migration to the two opposite poles of the cell. The last part of the migration is driven by the central spindle, assembled at the future site of cell division. A constriction ring is assembled around the cell at the exact same location that will contribute to the separation of two volumes corresponding to the two daughter cells [5]. The physical separation of the two cells, abscission, will occur later on during the following interphase.

Aurora-A localizes at both the centrosomes and the spindle poles. The kinase activity is required for microtubule nucleation during bipolar spindle assembly and during central spindle formation [6]. Aurora-B is part of the Chromosome Passenger Complex (CPC), it localizes at chromosome kinetochores from prophase to metaphase and at the midbody from anaphase to telophase [7]. Its kinase activity is responsible for the massive phosphorylation of the Ser10 of histone H3 in mitosis. Aurora-B corrects the wrong attachments of microtubules to kinetochores during prometaphase [8]. During exit from mitosis, Aurora-B is required for cytokinesis. Aurora-C that is mainly involved in meiosis can replace Aurora-B during mitosis [9].

In vitro, the three kinases share the same substrates, such as histone H3. *In vivo*, to fulfill their function, the three kinases are differentially localized and also locally activated, and the control of their activity is then tightly regulated in time and space during mitosis. For instance, Aurora-A is initially active in the cytoplasm and at the centrosomes in the end of G2, then on microtubules at the spindle pole during prometaphase. Aurora-B is active in the nucleus by the end of G2, then at chromosome kinetochores in prometaphase and later at the midbody during anaphase and telophase. To achieve such regulation in space and time Aurora kinases are locally activated by binding to activators.

2. Bipolar spindle assembly

2.1. Aurora-A and TPX2

Targeting protein for *Xenopus* kinesin-like protein 2 (TPX2) is the best-characterized Aurora-A activator. It is a 100-kDa protein expressed from G1/S transition to cytokinesis and then rapidly degraded [10, 11]. TPX2 was first identified as a binding partner of the plus end-directed

Xenopus kinesin-like protein 2 (Xklp2) [12]. TPX2 helps localizing Xklp2 to the spindle pole in prometaphase and metaphase [13, 14].

In interphase, from S to G2, TPX2 localizes to the nucleus where it is sequestered by importin alpha. In mitosis, TPX2 is released from the importin by RanGTP in the vicinity of the spindle. RanGTP produced by the chromosome protein RCC1 (RanGEF) is localized as a gradient around the chromosomes. It is only when the nuclear membrane breaks down that TPX2 can reach the centrosome to bind Aurora-A, to activate and re-localize Aurora-A protein on microtubules at the spindle pole [15–17].

The binding of TPX2 to Aurora-A induces a conformational change in the kinase in a way that the phosphorylated threonine of the activation loop (T288 in human) is better protected from phosphatase activity (PP1 in particular) [18]. Phosphorylation of T288 or binding to TPX2 triggers activation of Aurora-A activity independently, but both the events are synergistic [19]. Most importantly, because binding of TPX2 to Aurora-A changes the conformation of the kinase, it modifies the affinity of the substrates for the kinase as well as the affinity of kinase inhibitors [20].

2.2. Aurora-A and CEP192

Centrosomal protein of 192 kDa (Cep192) was named after a proteomic analysis of the centrosome composition [21]. Cep192 is a protein involved in centrosome maturation and bipolar spindle assembly [22]. These functions correspond to those described for Aurora-A [23, 24], and indeed, Cep192 activates Aurora-A at the centrosome to control mitotic spindle assembly [25]. The mechanism by which Cep192 activates Aurora-A is different from TPX2. Cep192 is a scaffold protein that brings together two molecules of Aurora-A. Within the dimer, each Aurora-A molecule phosphorylates its neighbor on T288, leading to the complete activation of the kinase. This activation takes place at the centrosome early in mitosis. Aurora-A is then presumably released from Cep192 to bind to TPX2 and to move on the spindle poles [25].

2.3. Aurora-A and Maskin/TACC3

Maskin is a *Xenopus laevis* protein that got its name by the fact that it regulates RNA translation. Maskin links the 5′cap and the 3′UTR of the mRNA, creating a closed structure that cannot be translated [26]. During *Xenopus laevis* oocyte maturation, phosphorylation of Maskin by Aurora-A is required for the control of sequential mRNA translation [27]. Maskin is not only a substrate of Aurora-A, but it is also an activator of its kinase activity as binding of Maskin to Aurora-A induces a sevenfold stimulation of its kinase activity [27]. Whether Maskin affects the phosphorylation of threonine in the activation loop of the kinase has not been investigated yet.

The human homologue of Maskin is transforming acidic coiled-coil containing protein 3 (TACC3) and D-TACC in *Drosophila melanogaster*. Phosphorylation of D-TACC or TACC3 by Aurora-A is required for its centrosome localization [28], microtubule nucleation during bipolar spindle assembly [29–31] and during central spindle assembly [32]. Conversely, the activation of Aurora-A by TACC3 or D-TACC has not been demonstrated yet.

2.4. Aurora-B and INCENP

INner CENtromer Protein (INCENP) participates to the chromosome passenger complex (CPC) together with Aurora-B, Survivin and Borealin [33]. The complex controls multiple events during mitosis: from chromosome condensation and segregation to cytokinesis [34]. Aurora-B is carrying the kinase catalytic activity of the CPC, while INCENP is the activator of the kinase.

Binding of INCENP to Aurora-B is essential to the function of the kinase such as chromosome segregation and cytokinesis [35, 36]. Just like binding to TPX2 triggers activation of Aurora-A through autophosphorylation of T288 in its activation loop, binding to INCENP triggers activation of Aurora-B through autophosphorylation of T232 in its activation loop [37, 38]. Both the kinases and the modes of activation are so closed that a single amino acid change (G198 to N) transforms the activator of Aurora-A from TPX2 to INCENP. The demonstration has been made in *Xenopus* [39] and human [40].

In term of evolution, it is striking to note that *Drosophila melanogaster* genome do not contain any gene coding for a TPX2. Ssp1/Mei-38 would be the closest TPX2-related protein in *Drosophila*. Ssp1/Mei-38 possesses a microtubule-binding domain but lacks the Aurora-A-binding domain, indicating that it cannot activate Aurora-A [41].

3. G2/M transition

3.1. Aurora-A and Ajuba

Ajuba means curiosity in Urdu, an Indian dialect. Ajuba is a LIM domain-containing protein that serves as a scaffold to build numerous protein complexes. The LIM domain is a Zinc finger structure [42]. Ajuba was first reported to bind to Aurora-A at the centrosome in late G2 and to trigger the kinase activation through autophosphorylation of T288. Ajuba would then participate to the activation of Aurora-A and the commitment to mitosis (**Figure 1**) [43] . It was also suggested that Ajuba interacts with the N-terminal domain of Aurora-A to release its inhibitory binding to the C-terminal catalytic domain of the kinase [43]. Aurora-A activation by Ajuba would be a two-step mechanism, binding to the N-terminal domain of the kinase and triggering autophosphorylation of T288 [44]. The activation of Aurora-A by Ajuba has not been observed In *Xenopus laevis* [45]. In *Drosophila melanogaster*, although Ajuba does not activate Aurora-A, the protein is necessary to maintain Aurora-A at the centrosome [46].

3.2. Aurora-A and Nucleophosmin

Nucleophosmin (NPM) is a nucleolar protein involved in multiple functions: histone chaperoning, ribosome biogenesis, mitotic spindle assembly, genome stability, apoptosis and cancer [47]. Like Aurora-A, NPM localizes to the centrosome where it is required for centrosome duplication [48]. Depletion of NPM leads to the formation of disorganized spindles, a phenotype observed after Aurora-A depletion [49]. NPM is also a strong interactor of Aurora-A, and

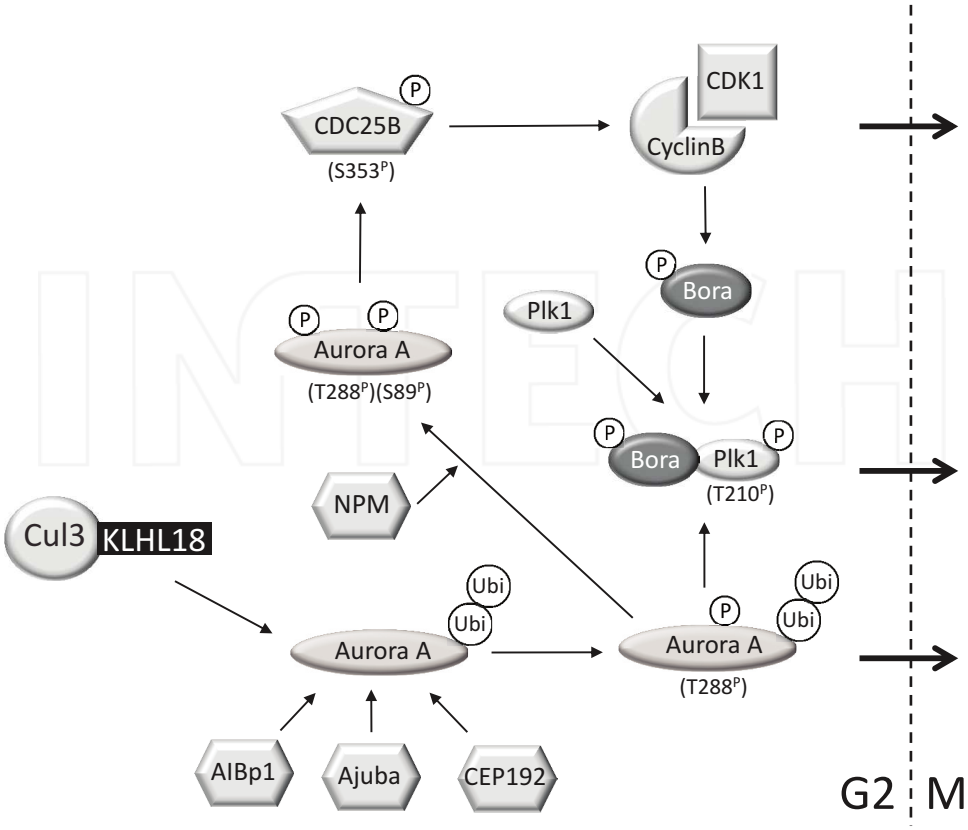

Figure 1. The three kinases CDK1/cyclinB, Plk1 and Aurora-A phosphorylate substrates required the G2/M transition. This scheme shows the pathways used by Aurora-A to activate Plk1 and CDK1/cyclinB.

both proteins interact at the centrosome late in G2. Binding of NPM triggers a phosphorylation event on the kinase. The active Aurora-A, already phosphorylated on T288, undergoes a second autophosphorylation on serine 89 which induces a very strong stimulation of its kinase activity [50]. This stimulation is required at the centrosome in particular for the phosphorylation of S353 on the phosphatase CDC25B involved in the activation of CDK1/cylin B for G2/M transition. Surprisingly, the stimulation of Aurora-A by NPM is not required for the phosphorylation of T210 that activates PLK1 in the end of G2 [50] (**Figure 1**).

3.3. Aurora-A and Bora

Bora was identified in a genetic screen setup to search for mutations affecting the development of *Drosophila melanogaster* external sensory (ES) organs [51]. The gene was named Bora for Aurora-A Borealis because the phenotypes of *Bora* and *Aurora-A* mutants were similar. Bora binds to Aurora-A *in vitro*, is phosphorylated but also activates the kinase in *Drosophila*

and human. *In vitro*, Bora can activate Aurora-A in the presence of PP1 (seven- to eightfold), suggesting that the mechanism used by Bora might be identical to TPX2 although it was not demonstrated that Bora triggers autophosphorylation of Aurora-A on threonine in the kinase activation loop [51]. However, when expressed at physiological levels, Bora does not co-immunoprecipitate with Aurora-A, and on the contrary, it immunoprecipitates with PLK1. Furthermore, depletion of Bora does not affect phosphorylation of T288 [52]. Eventually, it was demonstrated that Bora binds to the Polo-Box Domain of Plk1 (PBD) to relieve the auto-inhibition of PBD and to expose the T210 of the activation loop to Plk1-activating kinase. Aurora-A then binds to Bora, gets activated and activates Plk1 by phosphorylating T210 (**Figure 1**) [52, 53]. This activation of Plk1 by Aurora-A through the interaction with Bora occurs in G2.

3.4. Aurora-A and AlBp1

AIBp1 (AIK binding protein, AIK stands for Aurora/Ipl1-related kinase) is thus an Aurora-A binding protein but also a hNinein binding protein. Depletion of AIBp1 gives phenotypes typical of Aurora-A: bipolar mitotic spindle defects [54]. Binding of AIBp1 to Aurora-A increases its kinase activity *in vitro* [54]. *In vivo* expression of AIBp1 increases T288 phosphorylation on Aurora-A, whereas its depletion decreases T210 phosphorylation on Plk1 [55]. These data are reminiscent of the effect of Bora on both Aurora-A and Plk1 [52, 53]. It was then proposed that AIBp1 plays the same role as Bora but in a hNinein signaling pathway.

4. Actin network

4.1. Aurora-A and HEF-1/NEDD9/Cas-L

Human enhancer of filamentation 1 (HEF1) or neural precursor cell expressed, developmentally down-regulated 9 (NEDD9) or Crk-associated substrate related, lymphocyte-type (Cas-L) is a scaffolding protein that localizes to focal adhesions in interphase cells and to the mitotic spindle in M-phase. It participates in integrin-dependent signaling processes, such as cell attachments, cell migration and cell survival [56]. Cells depleted with HEF-1 show a decrease in T288 phosphorylation of Aurora-A, indicating that HEF-1 is required for activation of Aurora-A kinase *in vivo*. *In vitro* both proteins directly interact, and when increasing levels of HEF-1 are added to Aurora-A, an increase of T288 phosphorylation and its kinase activity are observed [57]. Interaction of HEF-1 with Aurora-A occurs in G2, during which the activation of Aurora-A by HEF-1 induces phosphorylation of HEF-1 and inhibition of the interaction. Interaction of HEF-1 with Aurora-A plays a critical role in primary cilia disassembly upon reentry in the cell cycle after Go arrest. In this particular case, HEF-1-activated Aurora-A phosphorylates and activates HDAC6, which in turn deacetylates the tubulin that is sufficient to provoke cilia resorption [58]. The activation of Aurora-A in the process of cilia disassembly is also dependent on Ca2+ and calmodulin (CaM) that are required for Aurora-A to bind to its activators [59].

4.2. Aurora-A and PAK-1

p21-Activated protein kinase-1 (PAK-1) regulates cell motility and morphology [60, 61] and is involved in focal adhesion disassembly through the PAK-PIX-GIT complex, PIX is a Rac

GTP exchange factor and GIT is a G-protein-coupled receptor kinase-interacting protein [62]. This complex is also active at the centrosome, and when Pak1 is activated it dissociâtes from the PIX-GIT to phosphorylate and activate Aurora-A [63]. The activation goes through phosphorylation of the T288 in the activation loop, but there was also a phosphorylation of S342 in the C terminal end of the kinase. Although T288 is known to be an activation site, S342 is rather known to inhibit Aurora-A kinase activity when phosphorylated. This has been shown in *Xenopus laevis* where the phosphorylation of S349 (human S342) downregulates Aurora-A *in vitro* [64] and *in vivo* during oocyte maturation between Metaphase I and Metaphase II of meiosis [65]. The same data have been reported in human where phosphorylation of S342 was observed in G2 upon DNA damage to inhibit Aurora-A, avoiding mitosis entry in the presence of lesions [66]. This discrepancy has not been solved yet.

4.3. Aurora-A and ILK

Integrin-linked kinase (ILK), like PKA-1, is a protein kinase involved in cell adhesion, and the kinase links the extracellular matrix to the actin cytoskeleton [67, 68]. ILK has also been observed in centrosome where it associates with TACC3/Ch-TOG, and its kinase activity is required for Aurora-A interaction with TACC3 [69]. ILK acts upstream of Aurora-A that in turn phosphorylates TACC3 on S558, to control microtubule nucleation [70, 71]. How ILK controls Aurora-A activity toward TACC3 is unknown.

4.4. Aurora-A and Arpc1b

Arpc1b is a component of the seven-subunit protein Arp2/3 complex involved in new actin filament nucleation and polymerization [72]. Arpc1b localizes on centrosome in G2 and interacts with Aurora-A only if Arpc1b has been previously phosphorylated on T21 by Pak-1. Arpc1b is also a substrate of Aurora-A, and the kinase phosphorylates wild-type Arpc1b but not the T21A mutant. This phosphorylation by Aurora-A is required for the interaction of Arpc1b with Arpc2 [73]. On the other hand, Arpc1b is an activator of Aurora-A *in vitro* and *in vivo*, and binding to Arpc1b triggers T288 autophosphorylation just like TPX2 does. Depletion of Arpc1b leads to a decrease on T288 as well as a decrease of its activity toward substrates such as PLK1 or histone H3 [73].

5. Ubiquitylation

Ubiquitination corresponds to a posttranslational modification (PTM) of proteins during which 76 amino-acid peptides are covalently linked to a protein, usually on lysine residues. It requires a multistep reaction: it needs 1) an E1 enzyme that will activate the ubiquitin, then 2) an E2 enzyme that will conjugate the ubiquitin and finally 3) an E3-ligase that will catalyze the transfer of ubiquitin peptide on the protein substrate [74].

5.1. Aurora-A and CUL3-KLHL18

The multiprotein complex E3 ubiquitin ligases of the cullin-RING-type ubiquitin ligase family include eight members in human. In the case of Cul3, the broad-complex, tramtrack and

bric-a-brac (BTB) domain-containing proteins like KLHL18 (*Kelch-like*) serve to recognize the ubiquitin substrate [75]. There are about 200 BTB proteins in human with various functions not all being Cul3 adaptors [76]. Cul3 and KLHL18 localize at the centrosome in late G2, and the depletion of each of the protein provokes a delay in the G2/M transition that has been attributed to a default in Aurora-A phosphorylation on T288 and consequently a default in the kinase activation at the centrosome [77]. The activation of Aurora-A by Cul3-KLHL18 involved a nonproteasomal ubiquitination of the kinase; however, the activation is not a direct effect of ubiquitination (**Figure 2**). Although the mechanism of activation is not fully understood, the hypothesis is that ubiquitination of Aurora-A could facilitate the interaction of the kinase with its activators in late G2, such as Ajuba or Cep192 for instance (**Figure 1**).

5.2. Aurora-B and CUL3-BTB proteins

Aurora-B unlike Aurora-A binds to three different BTB proteins KLHL9, KLHL13 and KLHL21, and all three substrate adaptors participate to Cul3 complexes that ubiquitinate Aurora-B *in vivo* and *in vitro* [78, 79]. Like for Aurora-A, the ubiquitination of Aurora-B does not lead to any degradation of the kinase, although depletion of Cul3, KLHL9 and KLHL13 mimics depletion of the 26S proteasome [78]. On the contrary, ubiquitination by Cul3-KLHL9, –KLHL13 or KLHL21 regulates the kinase localization during mitosis. In the absence of KLHL9 or KLHL13, instead of moving to the kinetochore region, Aurora-B remains on chromosome arms during

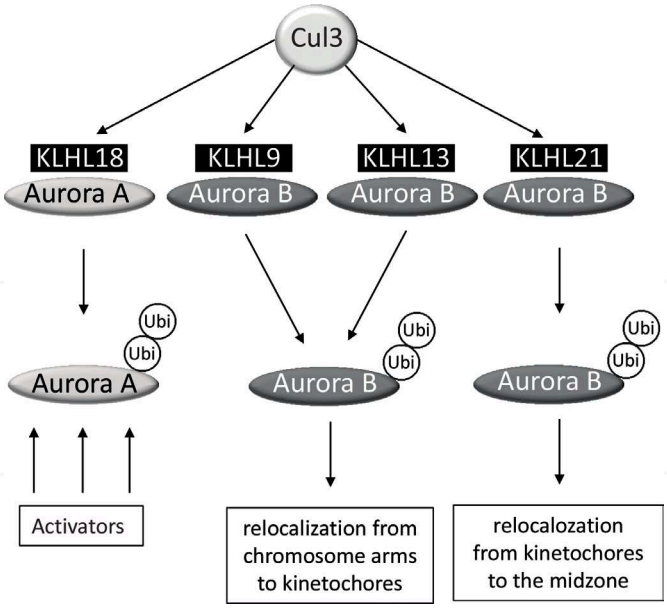

Figure 2. Cul3-dependent ubiquitination of Aurora kinases. Aurora-A is targeted by KLHL18, while Aurora-B by KLHL9, KLHL13 and KLHL21. Ubiquitination of Aurora-B localizes the protein, while ubiquitination of Aurora-A stimulates binding of the activators.

prometaphase/metaphase [78]. In the absence of KLHL21 instead of moving to the midzone, Aurora-B remains on anaphase chromosomes (**Figure 2**). In this last case, it is the whole CPC complex with Aurora-B, INCENP, Survivin and Borealin that remains on anaphase chromosomes [79].

Although KLHL9, KLHL13 and KLHL21 are substrate adaptors, their localizations do not really fit to their function. KLHL9 and KLHL13 have not been found on chromosomes for instance. On the other hand, KLHL21 does localize to the midzone where it could bring Cul3 to ubiquitinate Aurora-B, but even in this case KLHL21 should be at the kinetochore to ubiquitinate Aurora-B to target it to the midzone. The proposed hypothesis to explain this discrepancy is the high turnover of Aurora-B on its localization.

5.3. Aurora-A, Aurora-B and FBXW7

FBXW7 is an F-box protein participating to Skp, Cul1, F-box containing complex (SCF), a multiprotein E3 ubiquitin ligase complex that ubiquitinates proteins to be degraded by the proteasome. There are 69 F-box proteins coded by the human genome. Like KLHL proteins for Cul3, F-box proteins target the ubiquitin ligase complex to Cul1 substrates. FBXW7 binds to Aurora-A and Aurora-B and participates to their ubiquitination *in vivo* and *in vitro* [80, 81]. Depletions of FBXW7 stabilize both protein kinases levels *in vivo,* indicating that the FBXW7-dependent ubiquitination leads to the degradation of the kinases by the proteasome [81, 82]. F-box proteins usually recognize phosphorylated proteins, and FBXW7 for instance binds to Aurora-A previously phosphorylated by GSK3β on S245 and S387 [81] (**Figure 3**). Phosphorylation sites involved in Aurora-B/FBXW7 have not been identified, and the kinase involved is not known.

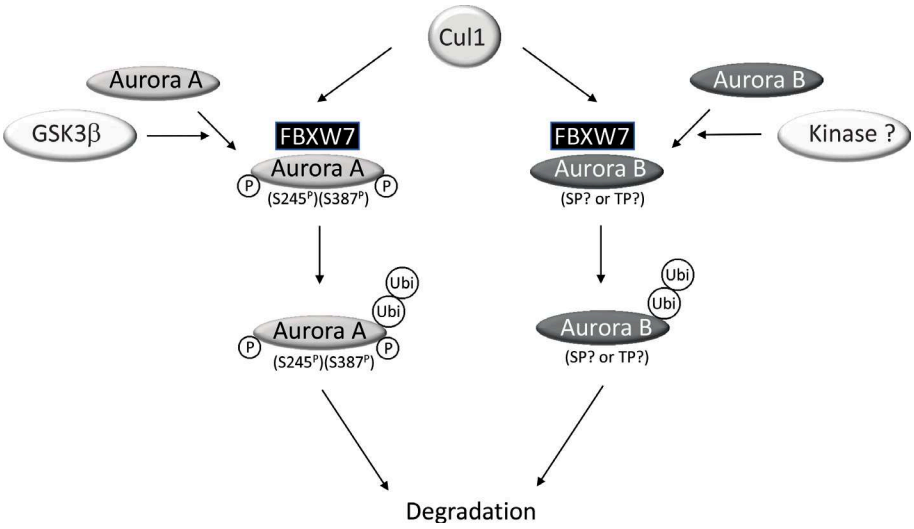

Figure 3. Cul1-dependent ubiquitination of Aurora kinases. Both Aurora-A and Aurora-B are targeted by FBXW7 F-box for ubiquitination. Aurora-A must be previously phosphorylated by GSK3β, while the kinase phosphorylating Aurora-B is unknown. Ubiquitination triggers degradation.

5.4. Aurora-A, Aurora-B and Cdh1

Cdc20-homologue 1 (Cdh1) is an activator and substrate adaptor of the E3 Ubiquitin ligase APC/C (Anaphase Promoting Complex/cyclostome). Cdh1 recognizes proteins containing a D-box (destruction box) (motif…RxxL…) and a KEN-box (motif …KEN…). All three Aurora kinases (A, B and C) contain a D-box in the carboxy end of the protein [83]. Both Aurora-A and B are ubiquitinated and degraded in a D-box-dependent manner [83, 84] although there are conflicting reports regarding Aurora-B [85]. Whether Aurora-C is degraded through its D-box remains also an open question. The Cdh1-dependent degradation of Aurora-A was demonstrated in both *Xenopus* and *human* [86, 87]. A new sequence required for the Cdh1-dependent degradation was discovered in Aurora-A from different species that was absent in Aurora-B and C. This sequence in the NH2 terminal end of the protein was named simultaneously A-box (for only in Aurora-A) [88] and DAD-box (for D-box-activating domain) (**Figure 4**) [89]. Interestingly, the A-box of Aurora-A contains a Serine at position 51 that when phosphorylated stabilizes the protein by inhibiting the functionality of the A-box [86, 90].

When tested in *Xenopus laevis* extracts Aurora-B was not found to be degraded in a Cdh1-dependent manner, it does contain a D-box but no A-Box, and only a chimera protein Aurora-A/Aurora-B containing Aurora-A A-box and Aurora-B D-box could be degraded in the extract [88, 89]. However, study in human finally revealed that Aurora-B contains also a functional D-box recognized by Cdh1 [84]. Interestingly, the same authors report that the KEN-box in

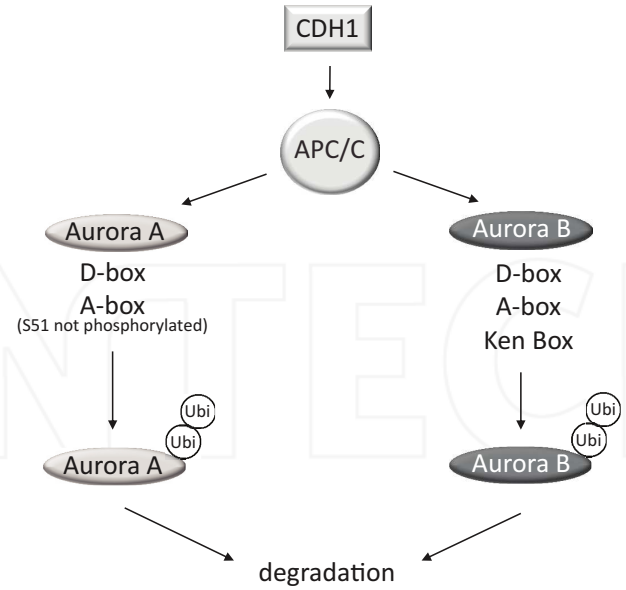

Figure 4. CDH1-dependent ubiquitination of Aurora kinases by APC/C. Both Aurora-A and Aurora-B are targeted by APC/C to be degraded. The ubiquitination required the presence A- and D-boxes in Aurora-A as well as the unphosphorylated state of S51 and the presence A- and D- and KEN-boxes in Aurora-B.

Aurora-B is required for Cdh1-dependent degradation. The discovery of a functional A-box in Aurora-B was more surprising, and since the box is not only present in Aurora-A, the name DAD-box seems now more adequate than A-box to name it. Finally, Aurora-B needs three functional boxes to be degraded in a Cdh1-dependent manner from the NH2 to the COOH end: a KEN-Box (KEN), an A-box (QRVL) and a D-box (RxxL) [85].

6. Sumoylation

Sumoylation resembles ubiquitination, and it is a posttranslational modification corresponding to a covalent attachment of a one or several SUMO proteins (100 amino acids) to a substrate. SUMOs stands for small ubiquitin-related modifiers, and there are now about ten different ubiquitin-like modifiers including ubiquitin and SUMO [91]. Like for ubiquitination, the sites of sumoylation are lysine residues, and the modification occurs also in a three-step reaction by E1, E2 and E3 ligases.

Both Aurora-A and Aurora-B are sumoylated *in vivo* on a lysine residue located in the sequence …IHDRIKPEN… conserved in all Aurora kinases [92, 93].

Aurora-A is sumoylated on K249, and expression of the Aurora-A mutant K249R that cannot be sumoylated affects spindle assembly and potentiates the oncogenic property of the kinase [93]. Aurora-B is sumoylated on lysine 207, and expression of the Aurora-B mutant K207R affects chromosome segregation and cytokinesis [92]. Interestingly enough, sumoylation of Aurora-A or Aurora-B does not affect the kinase activity in vitro indicating that sumoylation is probably playing required for the localization of the protein or for protein-protein interaction. The exact function of Aurora sumoylation remains to be found.

7. Poly(ADP-ribosyl)ation

Poly(ADP-ribosyl)ation (PARylation) is a covalent modification of protein that can be catalyzed by 16 different Poly(ADP-ribose) polymerases (PARP) that attach multiple ADP-ribose units on substrate proteins by hydrolyzing NAD+. The reverse reaction is insured by Poly(ADP-ribose) glycohydrolase (PARG) (three isoforms) and several ADP ribose hydrolases [94, 95]. Only Aurora-B is PARylated *in vivo*, and interestingly enough, the modification occurs in the presence of DNA damage [96]. PARylation of Aurora-B leads to a decrease of Serine 10 histone H3 phosphorylation, indicating a loss of Aurora-B kinase activity. The kinase directly interacts with PARP-1 and PARP-2, and both enzymes can PARylate Aurora-B and inhibit its activity [96].

8. Phosphorylation

Aurora kinases belong to a family of protein kinases that need to be phosphorylated on a threonine residue in its T-loop to be active [97]. Aurora-A must be phosphorylated on T288,

Aurora-B on T232 and Aurora-C on T198 and T202 [37, 98, 99]. These phosphorylations are autophosphorylation events that occur in the presence of an activator such as TPX2 for Aurora-A or INCENP for Aurora-B and C [16, 99]. Aurora-A has two other levels of regulation controlled by phosphorylation. The kinase activity can be upregulated by autophosphorylation of S89 in the presence of nucleophosmin, as described above [50]. Aurora-A can also be downregulated by phosphorylation of S342 [64]. *In vivo*, phosphorylation of S342 occurs in the presence of DNA damages during G2 and depends on the activation of the checkpoint kinase Chk1 [66, 100]. Is Aurora-A a direct target of Chk1 or the target of a kinase downstream of Chk1 remains an open question.

Since Aurora kinases are phosphorylated, they are obviously targeted by phosphatases. T288 in Aurora-A and T232 in Aurora-B for instance are dephosphorylated and inactivated by type 1 protein phosphatase [101, 102]. T288 is also dephosphorylated by PPP6 that specifically target Aurora-A when bound to TPX2 [103]. These dephosphorylations inactivated Aurora kinase activity.

As discussed above, Aurora-A degradation by APC/C-CDH1 depends on the presence of an A-box in the kinase and phosphorylation of S51 within the human A-box inhibits this degradation process [64]. Although the kinase responsible for this phosphorylation remains to be identified, the phosphatase PP2A insures its dephosphorylation [104].

9. Conclusion

Since their discovery, Aurora kinases have become priority targets for the development of inhibitors for cancer treatments [105]. But their regulation takes multiple forms, adding difficulties in developing the efficient drugs targeting the kinases. This review tends to report a nonexhaustive list of posttranslational modifications (PTMs) affecting the functions of the kinases. These PTMs can be used as biomarkers, like the phosphorylation of T288 in Aurora-A frequently used to measure the kinase activity *in vivo*, and this test is currently questioned [106]. More interestingly, these PTMs can be used to design original inhibitory strategies different from those targeting the kinase active site. The binding of TPX2 to Aurora-A for instance has been targeted to search for Aurora-A inhibitor [107]. This kind of approach targeting PTMs offers broad prospects for specific inhibition of Aurora kinases. Many new inhibitors should then be discovered in the coming years.

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1.3.1.2. Aurora-A as a therapeutic target in cancer

The role of Aurora-A in cancer and the possible use of Aurora-A as a therapeutic target and its consequences are described in the review work titled “Aurora A Kinase Is a Priority Pharmaceutical Target for the Treatment of Cancers” (Section 1.3.1.2.1).

Trends in Pharmacological Sciences

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Review

Aurora A Kinase Is a Priority Pharmaceutical Target for the Treatment of Cancers

Arun Prasath Damodaran,[1,2] Lucie Vaufrey,[1,2] Olivia Gavard,[1,2] and Claude Prigent[1,2,*]

Aurora kinases control multiple events during cell cycle progression and are essential for mitotic and meiotic bipolar spindle assembly and function. There are three Aurora kinases in mammals, some of which have oncogenic properties and all of which are overexpressed in multiple cancers. Pharmaceutical companies quickly made these kinases priority targets for the development of inhibitors to be used as cancer treatments. In this review, we focus on Aurora A, against which several inhibiting compounds have been discovered and made available; however, even though some of these compounds underwent clinical trials, none have yet gone beyond Phase III trials. The varying efficiencies and particularities of these drugs raise several questions that are explored in this review: is Aurora A even a good target? What biomarkers can we use to measure its activity *in vivo*? How can we improve the Aurora A-inhibiting drugs?

Aurora Kinases

Ipl1 was the first known member of the Aurora kinase family, discovered in yeast as a gene product involved in the control of ploidy (Ipl1 stands for 'increase in ploidy #1′) [1]. Aurora kinase was then found in multicellular organisms, initially in *Drosophila*, where it was shown to control centrosome separation and bipolar spindle formation [2]. Three different Aurora kinases were then discovered in mammalian cells, and named Aurora A, B, and C [3].

Aurora kinases were initially identified as protein kinases essential for error-free chromosome segregation during mitosis and meiosis. Aurora A appears to control premetaphase events, such as bipolar spindle assembly [3,4], with Aurora B and C directing metaphase and post-metaphase events, including cytokinesis [5,6]. Aurora B and C were later found to be responsible for the massive mitotic phosphorylation of serine 10 in histone H3 *in vivo* [7,8].

All three kinases show oncogenic properties and were found to be overexpressed in many different cancers [9–24]. Aurora A was the first member identified as one of the main protein kinases regulating cell proliferation. As soon as a strong link between Aurora A and cancer was found, most pharmaceutical companies started trying to develop Aurora A inhibitors to be used in treating cancer. Thus, here we focus only on Aurora A inhibitors.

Aurora A Inhibitors

There are currently many commercially available Aurora A inhibitors, with several involved in dozens of clinical trials. Three main Aurora A inhibitors are presented here and in Table 1.

Trends

Aurora A is a serine threonine kinase regulating cell division and cell cycle progression. It also has a major role in carcinogenesis.

Aurora A gain of function leads to genomic instability and cancer in mammals, while loss of function affects neuroblast renewal, leading to uncontrolled proliferation and cancer in *Drosophila*.

A first threshold of Aurora A is required for bipolar spindle assembly and chromosome segregation, while a second threshold is required for cell polarity, spindle orientation, and cell fate during asymmetric cell division. Aurora A is essential for stemness.

Aurora A is both an oncogene and a tumor suppressor; its inhibition leads to cancer cell death, whereas it may induce carcinogenesis in other sites of the body.

Development of Aurora A inhibitors started during early 2000, but none have yet progressed beyond Phase III trials.

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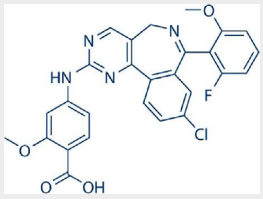
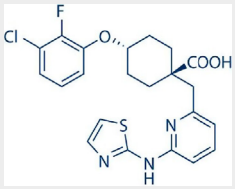
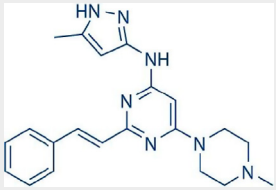
Table 1. Chemical Structure of the Three Main Aurora A Inhibitors Currently Used in Clinical Trials[a]

Name	Compound	Company	IC_{50} *in vitro* Aurora A
MLN8237 (Alisertib)		Millenium	1 nM
MK-5108/VX-689		Merck/Vertex	0.064 nM
ENMD-207		EntreMed	14 nM

[a]MLN8237 from Millenium, MK-5108 from Merck/Vertex, and ENMD-207 from EntreMed.

MLN8237

MLN8237 (Alisertib) was developed from MLN8054 by Millenium [25–27]. It has an IC_{50} of 1 nM and is approximately 200-fold more selective for Aurora A than for Aurora B. The compound was tested in more than 50 clinical trials [28], including Phase I trials for several solid tumors and lymphomas. Upon treatment, mitotic abnormalities and cell death were found in tumor specimens, but so were adverse effects, such as fatigue, nausea, and neutropenia. Alone or in combination, several dozen Phase II clinical trials have been completed, and MLN8237 showed some beneficial effect on tumor cells [28]. A Phase III trial was begun for patients with relapsed or refractory peripheral T cell lymphoma, but the company responsible (Takeda) terminated it due to insufficient effects on patient survival[i].

MK-5108

MK-5108 from Merck (VX-689 from Vertex) has an IC_{50} of 0.064 nM, and is approximately 200-fold more selective for Aurora A than for Aurora B. The results of a Phase I study of MK-5108 administrated alone or in combination for solid tumors were recently published [29]. The compound is the least-toxic Aurora A inhibitor studied so far, making it the most promising one for use in future trials.

ENMD-2076

ENMD-2076 is an interesting dual kinase inhibitor from EntreMed. It targets Aurora A and FMS-related tyrosine kinase 3 (Flt3) with an IC_{50} of 14 nM and 1.86 nM, respectively. The inhibitor is 25-fold more selective for Aurora A than for Aurora B. Phase I trials showed minimal cytotoxicity, and several Phase II trials were performed. ENMD-2076 did show some efficacy, particularly in association with other anticancer treatment (drugs or radiation) [30,31].

Why are there so many Aurora A inhibitors available that are so inefficient at killing cancer cells? Here, we explore a few ways in which Aurora A inhibitors could be improved.

The Role of Aurora A in Carcinogenesis

Aurora A Is An Oncogene

Aurora A acts as an oncogene. Its overexpression is sufficient to transform NIH/3T3 cells that then induce tumor formation when implanted in nude mice [9,10]. To test whether overexpression of Aurora A was sufficient to induce cancer in an animal, its encoding gene was placed under the control of CRE recombinase to induce its expression in mouse mammary glands. While the initial study indicated that increased Aurora A activity only caused mitotic defects and hyperplasia [32], a second study clearly demonstrated that, after a long period of genetic instability (20 months), the overexpressed kinase induced mammary tumor formation [33]. More recently, in a new mouse model, a β-lactoglobulin promoter activated by pregnancy and lactation was used to drive Aurora A overexpression in mammary glands. These mice then developed mammary adenocarcinoma with deregulation of gene expression identical to breast cancer in humans [34]. As indicated by the authors, this mouse model will be useful to better understand the mechanisms involved in the appearance of breast cancer due to overexpression of Aurora A.

Aurora A Is A Tumor Suppressor

In addition to its behavior as an oncogene, Aurora A has been reported to act as a tumor suppressor in cells dividing asymmetrically to self-renew. In *Drosophila,* for instance, Aurora A mutants with a loss of activity showed an increase in brain size attributed to neuroblasts overproliferation. How can *Drosophila* neuroblasts divide symmetrically or asymmetrically without any Aurora A activity? The implantation of a larval brain fragment of aur^{37} mutants in the abdomen of an adult wild-type fly induced the formation of an aggressive tumor that invades the entire abdomen and eventually kills the fly [35]. In normal conditions, each neuroblast divides asymmetrically, giving rise to another neuroblast (self-renewal) and to a ganglion mother cell. In the absence of Aurora A kinase activity, each neuroblast instead divides symmetrically, producing two neuroblast-like cells (proliferation) [33,36]. This has not been observed in the case of asymmetric cell divisions that lead to two daughter cells different from the mother cell, as seen during the formation of external sensory organs in *Drosophila* [37].

Asymmetric division of neuroblasts is often compared with stem cell division because of their self-renewal at each division. Tumor formation in the abdomen of *Drosophila* described above was attributed to overproliferated neuroblasts lacking Aurora A kinase activity. However, since implantations are performed with a piece of brain containing several cell types, it remains to be demonstrated that tumor formation is only due to neuroblast overproliferation. In vertebrates, mice heterozygous for Aurora A (carrying only one allele encoding active Aurora A) also show a significant increase in tumor formation, suggesting again a tumor suppressor activity of Aurora A [38]. However, it remains to be shown whether the tumors that appear in these heterozygous Aurora A mice are due to the proliferation of cells that would normally divide asymmetrically.

What then could be the link between the tumor suppressor activity of Aurora A, symmetric/asymmetric cell divisions, stem cell renewal, and uncontrolled proliferation? There are three major mechanisms required to organize asymmetric cell division: (i) the establishment of cell polarity; (ii) the orientation of the mitotic bipolar spindle; and (iii) the determination of the fate of the two daughter cells. Aurora A phosphorylates the Par complex, triggering Numb phosphorylation by aPKCζ and its asymmetric localization, thus organizing cell polarity [39] (Figure 1). Aurora A phosphorylates Lgl, disrupting its binding to the plasma membrane to allow localization of the Pins/LGN/Dlg complex; Aurora A also phosphorylates Pins, thus regulating mitotic spindle orientation [40,41] (Figure 1). Aurora A phosphorylates p53, leading to its degradation through MDM2-dependent ubiquitination, maintaining the pluripotency and self-renewal of embryonic stem cells (ESCs) by asymmetric cell division [42] (Figure 2). ESCs with high level of Aurora A contain a low level of p53, are pluripotent, self-renew, and divide asymmetrically; the

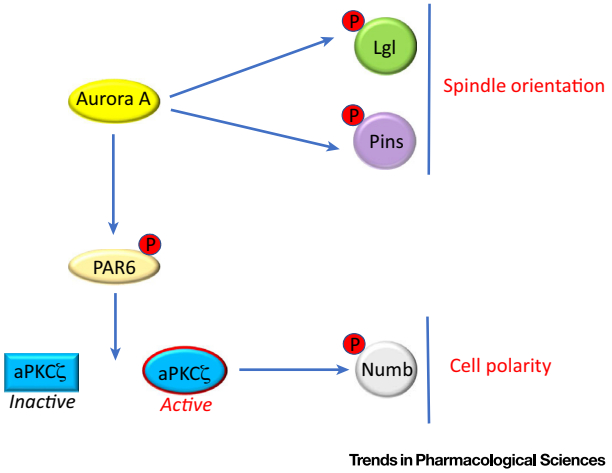


Figure 1. Aurora A and Asymmetric Division. Aurora A controls asymmetric cell division through the regulation of bipolar spindle orientation and cell polarity. Aurora A phosphorylates PAR6, which in turn activates aPKCζ, which phosphorylates Numb that then localizes asymmetrically, contributing to cell polarity. Numb is then inherited by one of the daughter cells, in which it represses the Notch pathway defining cell fate [39]. Aurora phosphorylates Lgl and Pins, which both participate in spindle orientation [41].

induced pluripotent stem cells (iPSCs) can also be reprogrammed. By contrast, ESCs with low level of Aurora A contain a high level of p53, differentiate, do not self-renew, and divide symmetrically, but the iPSCs lose reprogramming capacity. However, iPSC reprogramming can be rescued by p53 depletion (Figure 2) [42]. Numb also binds to and stabilizes p53, but the heterodimer is disrupted through Numb phosphorylation by aPKCζ [43]. A search for protein kinases and phosphatases that maintain ESC renewal and pluripotency identified Aurora A as a major player, according to the authors: 'milder knockdown of Aurora A in mESCs (mouse Embryonic Stem Cells) appears to preserve its basic proliferative and viability functions but directly impairs pluripotency and promotes differentiation' [42]. These results are important since inhibition of Aurora A in this cell model appears to affect the self-renewal capacity of stem cells without affecting their proliferation capacity. A first threshold of Aurora A activity appears to be required for cell division, while a second threshold is necessary for asymmetric cell division. More in-depth investigations of the effect of Aurora A inhibition on stem cell division in mammalians are needed to measure the effect of Aurora A inhibition in patients with cancer. However, could the use of Aurora A inhibitors provoke carcinogenesis in mammals?

Should Aurora A Inhibitors Be Developed?

To prepare effective Aurora A inhibitors, three things need to be taken into account: (i) increased Aurora A activity leads to cancer; (ii) Aurora A controls mitosis; and (iii) decreased Aurora A activity leads to cancer. In other words, Aurora A inhibitors might be efficient against some cancers, but may have the 'adverse effect' of triggering cancer development in other parts of the body if the inhibitors developed are not specific to the original cancer cells.

The first inhibitors were discovered more than 12 years ago and, although some efficiently inhibited kinase activity *in vitro*, none have progressed beyond a Phase III clinical trial. Nevertheless, there are several reasons why the development of Aurora A inhibitors should continue. Most importantly, when used in association with certain chemotherapeutic agents, such as taxanes (microtubule stabilizers), the results achieved have been promising [44–47]. Furthermore, a new approach based on kinome-wide small interfering (si)RNA) screening followed by kinase inhibitors has also yielded promising results (Box 1). Briefly, this approach takes advantage of the fact that tumor cells are not normal but instead have diverged and

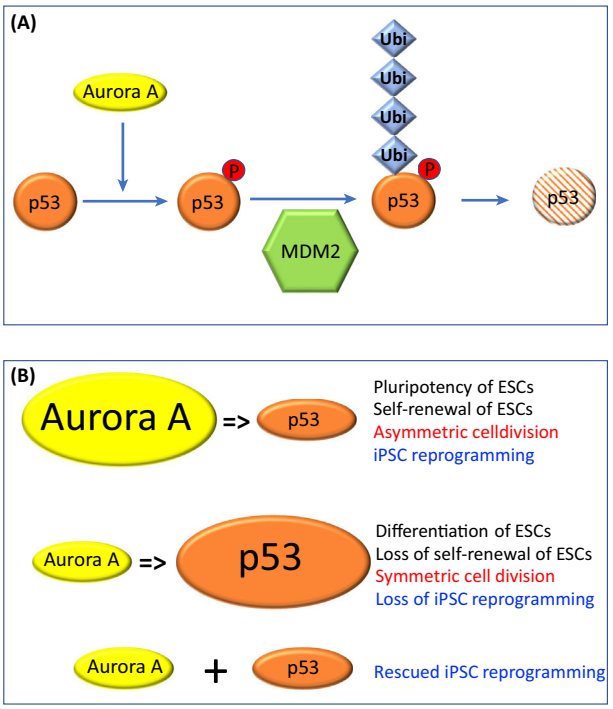


Figure 2. Aurora A, p53, and Pluripotency. (A) Aurora A directly phosphorylates p53, which becomes a target for the ubiquitin ligase MDM2, leading to its degradation by the proteasome. (B) Pluripotent embryonic stem cells (ESC) capable of self-renew show high levels of Aurora A and low levels of p53. A loss of Aurora A activity stabilizes p53, whose level then increases; this causes the ESCs to differentiate and lose their self-renewal capacity. Induced pluripotent stem cells (iPSC) with high Aurora A levels and low p53 levels can be reprogramed, while those with low Aurora A levels and high p53 levels cannot. Depletion of p53 in these latter cells rescues their reprogramming ability, highlighting the essential role of p53 and Aurora A in the pluripotency of stem cells [42].

adapted new signaling pathways to their advantage, adapting so that they can proliferate [48]. By doing so, some protein kinases become essential for the tumor, while remaining superfluous for the normal surrounding cells. Identifying these kinases and inhibiting them with low doses of inhibitors would be an efficient way to kill tumor cells without affecting normal ones. This approach has been successfully used in pancreatic cancers, opening new therapeutic possibilities [49,50]. Note that this approach is predicated on access to a toolbox containing specific inhibitors for each protein kinase, inhibitors that can be used as a drug in chemotherapy (including an Aurora A inhibitor) (Figure 3).

Improving the Search for Aurora A Inhibitors

Searching *In Vitro*

The classic *in vitro* approach is to look for kinase inhibitors using a simple enzymatic assay in which a substrate and radioactive ATP are incubated with active recombinant Aurora A [51]. The different compounds identified as inhibitors are then tested *in vivo*. In the case of Aurora A, this *in vitro* assay represents the first difficulty: Aurora A is a kinase that is activated by contact with a protein partner. For instance, the best-described Aurora A activator is the microtubule-associated protein TPX2 [52]. *In vivo*, TPX2 binds to Aurora A and triggers a conformational change around the active site of the kinase, allowing autophosphorylation of T288 in the T-loop. The binding of TPX2 also protects the phosphorylated T288 against dephosphorylation by the phosphatase PP1 [53,54]. When Aurora A is bound to TPX2, the interaction of the kinase with its substrates and its inhibitors appears to be altered [55,56]. Thus, we suggest that TPX2 should be included in *in vitro* assays to screen for Aurora A inhibitors that would target spindle assembly. This approach was recently used successfully to isolate inhibitors that disrupt the interaction between TPX2 and Aurora A [57].

A second complication then needs to be addressed: TPX2 is not the only Aurora A activator. Transforming acidic coiled-coil-containing protein 3 (TACC3), and its homolog Maskin in

Box 1. Killing Cancer Cells by Targeting Essential Kinases.

Cancer cells have modified their signaling pathways to survive. During this process, some of the protein kinases that are not essential for normal cells have become essential for cancer cells. Inhibiting these essential kinases with low doses of specific inhibitors has proved to be an excellent way to kill cancer cells without affecting normal cells [49,50].

In practice, normal and cancer cells from a patient must both be cultivated and used to perform differential screening using siRNA to identify these kinases. The human genome includes more than 500 kinase genes, which must be eliminated one by one by siRNA. The challenge here is then to have access to a library of inhibitors that would be specific for each of the kinases. The use of low dose of inhibitors has two benefits: it avoids both adverse effects and the appearance of drug resistance.

Xenopus laevis, also act in a similar way to TPX2, binding to Aurora A and triggering T288 autophosphorylation [58]. Adding TACC3 rather than TPX2 to the *in vitro* assays might yield different results. Furthermore, other activators use different mechanisms than those used by

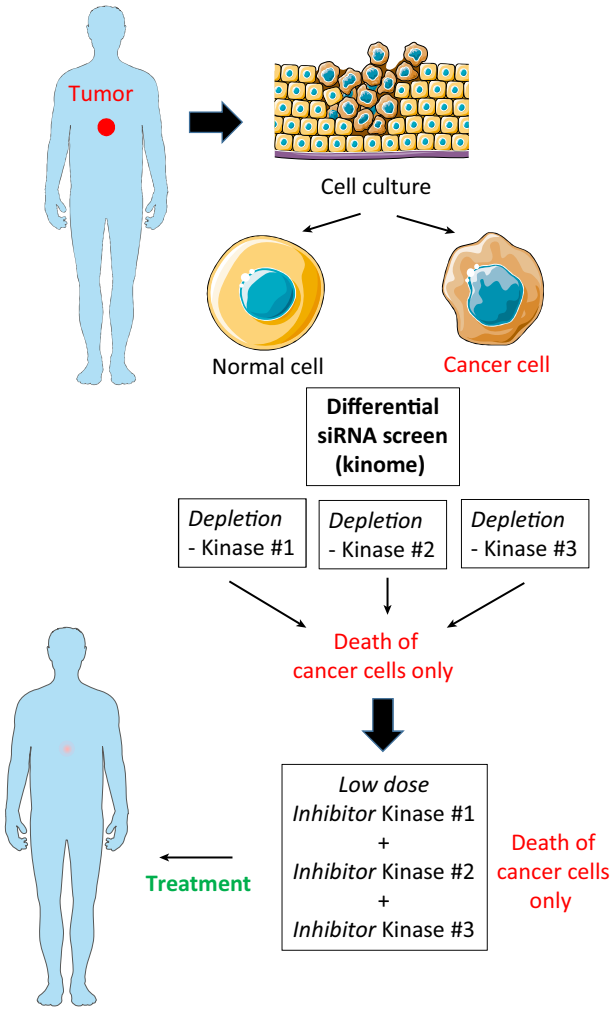


Figure 3. Signaling Pathways in Tumor Cells Have Been Adapted to the Advantage of the Tumor. Some protein kinases that are not essential for normal cells become essential only for tumor cells. These kinases can be identified by differential screening (normal cells versus tumor cells from the patient) using kinase depletion by small interfering (si) RNA. Inhibitors of each the kinase whose depletion leads to the death of tumor cells are used at low doses to design a personalized cocktail of inhibitors for chemotherapy [49,50].

TPX2 and TACC3. For instance, centrosomal protein of 192 kDa (CEP192) triggers T288 phosphorylation, but unlike TPX2, the molecular mechanism involved does not appear to be a conformational change. Instead, CEP192 triggers dimerization of the kinase that allows *trans* autophosphorylation on T288 [59]. Many other partners have also been identified, including Ajuba [60], HEF1 [61], and Bora [62].

Nucleophosmin (NPM) stimulates Aurora A kinase activity *in vivo* at the centrosome, but only if Aurora A is previously phosphorylated on T288. When bound to Aurora A phosphorylated on T288, NPM triggers the autophosphorylation of Aurora A on another site: S89. The consequence of this latter phosphorylation is amplification of kinase activity [63]. The mechanism involved at the structural level is unknown but NPM is thought to act as a chaperone for Aurora A. Adding both NPM and TPX2 to *in vitro* assays should help achieve high levels of kinase activity and reveal inhibitors that would be more efficient against spindle assembly *in vivo*. *In vitro* kinase assays should be specifically designed against a cellular mechanism; for example, the approach used to target Aurora A for its function during G2/M transition (Aurora A/CEP192) should be different than that used to target it during spindle assembly (Aurora A/TPX2), and so on. Is it possible to target one and only one event controlled by Aurora-A by inhibiting the binding of Aurora A to one of its activators?

In Silico Investigations

In recent years, high-resolution 3D protein structures and the development of an enormous number of docking tools and algorithms have paved the way for high-throughput virtual screening and the development of new drugs. Various docking software (used to find a 'key' to a 'lock') and criteria to be considered for reliable and accurate computer-aided drug design are listed elsewhere [64].

To date, 112 3D structures have been deposited for human Aurora-A in the Protein Data Bank (PDB[ii] [65]) as cited in 48 publications. Aurora-A structures have been resolved to a high resolution of 1.67 Å (PDB ID: 5L8L) [66], but none of the 112 structures were developed for the full-length kinase. This makes it difficult for computer-aided drug design to target N-terminal functions, such as the E3 ubiquitin ligase activity of the Aurora A Phe31 variant [67]. At its N terminus, Aurora A has an autoinhibitory non-Aurora box (aa 64–128) that interacts with the C terminus and keeps the kinase inactive [68]. The Aurora A activator Ajuba was reported to interact with its N terminus, preventing the interaction between it and the C-terminal domain. Ajuba then leads to Aurora A autophosphorylation and activation [60,69]. Therefore, resolving and targeting the Aurora A N-terminal structure should yield a new class of Aurora A inhibitors.

Most Aurora inhibitors have been developed to target the ATP-binding site, thus raising the question of inhibitor specificity. High-throughput screening identified lead compound 8a, a novel pyrazole that binds to the ATP-binding site of Aurora A ($IC_{50} \approx 15$ μM). This compound was later used to design a new compound called 12 w ($IC_{50} \approx 0.033$ μM), which inhibited Aurora A 450-fold more than the original lead compound. 12 w also showed more specificity towards Aurora A than to Aurora B or C [70].

Binding of the kinase activators has also been targeted. For instance, docking and molecular dynamic studies showed that Withanone, isolated from the plant ashwagandha (*Withania somnifera*), disrupts the interaction between Aurora A and TPX2, the targeting protein for *Xenopus* kinesin-like protein 2 (Xklp2). Withanone is a potential anticancer drug, because it disrupts the mitotic spindle and prevents cancer cell division [71]. In another study, Kilchmann and colleagues used the xLOS 3D shape and pharmacophore similarity algorithm to discover 'Inhibitor 9′, a highly specific Aurora A inhibitor with an IC_{50} of 2 nM [72]. Inhibitor 9 competes with TPX2 by locking Aurora A in an inactive conformation.

Many attempts have been made to develop pharmacophore models that target Aurora A [72–78]. Recently, a new approach to the search for Aurora A inhibitors was developed based on a general Ser/Thr kinase pharmacophore model, and identified compounds that showed little activity against Haspin, Aurora A, or Aurora B. The general model was adjusted to only Aurora A, and this optimization yielded hits showing higher specificity, in the 3–5 nM activity range, with good absorption, distribution, metabolism, and excretion (ADME) properties. These compounds also showed 10–30-fold selective cytotoxicity on 16 cancer cell lines relative to the primary cells [79]. This new approach is useful, since the same general pharmacophore model can be adjusted to target any Ser/Thr kinase. In the near future, advances in computational tools will aid in the development of drugs that target different Aurora A domains by taking into account their interactions with various activators.

In Vivo Explorations

Once an inhibitor has been identified *in vitro*, it must be validated *in vivo* in cell cultures. To evaluate the specificity of an Aurora A inhibitor, the usual control is to compare the phenotype obtained by the inhibitor with that obtained after depletion of Aurora A by RNAi or gene knockout [80–85]. However, in the case of Aurora A, this might not be appropriate, because it has been observed that Aurora A performs functions independently of its kinase activity. In *Caenorhabditis elegans*, for instance, abnormal spindle assembly after Aurora A depletion was rescued by an inactive kinase [86]. In another example, both active and inactive Aurora A forms interacted with heterogeneous nuclear ribonucleoprotein K (hnRNP K) to transactivate the MYC promoter [87].

These data suggest that cells without Aurora A protein (due to RNAi) cannot be compared with cells with inactive Aurora A kinase (due to inhibitors) when the protein itself is present. In addition to having enzymatic activity (as a kinase), the Aurora A protein might also serve as a platform for complexes.

With this idea in mind, a method was developed for evaluating the specificity of Aurora A inhibitors. An allele-sensitive (AS) Aurora A isoform was generated by mutating the kinase gatekeeper [63] (Box 2). The AS isoform can be specifically inhibited by an ATP analog (1-Na-PP1) that has no other target in the cells. Two stable U2OS (Human bone osteosarcoma cells) cell lines were prepared, one expressing a wild-type (WT) isoform of Aurora A, and the other expressing an AS isoform. Both were resistant to siRNA targeting the endogenous Aurora A and, when treated by siRNA to deplete this resistance, both cell lines survived either with only the WT or only the AS isoform. Due to specific inhibition of Aurora A, only the AS cell line gave a phenotype in the presence of 1-Na-PP1. This phenotype could then be compared with any obtained after treatment U2OS cells with potential Aurora A inhibitors [63] (Figure 4).

Some Aurora A functions might also differ depending on the cell type. Thus, exposing a whole organism to an Aurora A inhibitor might lead to different effects, which could explain why many

Box 2. Design of Allele Sensitive Kinases.

An AS kinase has been mutated in its active site to change the amino acids in the gatekeeper of the kinase. A point mutation is usually sufficient to achieve this. The mutation increases the size of the active site to give access to a larger ATP molecule. The catalytic activity of the kinase should not be affected and the AS kinase should still use normal ATP molecules as phosphate donors.

To inhibit the AS kinase, an ATP analog is modified chemically to increase its bulk so that it can enter only the AS kinase active site. These modified ATP analogs do not affect other kinases. The AS kinase must be expressed *in vivo* at the physiological level, and this is achievable via the use of the CRISPR/Cas9 gene-editing method. The use of the AS kinase together with its ATP-analog inhibitor offers a specific way to inhibit a kinase *in vivo*.

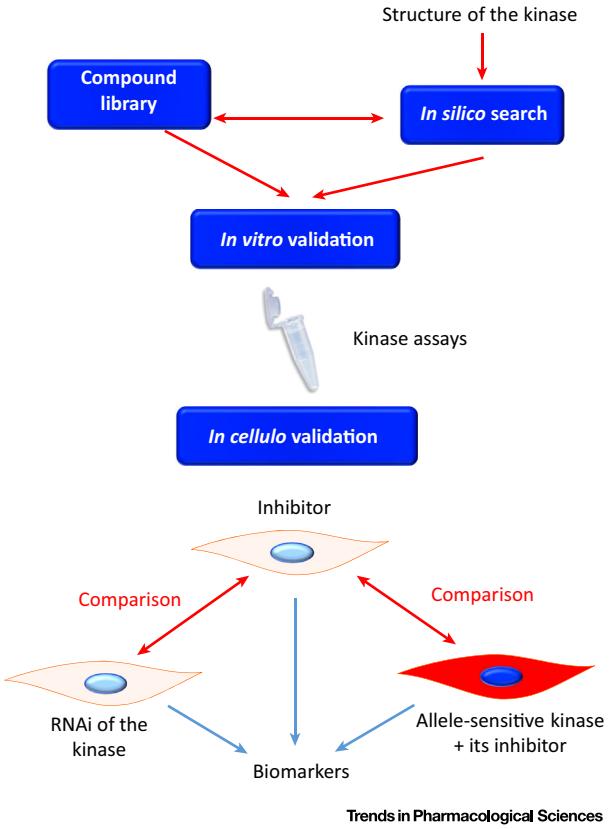


Figure 4. Preparation of Aurora A Kinase Inhibitors. The structure of the kinase can be used for *in silico* searches of potential drugs to be tested *in vitro*. A compound library can be screened using a classic *in vitro* kinase assay. Hits are then assayed in cell cultures. The effect of the inhibitor is compared with the effect of kinase depletion achieved by small interfering (si)RNA (i.e., inhibition is compared with depletion). An alternative is to compare the effect of the inhibitor with the effect of inhibition of an allele-sensitive (AS) Aurora A (i.e., inhibition is compared with inhibition). In both cases, a biomarker has to be selected to follow *in vivo* Aurora A inhibition.

clinical trials have failed. Besides its role in mitosis, the kinase is also involved in multiple cell mechanisms, including cell polarity, asymmetric cell division (differentiation during development or stem cell self-renewal), and epithelial-mesenchymal transition (EMT), for example in glioma [88–90]. The specificity of an inhibitor should then be defined using at least three criteria: (i) the kinase Aurora A; (ii) the cell type; and (iii) the mechanism.

How to Detect Aurora A Kinase Activity *In Vivo*

To be catalytically active, Aurora A must be phosphorylated at T288 in the activation loop, a phosphorylation thought to be due to a *trans* autophosphorylation mechanism [53,54]. In this process, a binding partner activates a reaction wherein an Aurora A kinase molecule phosphorylates another Aurora A molecule at T288. Consequently, any *in vivo* antiphosphoantibody detection of T288 phosphorylation will indicate that Aurora A is active. However, the method has some drawbacks. For instance, T288 phosphorylation may not be exclusively due to autophosphorylation. *In vitro,* protein kinase A (PKA) can phosphorylate Aurora A at T288 located in a PKA consensus sequence: RRXTL [91]. However, it is unlikely that PKA phosphorylates T288 *in vivo* [92]. p21 protein (Cdc42/Rac)-activated kinase 1 (PAK-1) can also phosphorylate Aurora A on T288 *in vivo* when localized at the centrosomes [93]. In this particular case, the T288 phosphorylation state reflects either PKA or PAK-1 activity rather than that of Aurora A. Aurora A has another threonine residue next to T288, and

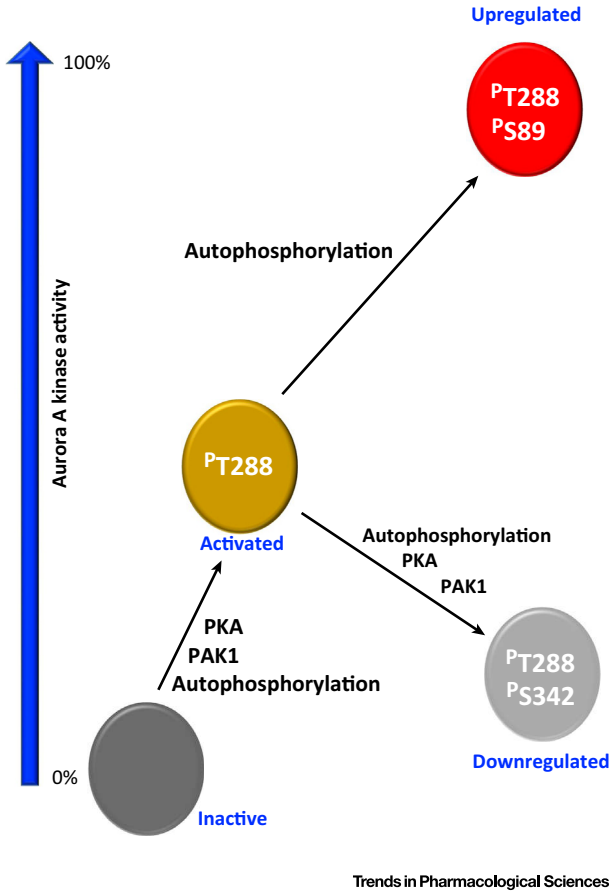


Figure 5. Regulation of Aurora A Activity by Phosphorylation. Unphosphorylated Aurora A is inactive. The first step in its activation is the phosphorylation of Threonine 288 (T288) [53,54]. Aurora A phosphorylated on T288 can be downregulated by phosphorylation of Serine 342 [95,96]. However, Aurora A phosphorylated on T288 can also be upregulated by phosphorylation of Serine 89 [94].

phosphorylation of T287 also activates Aurora A; this epitope is not detected by antiphospho-T288. In addition, at least *in vitro*, Aurora A activity is suppressed when both T288 and T287 are phosphorylated [94]. Aurora A remains inactive if dephosphorylated on T288, indicating that T288 phosphorylation is the first requirement for an active Aurora A kinase. However, T288 phosphorylation does not necessarily indicate that Aurora A is active, because the protein kinase activity is modulated by several other phosphorylation events. For instance, the kinase activity of Aurora A phosphorylated on T288 is strongly enhanced in contact with nucleophosmin. This increase is due to an autophosphorylation event on Aurora A S89 [95]. By contrast, the activity of Aurora A, previously phosphorylated at T288, can also be downregulated by S342 phosphorylation [92,96]. Similar to T288, this serine is in a PKA consensus sequence, and it can also be phosphorylated by PAK-1 [93,97] (Figure 5). The S342 in human Aurora A is conserved in *X. laevis* at position 349. It has been reported that S349 phosphorylation in *Xenopus* could be triggered by priming Aurora A, phosphorylated on T295 (equivalent of human T288), through GSK3 phosphorylation of two amino acid residues, S290 and S291, upstream of S349 [98]. This priming event appears to trigger S342 autophosphorylation. However, these data have only been obtained so far in *X. laevis*.

The take-home message here is that, although Aurora A phosphorylated on T288 is the activated isoform of Aurora A, measuring the level of phosphorylation of T288 does not reflect Aurora A kinase activity (Figure 5).

What would be the most appropriate readout for an *in vivo* Aurora A kinase activity measurement? Although the Aurora A substrate LATS2 phosphorylated on Ser 83 has been reported to give good data, a comparison with an Aurora B substrate is need, and histone H3 phosphorylated on Ser 28 would be a suitable choice. Antiphosphoantibodies are also commercially available for both substrates [56].

Concluding Remarks

The development of protein kinase inhibitors to be used in cancer treatments is a long, expensive process. The cost of Phase III trial failures was recently evaluated [99] and the authors observed that cancer trials lead to failure more frequently than those for other diseases. Based on conference presentations at a European Center for Pharmaceutical Medicine seminar, the authors compiled a list of 'drivers of failure'. These included inadequate basic science, flawed study design, suboptimal dose selection, flawed data collection and analysis, and problems with study operation [99].

In the case of Aurora A, basic science problems may be most responsible for trial failures, since the different functions of the kinase are not yet fully understood (see Outstanding Questions). It has become increasingly obvious that Aurora A activity is not just restricted to centrosome maturation and bipolar spindle assembly. How does Aurora A control cell cycle progression? The Aurora A community must now concentrate its efforts on understanding the functions of the kinase during different stages of interphase [100]. More importantly, how does Aurora A deregulation trigger carcinogenesis? In a complex organism, too much Aurora A is no good, but not enough is not good either. It should now be considered that Aurora A has cell type-specific functions. Cells can be polarized or not; cell division can be symmetric or asymmetric, can go from one mode of division to the other, and can lead to proliferation, differentiation, or stem cell renewal. In all these examples, there are specific functions and substrates for Aurora A; instead of targeting the kinase activity of Aurora-A, targeting only one of its substrates could be a way to inhibit one of its specific functions. However, when Aurora A inhibition is used as a part of cancer treatment, what are the consequences on the whole organism? Given its pleiotropy, direct inhibition of the kinase activity results in multiple adverse effects; thus, the inhibition of a specific substrate phosphorylation might lead to less-dramatic adverse effects.

What are the kinase-dependent and −independent functions of Aurora A? A systematic comparison of these functions using gene knockdown and gene knock-in to create a gene encoding an inactive kinase mimicking the effect of an inhibitor could be performed. The resulting data might provide an answer to the question: which is the better way to target the kinase: inhibition or protein elimination? All of these questions should be carefully addressed before we can contemplate fighting cancer using Aurora A as a target; without the answers, clinical use of Aurora A inhibitors will remain risky.

Since cancer research is and will remain a priority, financial support of basic science is crucial to improve the chances of success and transfer to the clinic. Isaac Newton said: 'To explain all nature is too difficult a task for any one man or even for any one age. 'Tis much better to do a little with certainty, & leave the rest for others that come after you, than to explain all things by conjecture without making sure of any thing.' The development of effective and safe inhibitors depends on the knowledge of the target obtained through active exploration of fundamentals: 'In order to beat your enemy, you must first know your enemy' (The Art of War, Sun Tzu).

Outstanding Questions

How can *Drosophila* neuroblasts divide symmetrically or asymmetrically without any Aurora A activity?

Can neuroblasts without Aurora A, injected alone, induce cancer in the abdomen of *Drosophila*?

Are the tumors that appear in heterozygous Aurora $A^{+/-}$ mice due to the proliferation of cells that would normally divide asymmetrically?

Could the use of Aurora A inhibitors provoke carcinogenesis in mammals?

Is it possible to target one and only one event controlled by Aurora A by inhibiting its binding to one of its activators?

What is the most appropriate readout to measure Aurora A activity *in vivo*?

What are the kinase-dependent and −independent functions of Aurora A in mammals?

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Resources

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1.4. Objectives

Aurora-A is known to be important for the stability of a SR protein, ASF/SF2 and in turn alters the alternative splicing of few apoptotic genes (Moore et al., 2010). Similarly, Aurora-A regulates the level of ASF/SF2 and alters the splicing pattern of androgen receptor in castrate resistant prostate cancer (Jones et al., 2017). In an effort to identify the new interacting partners and new functions of Aurora-A, Olivia Gavard and Jean-Philippe Gagné (former members of our team) identified many splicing proteins in the interactome of Aurora-A. Given that the interactome of Aurora-A suggested its role in pre-mRNA splicing, I aimed at understanding the molecular mechanisms that underlie its precise function in splicing. Towards this goal, I set up the following objectives for my thesis,

1) To determine if the splicing proteins directly interact with Aurora-A and to identify if the splicing proteins are also a substrate of Aurora-A.

2) To demonstrate if Aurora-A is important for the stability of all SR proteins.

3) To determine if Aurora-A localizes to nuclear speckles, the store-house of splicing factors, which is implicated in pre-mRNA splicing.

4) To identify transcriptome-wide Aurora-A dependent pre-mRNA splicing.

2. Materials and Methods

2.1. Expression vectors and molecular cloning

The plasmid constructs were generated using Gibson Assembly Master Mix (New England Biolabs). The cDNAs of splicing proteins were PCR amplified and was sub-cloned to pGEX4T1 vector. All the 12 flag-tagged SRSF constructs for expression in mammalian cells were a kind gift from Dr. Wei Wu (Fu et al., 2013). All plasmid constructs were verified by sequencing. The list of primers used for cloning is shown in **Table 1**.

Table 1 | The list of primers used for cloning splicing factors

Gene name	Primer Sequence (5' → 3')	
hnRNP-C2	Forward	ATCTGGTTCCGCGTGGATCCGGAGGAGGAGGAGG AATGGCCAGCAACGTTACC
	Reverse	TCGAGTCGACCCGGGAATTCTTAAGAGTCATCCT CGCCATTG
PCBP2	Forward	TCGGATCTGGTTCCGCGTGGATCCGGAGGAGGAG GAGGAATGGACACCGGTGTGATTGAAG
	Reverse	CCGCTCGAGTCGACCCGGGAATTCCTAGCTGCTC CCCATGCCAC
RALY	Forward	CCGCTCGAGTCGACCCGGGAATTCTTACTGCAAG GCCCCATCATC
	Reverse	CCGCTCGAGTCGACCCGGGAATTCTTACTGCAAG GCCCCATCATC
PTBP1	Forward	TCGGATCTGGTTCCGCGTGGATCCGGAGGAGGAG GAGGAATGGACGGCATTGTCCCA
	Reverse	CCGCTCGAGTCGACCCGGGAATTCCTAGATGGTG GACTTGGAGAAG
RS26	Forward	TCGGATCTGGTTCCGCGTGGATCCGGAGGAGGAG GAGGAATGACAAAGAAAAGAAGGAAC
	Reverse	CCGCTCGAGTCGACCCGGGAATTCTTACATGGGC TTTGGTGG
EIF4A3	Forward	ATCGGATCTGGTTCCGCGTGGATCCGGAGGAGGA GGAGGAATGGCGACCACGGCCACG
	Reverse	TCACGATGCGGCCGCTCGAGTCGACTCAGATAAG ATCAGCAACGTTCATCGG

2.2. Expression and purification of recombinant proteins

E.coli strain BL21 (DE3) was transformed with GST fusion plasmids encoding hnRNP-C2, RALY, PCBP2, PTBP1, RS26 and eIF4A3 or HIS fusion plasmid encoding Aurora-A. Cells were grown till the OD600 reached 0.6 in 250 ml LB media

supplemented with antibiotics (ampicillin 100 μg/ml) and expression of the protein was induced for 4h with 1mM isopropyl 1-thio-B-D-galactopyranoside (Sigma) at 18°C. Cells were collected, sedimented by centrifugation for 20 min at 4000 rpm (4°C). The cell pellet for HIS-tagged protein purification was resuspended in 10 ml of Buffer A (20 mM Tris pH 7.5, 500mM NaCl, 20mM imidazole) containing 1X protease inhibitors (EDTA free protease inhibitor cocktail, Roche). Whereas for purification of GST tagged proteins, cell pellets were resuspended in 10 ml of Buffer B (0.1% Triton X100 in PBS) supplemented with 1X protease inhibitors. The cells were then sonicated at 50% amplitude for 3 min (30 sec pulses) and cell lysates were centrifuged at 10000 rpm for 30 min at 4°C. The supernatant was incubated with TALON his-tag purification resin (TaKaRa) or glutathione agarose beads (Sigma-Aldrich) for 30 min in a rocker at 4°C. Beads were then washed thrice with buffer and eluted with 250mM imidazole (for HIS tagged proteins) or 5mM reduced glutathione (for GST tagged proteins). Protein profiles from the eluted fractions were estimated by SDS-PAGE followed by Coomassie staining.

2.3. *In vitro* interaction assay

GST pull-down assay

The GST-tagged hnRNP and HIS-tagged Aurora-A were incubated in interaction buffer (containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 10 mM MgCl2 and 0.1% Triton X100) for 2 hours in a rocker at 4°C and the complex was pulled down using Glutathione Sepharose 4B beads. The interaction of splicing proteins with Aurora-A was analysed by western blots against anti-GST and anti-Aurora-A antibodies.

FLAG pull-down assay

The FLAG-tagged SR proteins (SRSF) were expressed in HEK cells and the cells were lysed with M2 lysis buffer (50 mM Tris-base (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100 and 1mM EDTA). The FLAG-tagged SR proteins were incubated with FLAG-M2 beads and were washed twice with M2 lysis buffer. The bound FLAG-tagged SR proteins were incubated with recombinant HIS-tagged Aurora-A for 2 hours in a rocker at 4°C. The beads containing protein complexes were again washed thrice and eluted with 100μM of FLAG peptide. The interaction of SR proteins with Aurora-A was analysed by western blots against anti-FLAG and anti-HIS or anti-Aurora-A antibodies.

2.4. *In vitro* kinase assay

Recombinant GST-tagged splicing proteins and HIS-tagged Aurora-A were incubated in kinase buffer (containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 10 mM MgCl2 and 100 μM ATP) in presence of [γ-32P]-ATP at 37°C for 30 minutes. The reaction was stopped by the addition of SDS sample buffer and heating at 95 °C for 5 min. Phosphorylated splicing proteins were separated by SDS-PAGE on 10% gels and visualized by autoradiography

2.5. *In vitro* splicing assay

In vitro splicing assay was carried out in a 25 μl splicing mixture containing 1ng of β-globin pre-mRNA, 50% nuclear extract, 1mM ATP, 3.2mM $MgCl_2$, 20mM creatine phosphate and 2.6% polyvinyl alcohol and 0.4 units/μl RNasin in buffer D at 30°C for 45 minutes. Aurora-A was added 10 minutes before the start of splicing reaction and was incubated on ice. The splicing mixture was subjected to proteinase K treatment followed by phenol: chloroform extraction of RNA. The RNA was reverse transcribed and the splicing was assessed by PCR. The primers used are listed in **Table 2**.

Table 2 | List of primers used for PCR analysis of *in vitro* splicing assay

Gene name	Primer Sequence (5' → 3')	
β-globin	Forward	GTTGGTGGTGAGGCCCTG
	Reverse	GCATCAGGAGTGGACAGATCCC

2.6. Mass spectrometry data analysis

To define specific interactors of Aurora-A and rank the interacting partners, MaxLFQ was employed, which is the label-free quantification (LFQ) module of the MaxQuant software (Cox and Mann, 2008; Cox et al., 2014). The algorithm uses the peak area of the elution profile of a peptide to calculate LFQ intensity. The relative fold enrichment was calculated as,

$$\text{Relative enrichment} = \frac{(\text{LFQ intensity of protein-X in Aurora-A IP}) - (\text{LFQ intensity of protein-X in control IP})}{\text{LFQ intensity of Aurora-A protein in Aurora-A IP}}$$

2.7. Cell culture and transfection

U2OS cells, HEK cells and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM)- GlutaMAX ™ -I (Sigma-Aldrich) supplemented with 10% fetal bovine serum (GE Healthcare) and 1% penicillin–streptomycin (GE Healthcare). For transfection of siRNA (siAurora-A : 5′-AAATGCCCTGTCTTACTGTCA-3′) and Flag-tagged SRSF plasmids, cells were grown till 60% confluence and were transfected using jetPRIME® (Polyplus-transfection S.A, Illkirch, France) or Lipofectamine-3000 (ThermoFisher scientific) transfection reagents according to the manufacturer's instructions.

2.8. Western blotting

The cells were washed with PBS twice and scraped directly in 2X laemmli buffer and boiled in 95°C for 5 minutes. The proteins concentration was quantified using Protein Quantification Assay kit (Cat.No: 740967.50, Macherey-Nagel) The protein samples were resolved using 4–15% Mini-PROTEAN™ TGX Stain-Free™ Protein Gels (Biorad) and transferred to PVDF membrane (GE Healthcare) and analysed by western blotting. The following primary antibodies were used: rat anti-α-tubulin (MAB1864, Millipore, 1:10000), mouse anti-Aurora A (clone 5C3, 1:100 (Cremet et al., 2003)), rabbit anti-phospho-Aurora A/B/C (clone D13A11, 2914, Cell Signaling Technology, 1:500) mouse anti-SR (clone 16H3, 33-9300, Invitrogen, 1: 1000 dilution), mouse anti-phospho-SR (clone 1H4, MABE50, Merck, 1:500 or clone 10H3, 1:20 (Cavaloc et al., 1999)), mouse anti-SC35 (1:100 (Cavaloc et al., 1999), mouse anti-ASF/SF2 (clone 96, 32-4500, Thermo Fisher Scientific, 1:10000). Following secondary horseradish peroxidase-conjugated antibodies anti-mouse and anti-rabbit (Jackson Immuno Research Laboratories) and anti-rat (A110-105P, Bethyl Laboratories) were used. Membranes were incubated with home-made enhanced chemiluminescence containing 100 mM Tris (pH 8.5), 13 mg/ml coumaric acid (Sigma), 44 mg/ml luminol (Sigma) and 3% hydrogen peroxide (Sigma). Chemiluminescence signals were captured on a film (CP-BU new, Agfa Healthcare) with a CURIX 60 developer (Agfa Healthcare).

2.9. Immunofluorescence staining and microscopy

The cells grown on coverslips were fixed with methanol (−20°C) for 5 minutes. The cells were then blocked with 5% FBS in PBS and incubated with primary antibody (SC35- S4045 Sigma) at 1:2000 dilution in blocking solution for 1 hour at room temperature. Cells were washed thrice in PBS and incubated with secondary antibody (anti-mouse antibody conjugated to Alexa 647 or Alexa 546, Thermo Fisher Scientific) at 1:4000 dilution and GFP nano-booster (chromotek) at 1:1000 dilution in blocking buffer for one hour at room temperature. The cells were then washed and the DNA was stained using Hoechst 33342 (Thermo Fisher Scientific) or DAPI. Finally, the coverslips were mounted using ProLong® Gold antifade reagent (Thermo Fisher Scientific). High-resolution images were taken with the new LSM800 Airyscan (Zeiss Inc.) and processed with FIJI (ImageJ). Lasers power and acquisition settings are the same for all conditions within an experiment.

2.10. Flow cytometry analysis and mitotic index

After the cell cycle specific synchronization, HeLa cells were washed twice with PBS and was resuspended in 70% ice-cold methanol and incubated at 4°C for 1 hour. Pellets were washed twice with PBS and resuspended in PI buffer containing 50 μg/ml propidium iodide, 10mM Tris pH7.5, 5mM $MgCl_2$, 200μg/ml RNase A and incubated at 37°C for 30 minutes. The percentage of cells in each cell cycle phase was calculated with a Fortessa X-20 LSR (Becton Dickinson).

The mitotic index of FACS samples was performed in order to discriminate the cells in G2 and mitotic phase. The samples prepared for FACS analysis were mounted on a glass slide and counted using a fluorescent microscope (DMRXA2 Leica). At least 100 cells were counted for each condition.

2.11. RNA isolation and RT-PCR

All the synchronized cell samples for RNA isolation were stored in -70° C prior to FACS (cell cycle profile) and western analysis to confirm the correct cell synchronization and inhibition of Aurora-A. The total RNA was isolated with the RNeasy Plus Mini Kit (QIAGEN- Cat No./ID: 74134) as per the manufacturer's protocol. While isolating total RNA for RNA-seq analysis, on-column digestion of DNA was additionally

performed using RNase-Free DNase Set (QIAGEN - Cat No./ID: 79254) as a precautionary measure. For RT-PCR experiments, cDNA was synthesized using (Invitrogen ™) SuperScript ™ III Reverse Transcriptase. Briefly, 500 ng total RNA was reverse transcribed in a 20µl reaction containing 200 units of Superscript III reverse transcriptase, 40 units of RNAseOUT^{TM} recombinant ribonuclease inhibitor, 1X First strand buffer, 5mM of DTT, 0.5mM of dNTPs, 1.25µM of oligo-dT (Sigma-Aldrich) and 125ng of random primers (Life Technologies). The synthesized cDNA was diluted 6 times and used for PCR.

PCR reactions were carried out using 2µl of cDNA in a 30µl reaction containing 1X GoTaq® Flexi Buffer, 1.5mM of $MgCl_2$, 0.2mM of dNTPs, 0.6µM primers and 0.5 units of GoTaq® DNA Polymerase. The PCR reactions were carried out in the following condition: 95°C for 1 minute followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and finally at 72°C for 1 minute. The list of primers used for RT-PCR is mentioned in **Table 3 and 4**.

Table 3 | The list of primers used for RT-PCR (splicing event-specific PCR)

Gene Name	Primer Sequence (5' → 3')	
CHEK2	forward	CAGCTCTCAATGTTGAAACAGAA
	reverse	CAGCCAAGAGCATCTGGTAA
MCL1	forward	AGACCTTACGACGGGTTGG
	reverse	ACCAGCTCCTACTCCAGCAA
CCNE1	forward	ATCCTCCAAAGTTGCACCAG
	reverse	ATGATACAAGGCCGAAGCAG
RAC1	forward	TGCCAATGTTATGGTAGATGGA
	reverse	CTTTGCACGGACATTTTCAA
MSTR1	forward	GCTTTACACTGCCTGGCTTT
	reverse	GCCACCAGTAGCTGAAGACC
PKM2	forward	GCTGGAGAGCATGATCAAGAA
	reverse	CAACATTCATGGCAAAGTTCA
MAP3K7	forward	GAATCTGGACGTTTAAGCTTGG
	reverse	TGACCAGGTTCTGTTCCAGTT
MINK1	forward	CCTCAGAGGACCTCATCTATCG
	reverse	CGTGAGAGGCTGGAAGGAC
NUMB	forward	CTCCCTGTGCTCACAGATCA
	reverse	CGGACGCTCTTAGACACCTC
CASP2	forward	GCTCTTTGACAACGCCAACT
	reverse	GGCATAGCCGCATATCATGT

BCL2L1	forward	CAGTAAAGCAAGCGCTGAGG
	reverse	AAGAGTGAGCCCAGCAGAAC
APAF1	forward	ATGCGACATCAGCAAATGAG
	reverse	CTCTGCAATCAGCCACCTTT
MADD	forward	AGCCTCTATCGGAACCACAG
	reverse	GGGGTGATGGAGGTTCTCTT
CFLAR	forward	TTCAAGGAGCAGGGACAAGTTAC
	reverse1	GAATGATTAAGTAGAGGCAGTTCCA
	reverse2	TCCCATTATGGAGCCTGA
FAS	forward	GAACATGGAATCATCAAGGAATGCAC
	reverse	AGTTGGAGATTCATGAGAACCTTGG
OLR1	forward	GGCATGGAGAAAACTGTTACCTATTTTC CTC
	reverse	CACTGTGCTCTTAGGTTTGCCTTCTTCTG
MAP4K3	forward	GGCCAAGTGAAATTTGATCC
	reverse	TTGCACCTAAAAAGTAACCACCT
PAX6	forward	CCGGCAGAAGATTGTAGAGC
	reverse	CTCCCGCTTATACTGGGCTA
CASP9	forward	CGGGCAGGCTCTGGATCTC
	reverse1	CCAGCACCATTTTCTTGG
	reverse2	TGGTCTTTCTGCTCGACA
GADD45A	forward	AATATGACTTTGGAGGAATTC
	reverse	TCACCGTTCAGGGAGATTAATC
MAP4K2	forward	GAGCTGACAGCGTCTGTGG
	reverse	GCGAGTCTTATCTCTCAGTTTGG
STAT3	forward	CCCCATACCTGAAGACCAAG
	reverse	ACTCCGAGGTCAACTCCATGTCAA
BIRC5	forward	GAGGCTGGCTTCATCCACT
	reverse	TCTCCGCAGTTTCCTCAAAT
DIABLO	forward	ACCATGGCACAAAACTGTGA
	reverse	TCCTATGATCACCTGCCACA
SMN	forward	ATAATTCCCCCACCACCTCCC
	reverse	TTGCCACATACGCCTCACATAC
PHF19	forward	CCTGCTCACACCTTGGTTCT
	reverse	CACCAAACCCATCTCCACTT
	reverse	CCGCAGTAGCAGTAGCATTG

Table 4 | The list of primers used for RT-PCR (validation of VAST tool identified splicing events)

Gene name	Primer Sequence (5' → 3')	
CLK1	forward	CCACAGTACTTCACATCGTCG
	reverse	TGATCGATGCACTCCACAAC
CLK4	forward	CGGGTATCGAATCCACTGCAG
	reverse	ATGCCATGATCAATGCACTCT

2.12. Capillary electrophoresis and native PAGE of DNA

The RT-PCR products were separated using native PAGE gel electrophoresis. The native PAGE gels were stained with GelRed stain (Biotium) and imaged using Gel Doc system (Bio-Rad). The bands were then quantified using FIJI tool.

Alternatively, a high throughput Capillary electrophoresis was performed using the Labchip GX Caliper workstation (Caliper, Perkin Elmer) and an HT DNA 5K LabChip (Perkin Elmer) for analyzing alternative splicing of selected cell proliferation and apoptotic events. The separated RT-PCR products were then quantified using LabChip GX software.

2.13. RNA-seq analysis (Differential gene expression and splicing analysis)

Differential gene expression analyses (edgeR)

The quality of the RNA sequencing data was evaluated using the MultiQC tool. The reads were aligned using the Bowtie short read alignment program to the hg19 human reference genome. The read counts were corrected using edgeR upper quartile normalization factors. Differential gene expression analysis between Aurora-A inhibited and control conditions were carried out using edgeR tool.

Alternative splicing analyses (VAST-TOOLS)

For all events, a minimum read coverage of 10 actual reads per sample was required, as described (Irimia et al., 2014). PSI values for single replicates were quantified for all types of events (cassette exons, alternative 3' and 5' splice sites and retained introns). A minimum ΔPSI of 15% was required to define differentially spliced events upon each knockdown, as well as a minimum range of 5% between the PSI values of the two samples. (VAST tool: https://github.com/vastgroup/vast-tools).

2.14. Statistical analysis

All statistical analyses were performed using two-tailed pairwise student's t-tests. The P value of < 0.05 is considered as statistically significant.

3. RESULTS

3. 1. Analysis of Aurora-A interactome data

The interactome study of Aurora-A was carried out previously by former members of the lab (Olivia Gavard and Jean-Philippe Gagné). Briefly, they established an U2OS stable cell line expressing GFP-Aurora-A protein together with shRNA to deplete endogenous Aurora-A. The GFP-Aurora-A encoding gene was placed under the control of its minimal endogenous promoter in order to avoid the induction of any atypical phenotype generated as a consequence of the overexpression of Aurora-A. Two negative controls, wild-type U2OS cells and U2OS cells expressing GFP were used to eliminate contaminants. All these three cells were synchronized in mitosis using taxol since Aurora-A protein level is maximum at mitosis. Affinity-purification was performed using anti-GFP monoclonal antibody coupled to Protein-G magnetic beads. Immunoprecipitated protein complexes were resolved using 4-12% gradient PAGE and stained with Sypro Ruby. Following in-gel trypsin digestion, peptides were subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS) (**Figure 23**). I used this interactome data to identify the potential interactors of Aurora-A using label free quantification (LFQ).

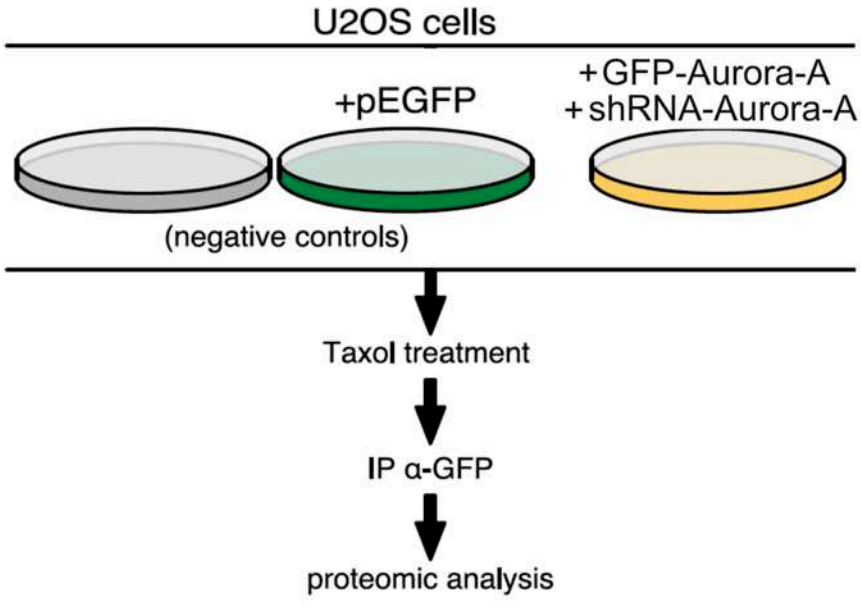


Figure 23 | Schematic of the strategy used to isolate and identify Aurora-A interacting proteins. Two negative controls (wild-type U2OS cells and U2OS cells expressing GFP) and U2OS cells with stable

expression of GFP-Aurora-A and shAurora-A were synchronized in mitosis by incubation with Taxol. GFP and GFP-AURKA were immunoprecipitated with anti-GFP antibody - coupled with magnetic beads. Immunoprecipitated proteins were identified by liquid chromatography tandem mass spectrometry (LC-MS/MS).

The complete list contained 722 proteins and was filtered for common contaminants (keratin, immunoglobin and ribosomal, proteins) to eliminate 103 contaminants. The remaining 619 proteins were further filtered with a stringent cut-off for sequence coverage less than 5%, which filtered out 137 proteins. The remaining 482 proteins were considered for label-free quantification (LFQ).

The label-free quantification using MaxQuant software provided the LFQ intensity of a protein in control and Aurora-A IP (immunoprecipitation). The fold enrichment was calculated as the ratio of LFQ intensity of a protein in Aurora-A IP to that of the control IP (IP from U2OS cells expressing GFP was used as control IP). However, the LFQ intensity of many proteins in control IP is zero. Though this increases the confidence of the data and implies that the interactors are in fact specific for Aurora-A, this made it impossible to calculate the fold enrichment as division requires non-null denominator. To overcome this problem, an alternate method was employed where "relative enrichment" was calculated,

$$\text{Relative enrichment} = \frac{(\text{LFQ intensity of protein-X in Aurora-A IP}) - (\text{LFQ intensity of protein-X in control IP})}{\text{LFQ intensity of Aurora-A protein in Aurora-A IP}}$$

All the proteins with a relative enrichment lesser than or equal to zero were filtered out and the remaining 444 proteins were considered as potential Aurora-A interacting partners and this data-set was used for global analysis such as gene ontology.

3.1.1. Gene ontology of Aurora-A interactome reveals the role of Aurora-A in pre-mRNA splicing

The enrichment analysis of the putative Aurora-A interacting partners was performed using Gene Ontology (GO) classification tool DAVID with default parameters. The DAVID tool mapped 441 out of 444 proteins for enrichment analysis and showed the significant enrichment of terms such as "mRNA splicing, via spliceosome (GO:0000398)", "mitochondrial respiratory chain complex I assembly (GO:0032981)" and other processes related to RNA processing (GO:0006364, GO:0006396, GO:0008380 etc.,) and mitochondrial biogenesis and function (GO:0007005, GO:0042776, GO:0006120 etc.,) (**Figure 24**). The enrichments were also observed for cell-cell adhesion and osteoblast adhesion. Most of the cell cycle related functions and microtubule-associated functions of Aurora-A have been well studied. The detailed study on the role of Aurora-A in mitochondrial biogenesis has also been recently published (Bertolin et al., 2018). Out of all enrichments, the mRNA splicing and spliceosome were very surprising as there were very few reports focusing on this functional aspect of Aurora-A. In particular Aurora-A has been shown to modulate alternative splicing of apoptotic genes by stabilizing the splicing protein ASF/SF2 (Moore et al., 2010). But it remains unclear how Aurora-A stabilizes ASF/SF2 levels and what are the global changes in splicing following Aurora-A inhibition. I decided to investigate further this potential function of Aurora-A.

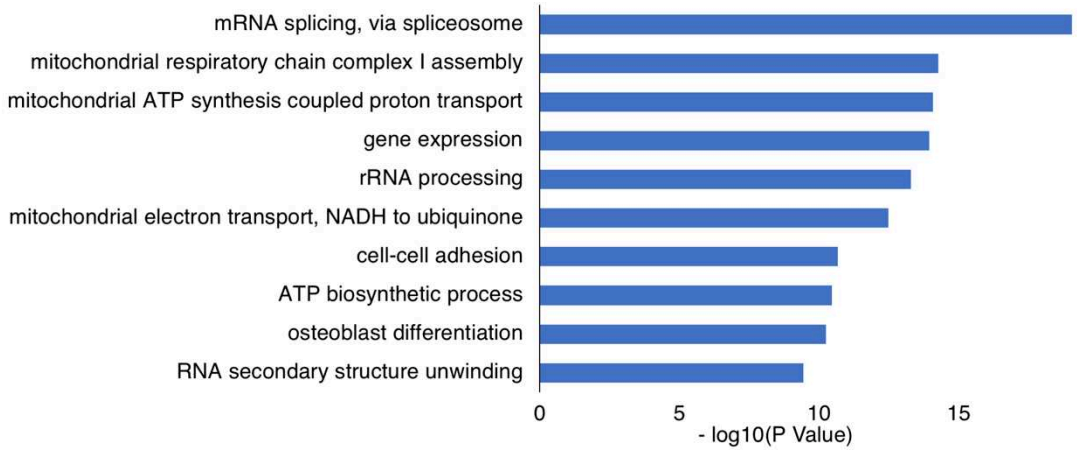


Figure 24 | Gene ontology of Aurora-A interactors. The histogram summarizes the enrichment analysis of Gene Ontology (GO) terms using annotations for the filtered AURKA protein interaction dataset (444 putative AURKA-interacting proteins). Top 10 predominant terms in Biological processes were plotted relative to their -log10(P values).

3.1.2. Aurora-A interacts with both the core and non-core components of the spliceosome

The splicing of pre-mRNA represents a crucial step in gene expression in eukaryotes. The splicing of pre-mRNA is carried out by a complex and dynamic machinery called 'spliceosome'. More than 200 proteins are known to be associated with spliceosome at different stages of splicing (Jurica and Moore, 2003; Schmidt et al., 2014; Wahl et al., 2009). Exploring the interaction of Aurora-A to the proteins in each sub-complexes of spliceosome is very important to understand the molecular mechanisms and the kind of regulation of splicing by Aurora-A. So I made use of the available proteomic data of the spliceosome complexes. Remarkably, the Aurora-A interactome contained 51 splicing proteins out of 263 known splicing proteins (**Figure 25A**). Agafonov et al categorized all the spliceosomal proteins into core and non-core components of spliceosome based on their abundance in the spliceosome. 141 highly abundant proteins were categorised as core-component of the human spliceosome and include the small nuclear ribonucleoproteins (U1, U2, U4, U5 and U6 snRNP). Conversely, 100 low abundant proteins were categorised as non-core proteins and include mostly regulatory proteins such as SR (Serine and arginine-rich) proteins and hnRNPs (Heterogeneous nuclear ribonucleoproteins) (Agafonov et al., 2011; Hegele et al., 2012). I discovered that out of 51 Aurora-A interacting splicing proteins, 11 proteins are from the core and 37 proteins are from the non-core components of the spliceosome. 3 out of 51 proteins were not categorized by Agafonov et al., 2011 (**Figure 25B**). Hegele et al showed 242 protein-protein interactions (PPI) between the core splicing proteins. These interactions form the basis for the formation of the active spliceosome complex that mediates the splicing reaction to remove introns from pre-mRNA (Hegele et al., 2012). They also had found 390 PPI between the non-core splicing proteins, which regulate the splicing machinery and links them to other cellular machinery such as transcription. This suggests that the Aurora-A could take part in splicing in two modes, one by modulating the PPI between the core components of the spliceosome and thus directly taking part in the splicing reaction and another one by modulating the PPI between non-core components in order to regulate the regulators of splicing machinery.

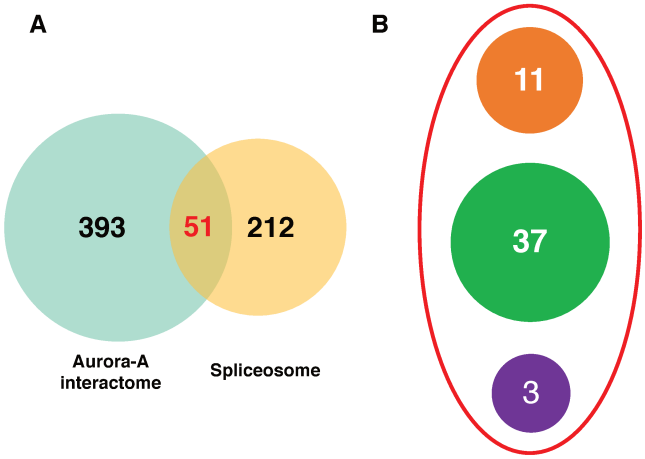


Figure 25| (A) Venn diagram showing identification of splicing proteins interacting with Aurora-A by comparing Aurora-A interactome with the 263 known splicing proteins. Numbers in the diagram refer to the number of proteins in each category. **(B)** Schematic drawing showing the categorization of Aurora-A interacting partners into core (orange), non-core (green) component of the spliceosome. The un-categorized proteins are in violet.

In order to determine if and how Aurora-A functions in splicing, the proteins in the Aurora-A interactome has been further assigned to distinct spliceosomal sub-complexes such as Sm proteins, U2 related, A complex, PRP19, B complex, SR proteins, hnRNP, MISC (Miscellaneous proteins), mRNP, EJC/TREX and C2 (potential C complex proteins) **(Figure 26A).** The relative enrichment analysis of the Aurora-A interactome indicated that proteins in the PRP19 complexes are the most abundant among the core-spliceosomal component, which suggest that the PRP19 complex proteins are strong interactors of Aurora-A. The PRP19 complex is known to mediate the non-proteolytic ubiquitination of PRP3 (a component of the U4 snRNP) and to stabilize the U4/U6.U5 tri-snRNP (Song et al., 2010).

The non-core component of the spliceosome is the group of proteins that are involved in the regulation of the spliceosome machinery in order to dictate the fate of pre-mRNA in response to different environmental cues. These include the two main classes of proteins called SR proteins and hnRNP proteins which are also called the trans-acting splicing factors. The trans-acting splicing factors include 12 SR proteins and 13 hnRNP families (Busch and Hertel, 2012; Manley and Krainer, 2010). The Aurora-A interactome contained 5 out of 12 SR proteins and 16 hnRNP proteins from the 13 hnRNP families (13 hnRNP families contain 37 proteins) (**Figure 26A**). These splicing factors recognize the cis regulatory elements present on the pre-mRNA and either promote or inhibit the recruitment of spliceosome. The relative enrichment analysis of the splicing proteins in

the interactome (**Figure 26A**) also implied that hnRNP and SR proteins are prominent interactors of Aurora-A.

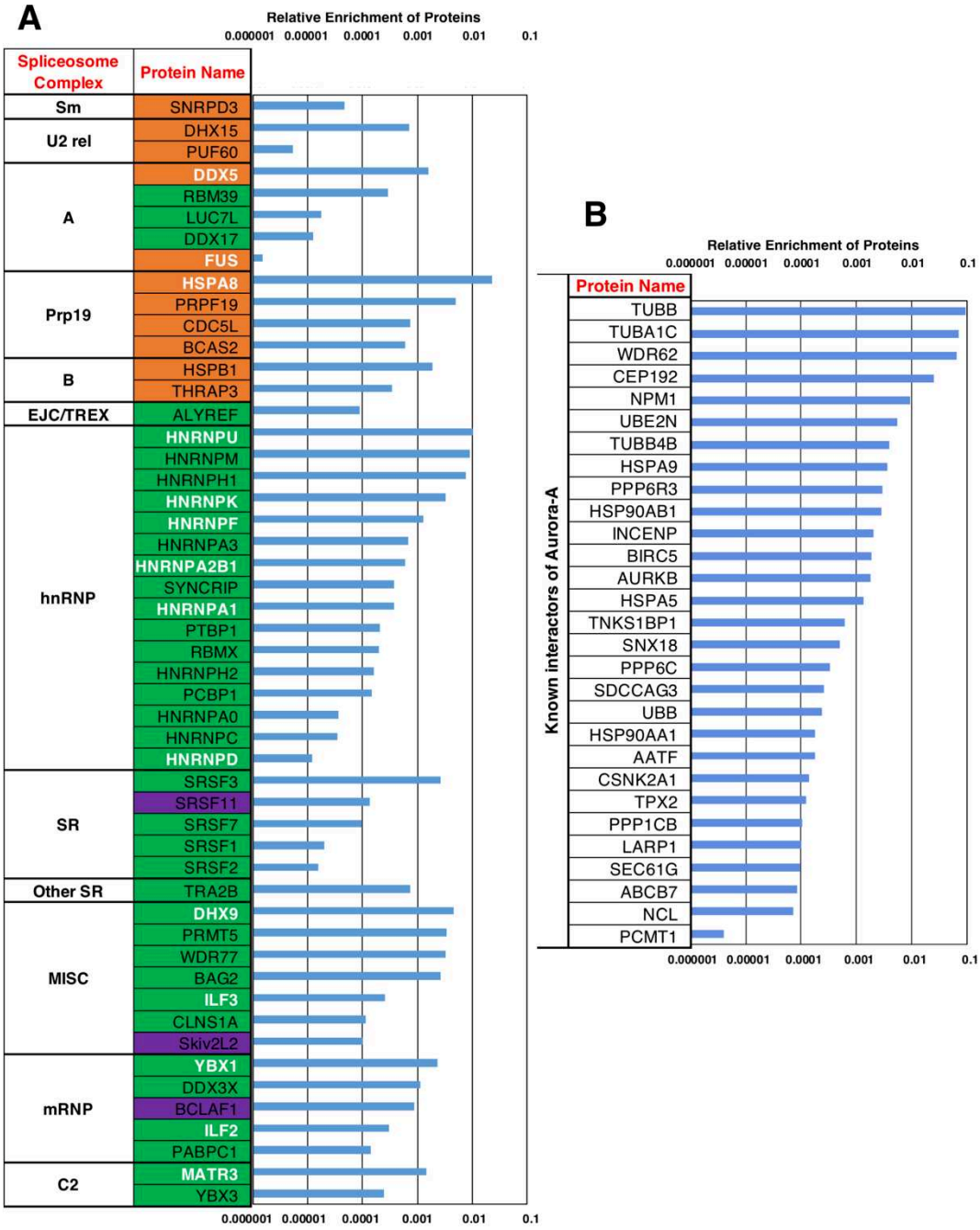


Figure 26 | Interaction of Aurora-A with core and non-core components of the spliceosome.
(A) Graphical representation showing the relative enrichment of the splicing proteins in the interactome of Aurora-A that are assigned to individual spliceosome sub-complexes. Green: non-core spliceosomal proteins; Orange: core spliceosomal proteins; Violet: un-categorized proteins. The proteins written in white are the known interactors of Aurora-A. **(B)** Graphical representation showing the relative enrichment of the non-splicing proteins in the interactome of Aurora-A, which are also known interactors of Aurora-A.

The relative enrichment of the splicing proteins in the Aurora-A interactome was similar to that of the non-splicing known interactors of Aurora-A (**Figure 26B**). The splicing protein in the interactome contained 14 known Aurora-A interactors, which had already been reported by others (**Figure 26A – protein names in white**). This provides additional support for the high-quality of the data. Thus, this data set is a very reliable resource that will promote further investigation of the function of Aurora-A in splicing. Overall the analysis of interactome data of Aurora-A revealed the interaction of Aurora-A with both the core and non-core splicing component which strongly suggested that Aurora-A could impact pre-mRNA splicing in multiple ways.

3.2. Aurora-A directly interacts with splicing proteins and phosphorylates them *in vitro*

3.2.1. Aurora-A directly interacts with splicing proteins *in vitro*

As with any interactome or proteome that identifies even the co-pulled down proteins, *in vitro* assays are required to validate the direct interaction between splicing proteins and Aurora-A. Often the proteomic data are observed with the presence of different class of proteins (Cytoskeletal/structural/motility proteins, DEAD-box proteins, Eukaryotic translation elongation, and initiation factors, Heat shock proteins, Histones, hnRNP proteins, Ribosomal proteins), which are often considered as common contaminants bound to the affinity matrices (beads) (Trinkle-Mulcahy et al., 2008). In the case of Aurora-A interactome, all these class of proteins were highly represented. In order to confirm that identified proteins in the interactome of Aurora-A are genuine interactors of Aurora-A, I tested the direct protein-protein interaction by performing *in vitro* GST or FLAG pull-down assays.

The GST pull-down assay was performed between recombinant Aurora-A and splicing proteins (hnRNP-C2, RALY, PCBP2, eIF4A3 and PTBP1) or the ribosomal protein RS26, which was filtered out as a contaminant. The assay revealed a direct interaction of Aurora-A with the positive control, NPM as well as with all splicing proteins tested (hnRNP-C2, RALY, PCBP2, eIF4A3 and PTBP1) **(Figure 27A)**. There was no interaction of Aurora-A with the GST protein, which confirms the specific interaction between Aurora-A and the splicing proteins. Surprisingly Aurora-A also interacted directly with RS26, a protein from ribosomal subunits, which was often considered as a common contaminant during proteomic analysis.

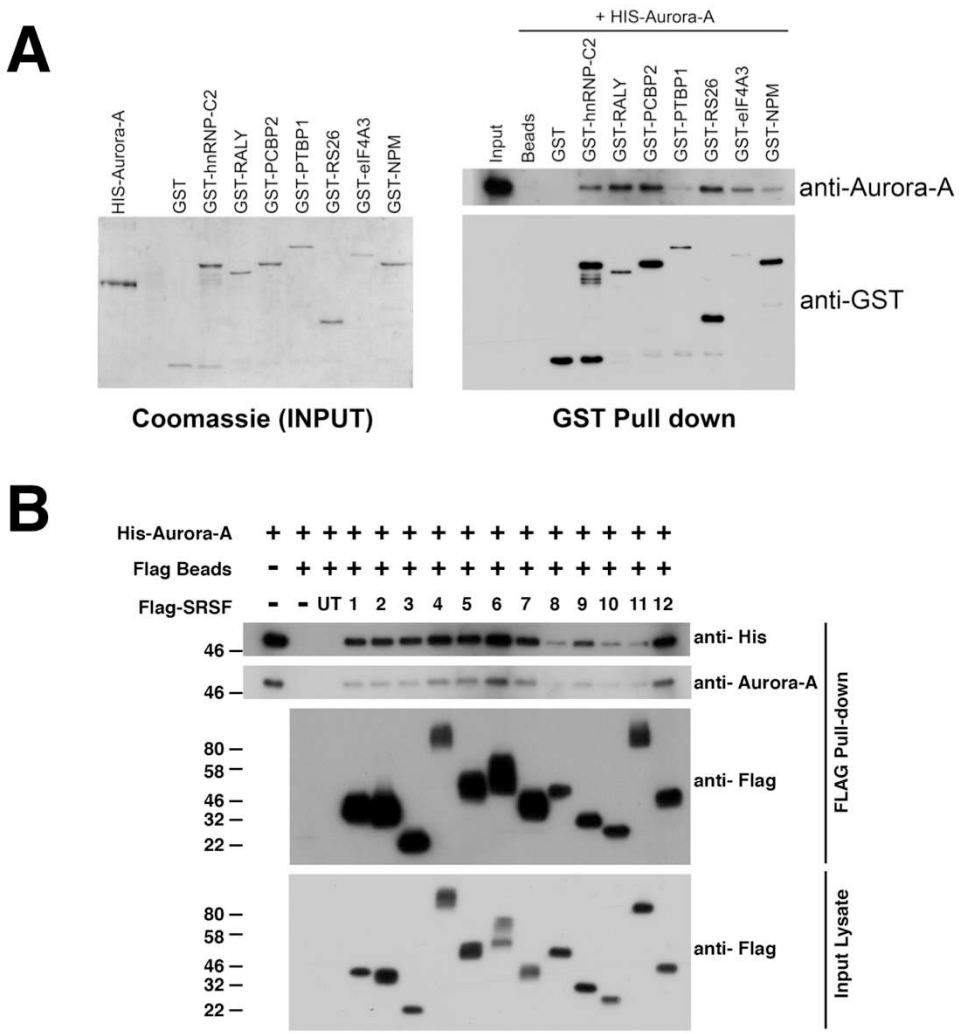


Figure 27 | Aurora-A directly interacts with the splicing proteins and phosphorylates them *in vitro*. **(A)** GST pull-down assay showing the direct interaction of the splicing proteins with Aurora-A *in vitro*. The left panel shows the coomassie staining of purified recombinant HIS-tagged Aurora-A and GST-tagged splicing proteins purified from E.coli. The right panel is the western blot showing the GST pull-down assay between HIS-tagged Aurora-A and GST-tagged splicing proteins. GST was used as negative control and NPM was used as positive control. **(B)** FLAG pull-down assay showing the direct interaction of the SR proteins with recombinant HIS-tagged Aurora-A *in vitro*. The bottom panel shows the western blot analysis of lysates from HEK cells that were overexpressed with FLAG-tagged SRSF proteins. The middle panel is the western blot analysis showing the FLAG pull-down of SRSF proteins. The top two panels are the western blot showing the interaction of Aurora-A with SR proteins.

In parallel, FLAG-tagged SR (SRSF) protein was overexpressed in HEK cells, purified using FLAG-M2 beads and used for *in vitro* FLAG pull-down assay. The assay revealed the direct interaction of Aurora-A with all the 12 canonical SR proteins tested **(Figure 27B**). However, I did not observe all the SR proteins in the interactome which could be because of the spatiotemporal dynamics of these proteins. It is well-known that

all the 12 SR proteins have one or two RNA Recognition Motif (RRM) and an arginine/serine (RS) domain (Manley and Krainer, 2010). On the other hand, hnRNP proteins are known not to contain RS domains but instead contain 4 types of RNA binding domains (RBD) such as RRM, aRRM, RGG box and KH domain (Busch and Hertel, 2012; Han et al., 2010). It is also evident from the domain enrichment analysis that there is an enrichment for RRM domain among the interactors of Aurora-A **(Figure 28)**. The enrichment was also observed for domains such as "nucleotide-binding, alpha-beta plait", "RNA helicases", "Tubulin". Collectively these interactome and *in vitro* interaction data indicate that Aurora-A can bind to the proteins that contain RRM. The RNA helicase domain containing proteins are known to be involved in spliceosome assembly process by controlling the conformational rearrangement between snRNAs.

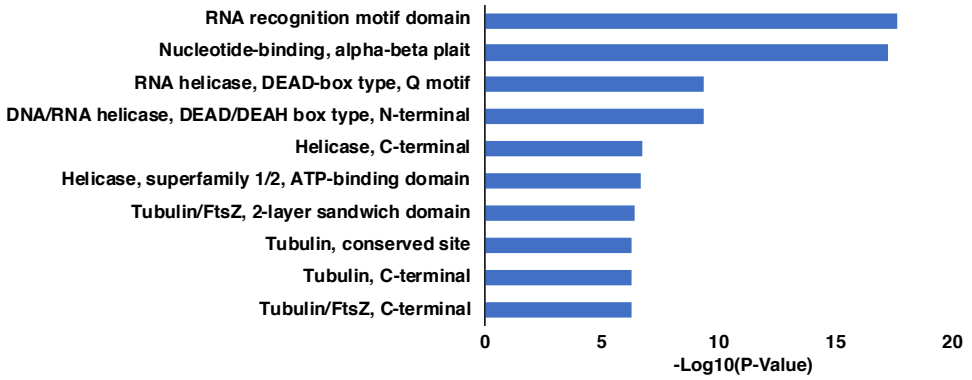


Figure 28 | The histogram summarizing the domain enrichment analysis of Gene Ontology (GO) against the InterPro database using the filtered AURKA protein interaction dataset (444 putative AURKA-interacting proteins). Top 10 predominant domain terms were plotted relative to their -log10(P values).

3.2.2. Aurora-A phosphorylates the splicing factors *in vitro*

Phosphorylation of splicing factors is known to alter multiple crucial steps during pre-mRNA splicing. Phosphorylation influences dynamic structural reorganization of spliceosome by modulating protein-protein interaction (Misteli and Spector, 1999). Xiao et al., 1997 showed that phosphorylation of ASF/SF2 in its RS domain affects both the protein-protein and protein-RNA interactions and reduces the non-specific binding towards RNA (Xiao and Manley, 1997). Phosphorylation event is important for pre-mRNA splicing. The concentration of various splicing factors on the site of splicing affects the alternative splicing. Phosphorylation of splicing proteins is also important for their localization. For example, phosphorylation of SR proteins promote their recruitment

to the site of transcription from nuclear speckles (Misteli et al., 1998). Phosphorylation is not only important for SR proteins but also for hnRNPs to alter their localization and splicing activity. Osmotic stress induces phosphorylation of hnRNP-A1 at F peptide leading to accumulation of hnRNP-A1 at cytosol (Allemand et al., 2005). Phosphorylation of hnRNP-L by AKT induces anti-apoptotic splicing of Caspase 9 by increasing its affinity for exon 3 of Caspase 9 and competing with hnRNP-U (Vu et al., 2013). It has to be noted that AKT consensus sequence targeted for phosphorylation is RxRxxS that contains an Aurora-A phosphorylation site RxxS.

Since phosphorylation plays such a crucial role during splicing and Aurora-A being a protein kinase, I tested whether the proteins interacting directly with Aurora-A could be phosphorylated by Aurora-A using *in vitro* kinase assay. I found that the splicing proteins hnRNP-C2, RALY, PCBP2, PTBP1 and eIF4A3 are phosphorylated by Aurora-A *in vitro* **(Figure 29)**. PTBP1 is phosphorylated more in spite of its weak interaction with Aurora-A **(Figure 27A and 29)** whereas RALY is less phosphorylated though interacting strongly with Aurora-A. This suggests that different splicing proteins interact with Aurora-A with different affinity and are phosphorylated at different efficiency, which may be attributed to the different number of sites phosphorylated by Aurora-A. However, I did not observe phosphorylation for the ribosomal protein, RS26 in spite of its interaction with Aurora-A, which indicates that interacting with Aurora-A is not sufficient for a protein to be a substrate of Aurora-A. Overall the *in vitro* interaction and kinase assay suggested that Aurora-A possesses some selectivity for splicing proteins.

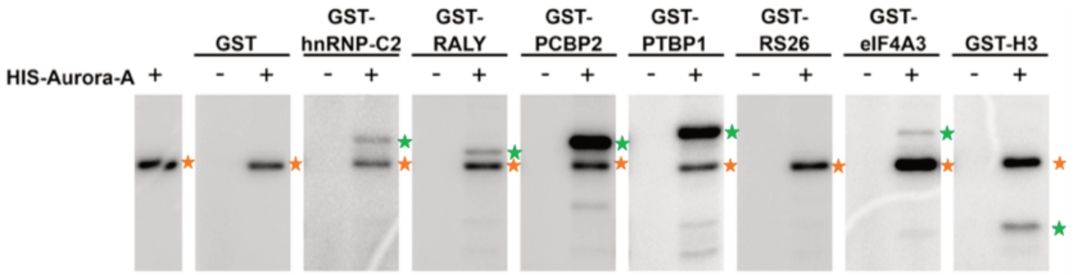


Figure 29 | *In vitro* kinase assay of splicing proteins. Phosphorylation of splicing proteins by Aurora-A was analyzed by autoradiography. Star (*) indicates the auto-phosphorylation of Aurora-A and star (*) indicates the phosphorylation of substrate (splicing proteins). GST was used as negative control and H3 was used as positive control. Coomassie staining of purified recombinant HIS-tagged Aurora-A and GST-tagged splicing proteins is shown in the left panel of **Figure 27A**.

3.3. Effect of Aurora-A on SR proteins *in vivo*

Many SR proteins and hnRNPs has been implicated in cancers either as tumour suppressor or proto-oncogenes. For instance, SR proteins such as SRSF1, SRSF3, SRSF6 and SRSF10 can act as proto-oncogenes/oncogenes. hnRNPs such as hnRNP-A1, hnRNP-A2, hnRNP-I, hnRNP-A2/B1 and hnRNP-H act as proto-oncogenes/oncogenes, whereas, hnRNP-K acts as a tumour suppressor in leukaemia (Dvinge et al., 2016). It has been reported that the inhibition or depletion of Aurora-A kinase reduced the ASF/SF2 (SRSF1) protein levels (Jones et al., 2017; Moore et al., 2010). Since Aurora-A interacted with many splicing proteins and phosphorylated few of them *in vitro*, I examined whether the levels of SRSF proteins were affected upon RNAi mediated depletion of Aurora-A. I observed that depletion of Aurora-A indeed leads to a dramatic decrease in levels of many SRSF proteins including ASF/SF2 (SRSF1) and SC35 (SRSF2) **(Figure 30)**. This result indicates that Aurora-A is important for maintaining the level of 'SR proteins'. However, it has to be further determined if this kind of regulation by Aurora-A on SR proteins is in RNA or protein level.

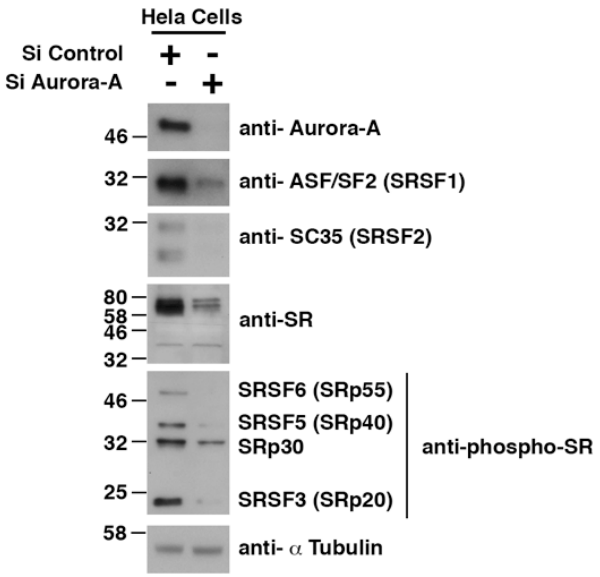


Figure 30 | Depletion of Aurora-A reduces phosphorylated SR proteins. HeLa cells were treated with 25 nM of siRNA against luciferase or Aurora-A for 48 hours. The whole cell lysates were prepared and western analysis was performed with antibodies against Aurora-A, ASF/SF2, SC35, SR, phospho-SR and α-tubulin. Western blot against α-tubulin acts as a loading control.

Moore et al., 2010 showed that the inhibition of Aurora-A reduced ASF/SF2 protein levels and argued that this reduction lies at post-translational level as Aurora-A

inhibition also reduced the exogenous GFP tagged ASF/SF2 levels similar to endogenous ASF/SF2 protein. Further investigations are now required to address if Aurora-A acts in RNA or protein level in order to maintain the level of 'SR proteins'.

3.4. Aurora-A localizes to nuclear speckles

The pre-mRNA splicing proteins are enriched in a specific domain located in the interchromatin region of nucleoplasm called "Nuclear speckles", which appear as 20-50 irregularly shaped structures of varying size (Spector and Lamond, 2011). Since many splicing proteins were identified in the interactome of Aurora-A, I decided to address whether Aurora-A localizes in the nuclear speckles. In that aim, U2OS cells were transfected with GFP and GFP-Aurora-A plasmids, fixed 24 hours after transfection and stained with SC35 antibodies, a nuclear speckle marker and nanobodies to reveal GFP (**Figure 31A**). Cells transfected with GFP plasmid does not show co-localization of GFP with SC35 speckles or nuclear speckles, whereas the cell transfected with GFP-Aurora-A plasmid shows a strong co-localization of GFP-Aurora-A with the SC35 speckles (**Figure 31A**).

To confirm this observation, U2OS cells expressing GFP-tagged Aurora-A under the control of its own promoter were stained with SC35 antibodies, a nuclear speckle marker and nanobodies to reveal GFP. Again GFP-Aurora-A staining was detectable not only at centrosome but also in the form of punctuated patterns, some of which co-localize with the SC35 staining (**Figure 31B**). The analysis of this image with line scan showed that the Aurora-A intensity peaks completely overlap and coincide with the SC35 peaks. **(Figure 31C)**. This result clearly shows the localization of Aurora-A to nuclear speckles.

It has to be noted that the splicing factors are stored in the nuclear speckles and are recruited to the site of active transcription in order to perform their functions in pre-mRNA splicing (co-transcriptional splicing) in a phosphorylation-dependent manner (Misteli and Spector, 1999; Misteli et al., 1997, 1998). Altogether, these observations (**Figure 29 and 31)** suggest that Aurora-A localizes to the nuclear speckles in order to regulate the splicing factors through its kinase activity. Girard et al showed that ~80 % of pre-mRNA splicing occurs co-transcriptionally at the periphery of nuclear speckles and remaining ~20 % of pre-mRNA splicing occurs post-transcriptionally within the nuclear speckles (Girard et al., 2012). The immunofluorescence image shows that Aurora-A localized within nuclear speckles (yellow area in the center) (**Figure 31D - bottom right**

panel) and also to their periphery (green) (**Figure 31D - right panels**) suggesting Aurora-A could regulate both co- and post-transcriptional splicing.

Altogether, these results indicate that Aurora-A kinase localizes to nuclear speckles and its periphery (the site of transcription/splicing) in full agreement with the notion that Aurora-A participates in pre-mRNA splicing.

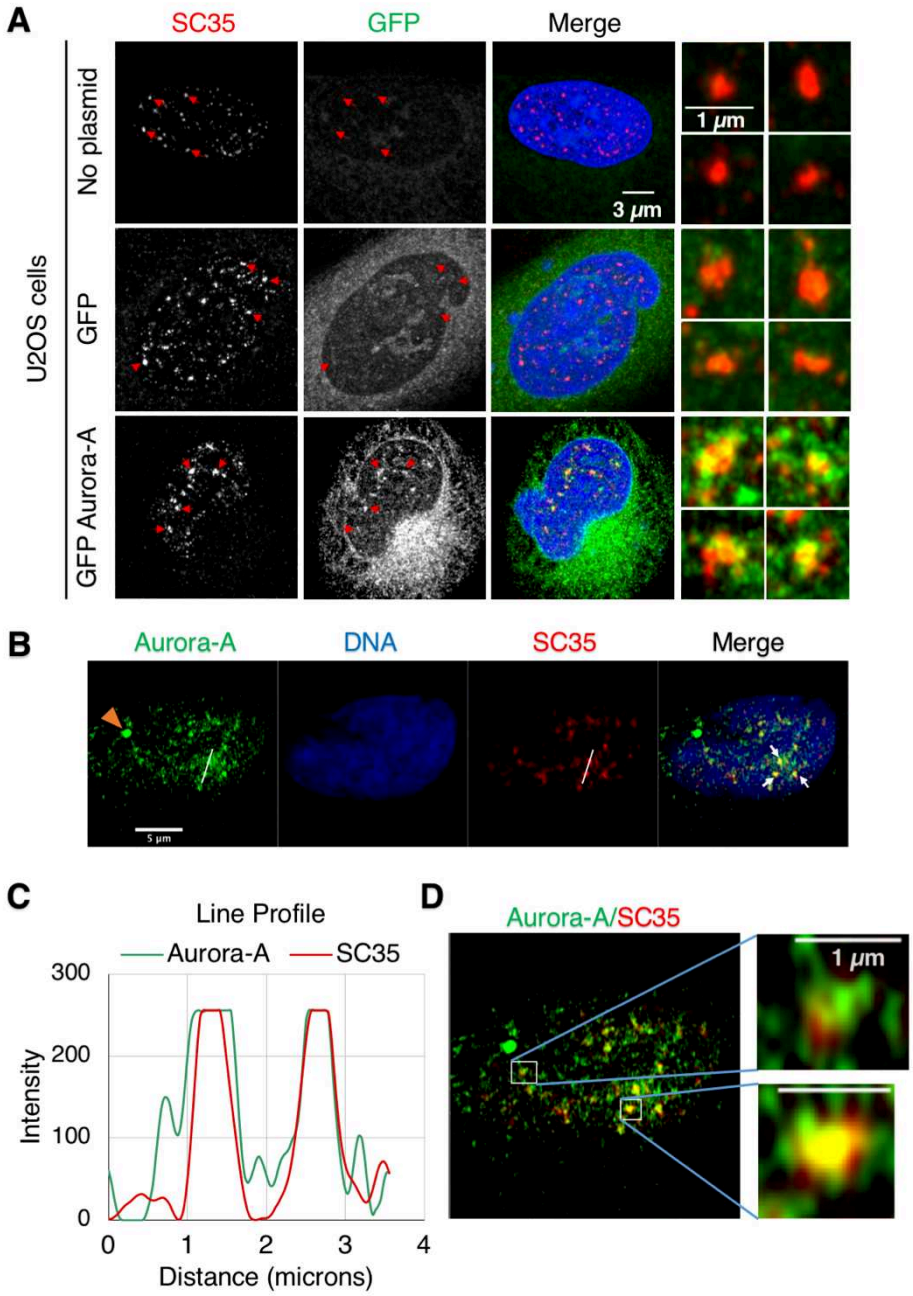


Figure 31 | Aurora-A localizes to nuclear speckles and periphery of nuclear speckles. (A) Immunofluorescence (IF) microscopy of U2OS cells transfected with GFP and GFP-Aurora-A plasmids. Nuclear speckles were visualized with the anti-SC35 antibody (red) and DNA with DAPI (blue). Scale bar, 3 μm. The extreme right panels show the magnification of the regions pointed with

red arrow marks in the first two left panels. Scale bar, 1 μm. **(B)** Immunofluorescence (IF) microscopy of U2OS cells expressing GFP-Aurora-A (green). Nuclear speckles were visualized with the anti-SC35 antibody (red) and DNA with DAPI (blue). The centrosome is indicated in orange arrowhead in the left panel and three white arrows indicate the co-localization of Aurora-A with SC35. Scale bar, 5 μm. The image represented here is from the single stack. **(C)** Line scans showing local intensity distributions of Aurora-A in green and of SC35 in red. A white line bar in **B** indicates the position of the line scans. **(D)** Left Panel: Magnification of the merged image in **B** excluding DAPI staining. Right panel: Magnification of the boxed region in the left panel**.** Scale bar, 1 μm.

3.5. Aurora-A promotes splicing of β-globin pre-mRNA *in vitro*

I revealed the localization of Aurora-A to nuclear speckles and its periphery where the post and co-transcriptional splicing are known to take place respectively. Also, Aurora-A interacts with and phosphorylates the splicing proteins *in vitro*. In order to investigate if Aurora-A could modulate pre-mRNA splicing, I performed an *in vitro* splicing assay using HeLa cell nuclear extracts and β-globin pre-mRNA (Kole *et al*., 1982). I observed that the addition of active Aurora-A to the assay increases the splicing efficiency of β-globin pre-mRNA in a dose-dependent manner (**Figure 32**). This result indicates that Aurora-A kinase participates in pre-mRNA splicing reaction and can potentially enhance the splicing efficiency.

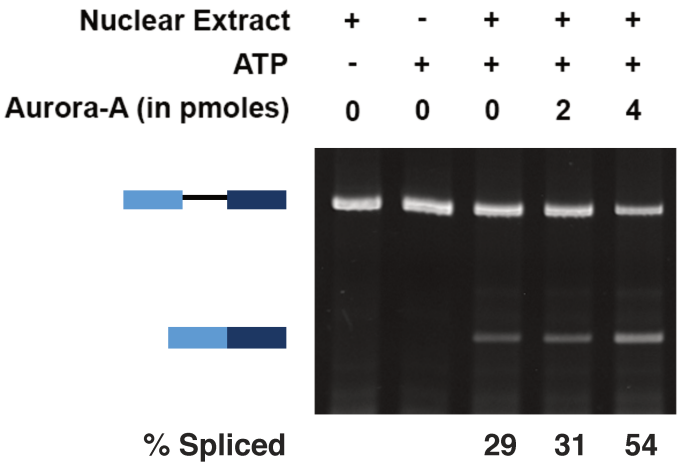


Figure 32 | Aurora-A promotes splicing of β-globin pre-mRNA. RT-PCR products of the splicing reaction separated on 8% native page. The top band on the gel indicates the un-spliced β-globin pre-mRNA and the bottom band indicates the spliced product (β-globin mRNA). The numbers below the figure represent the splicing efficiency of β-globin pre-mRNA calculated as percentage spliced.

Altogether, these results suggest that Aurora-A kinase participates in pre-mRNA splicing by modulating the splicing factor's phosphorylation and their protein levels.

3.6. Effect of Aurora-A inhibition on pre-mRNA splicing

I have shown that Aurora-A can regulate pre-mRNA splicing *in vitro*, interacts with many trans-acting splicing factors and phosphorylates at least a subset of them *in vitro*. The phosphorylation of the splicing proteins is important for their localization, function and activity. The *in vitro* results suggest that Aurora-A could modulate the splicing *in vivo*. In order to understand the functional aspect of these physical interactions *in vivo*, I examined whether inhibition of Aurora-A would affect pre-mRNA splicing in cultured cells. This experiment was carried out in two ways. The first method was focused only on the alternative splicing of genes involved in cell proliferation and apoptosis as Aurora-A is known to function in cell proliferation and anti-apoptosis in cancer. The second method involves the identification of transcriptome-wide pre-mRNA splicing alteration by Aurora-A. Both these experiments were carried out in collaboration with Dr. Juan Valcarcel's team from Centre for Genomic Regulation, Barcelona, Spain. For both these experiments, HeLa cells were used as Dr. Juan Valcarcel's team used HeLa cells as a model system for their splicing studies.

3.6.1. Effect of Aurora A inhibition on alternative splicing of cell proliferation and apoptotic events

3.6.1.1. Inhibition of Aurora-A with MLN8237

In order to evaluate the impact of Aurora-A on pre-mRNA splicing, HeLa cells were treated with a selective Aurora-A inhibitor, MLN8237 for 24 hours. MLN8237 (Alisertib) is a highly selective small molecule inhibitor of Aurora kinase A (Manfredi et al., 2011). The perturbation of Aurora-A is known to induce mitotic arrest (Park et al., 2008; Yang et al., 2005). Consistent with these publications, inhibition of Aurora-A for 24 hours increased the number of G2/M population in a dose-dependent manner (**Figure 33**). Since splicing is altered with respect to the cell cycle stage and to avoid the indirect effect of cell cycle on pre-mRNA splicing, in the following experiments, the splicing alterations were evaluated in synchronized cells.

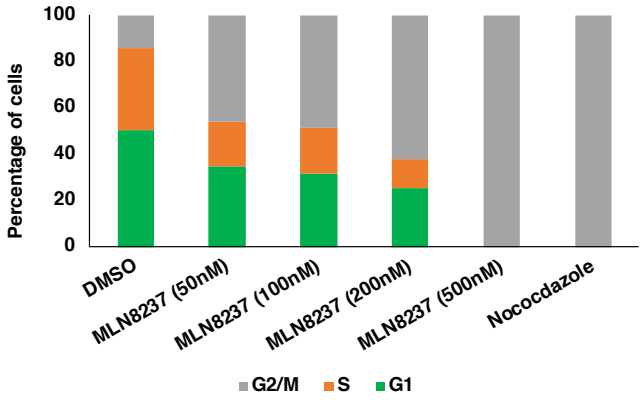


Figure 33 | Inhibition of Aurora-A alters cell cycle profile of HeLa cells. HeLa cells were grown for 24 hours and then inhibited for Aurora-A using the inhibitor, MLN8237 for 24 hours. The graph shows the cell cycle distribution upon treatment with different amount of Aurora-A inhibitor and nocodazole. DMSO acts as the control.

3.6.1.2. Inhibition of Aurora-A alters modestly alternative splicing of cell proliferation and apoptotic genes.

Aurora-A is well known to be involved in cell proliferation and inhibiting apoptosis of cancer cells (Wang et al., 2006). Aurora-A is also known to alter the alternative splicing pattern of certain apoptotic genes such as *Bcl-x*, *Mcl1*, and *caspase-9* (Moore et al., 2010). Thus I tested if inhibition of Aurora-A can alter other cell proliferation and apoptotic events by employing the methods described by (Papasaikas et al., 2015). Briefly, HeLa cells were synchronized in G1, G2 and mitosis and inhibited for Aurora-A and later the alternative splicing of 28 genes where evaluated by separating the RT-PCR products using capillary electrophoresis.

HeLa cells were synchronized at G1, G2 and mitosis as described in **Figure 34A, B and C** and the cell cycle distribution analysis shows that the cells are synchronized at G1, G2 and mitosis without any difference in cell cycle distribution between control (DMSO) and MLN8237 treatment condition **(Figure 34D, E and F**). The inhibition of Aurora-A at each cell cycle phase was examined using the antibody that recognizes the auto-phosphorylation of Aurora-A at threonine-288 residue. Upon inhibition of Aurora-A, there is a marked decrease in the levels of phosphorylation of thr288 levels but not in that of Aurora-B and Aurora-C **(Figure 35)**. It is evident from the western blot results that the drug MLN8237 effectively and selectively inhibited Aurora-A at all there synchronized conditions.

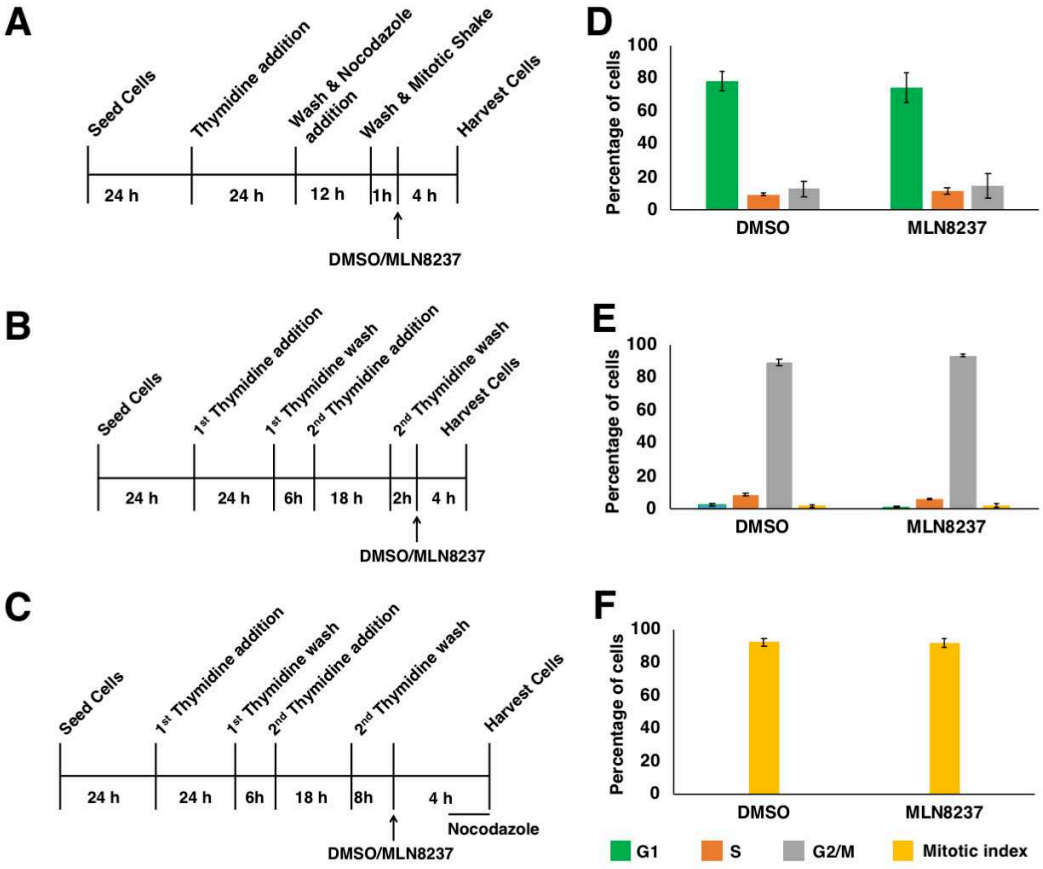


Figure 34 | Cell synchronization condition and cell cycle distribution of HeLa cells. (A) The scheme shows the condition used for synchronizing of HeLa cells at G1 phase using sequential treatment with thymidine and nocodazole. **(B)** The scheme shows the condition used for synchronizing of HeLa cells at G2 phase after releasing cells from double thymidine block for 6 hours. **(C)** The scheme shows the condition used for synchronizing of HeLa cells at mitotic phase by releasing cells from double thymidine block for 12 hours. Nocodazole was used in the last two hours of synchronization to prevent the release of cells from mitosis. Mitotic cells were collected by mitotic shake-off. **(D)** The Graph showing cell cycle profile from FACS indicating the synchronization HeLa cells at G1 phase. **(E)** The Graph showing cell cycle profile from FACS and mitotic index indicating the synchronization HeLa cells at G2 phase. **(F)** The Graph showing mitotic index indicating the synchronization HeLa cells at mitotic phase.

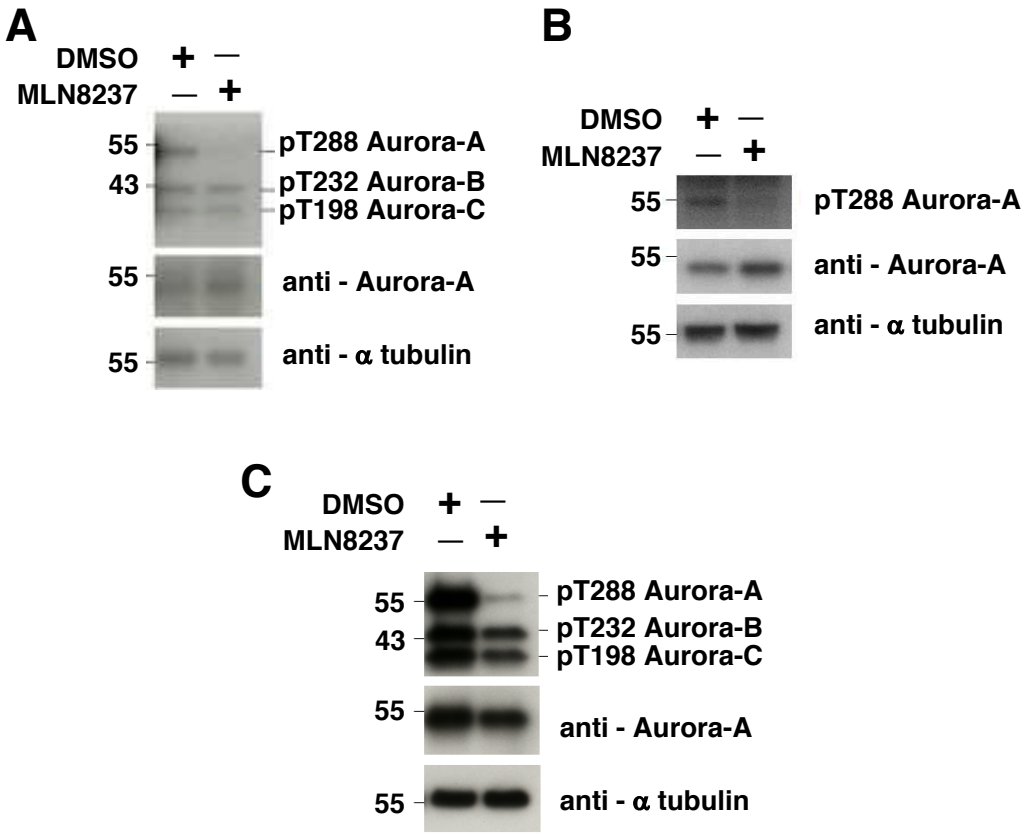


Figure 35 | Inhibition of Aurora-A in HeLa cells synchronized at G1, G2 and mitotic phases. (A) Western blot analysis of HeLa cells synchronized at G1 phase showing the decrease in the levels of T228 phosphorylation of Aurora-A. **(B)** Western blot analysis of HeLa cells synchronized at G2 phase showing the decrease in the levels of T228 phosphorylation of Aurora-A. **(C)** Western blot analysis of HeLa cells synchronized at mitotic phase showing the decrease in the levels of T228 phosphorylation of Aurora-A

Total RNA was extracted under these synchronized conditions and subjected to processing for splicing changes of genes involved in cell proliferation and apoptosis. The splicing event-specific PCR was carried out for 31 events spanning around 28 genes. The percent spliced index (PSI) is calculated as, PSI = (Inclusion / (Inclusion + Skipping))*100

The percent spliced index for control and MLN8237 treatment for genes involved in cell proliferation and apoptosis are shown in **Figure 36**. I observed the alternative splicing for only 5 events to be altered upon inhibition of Aurora-A in all three cell cycle phases (G1, G2 and mitosis). During G1 phase, The maximum splicing difference was observed for *MINK1* with a P value of 0.001 followed *MAP4K3*, *MSTR1* and *MAP4K2* with a P value of 0.002, 0.01 and 0.02 respectively. I did not observe any difference in splicing at G2 phase whereas *Rac1* (P value=0.01) was found to be altered at mitosis.

Overall, Aurora-A inhibition shows a modest difference in splicing events of cell proliferation and apoptotic events.

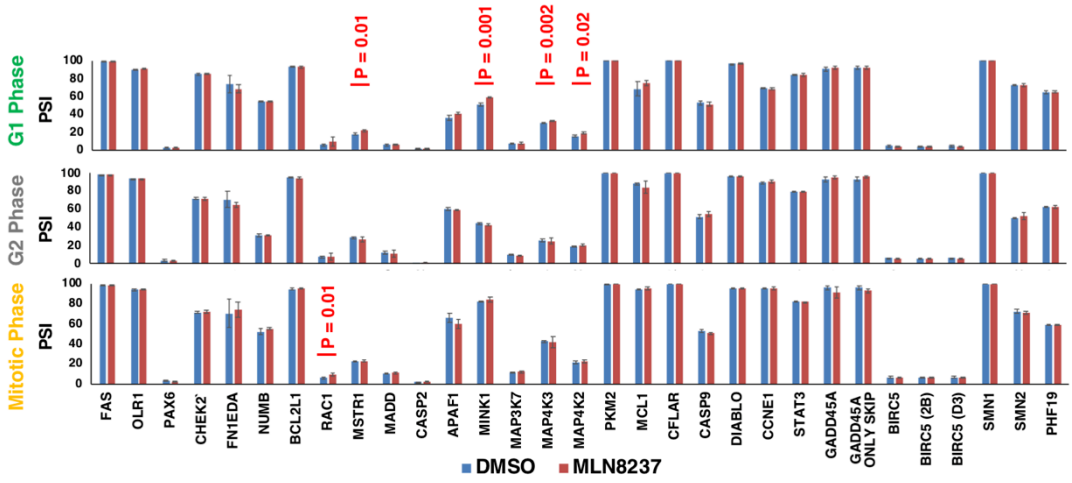


Figure 36 | Percent spliced index (PSI) of cell proliferation and apoptotic genes at G1, G2 and mitotic phase. Bar diagram showing the percent spliced index (PSI) calculated from the high throughput electrophoresis of RT-PCR samples (splicing event-specific PCR). Top panel showing PSI at G1 phase. Middle panel showing PSI at G2 phase. Lower panel showing PSI at mitotic phase. (n=3).

However, in our alternative splicing analysis of selected genes, we did not observe any difference in splicing of *Bcl-x* (*BCL2L1*), *Mcl1*, and *caspase-9*. I suspect the reason for this modest effect on splicing of cell proliferation and apoptotic events is due to the fact that inhibition of Aurora-A was carried out only for the duration of 4 hours. It is also known that the Aurora-A inhibitor, MLN8237 induces multiple abnormalities in a dose and time-dependent manner (Asteriti et al., 2014). However, using a higher dose of inhibitor in combination with cell synchronization was very challenging. So for further experiments, I decided to use low dose (50 nM) of MLN8237 to inhibit Aurora-A for 24 hours.

3.6.2. Impact of Aurora-A inhibition on transcriptome-wide gene expression and pre-mRNA splicing

In order to evaluate the impact of Aurora-A on transcriptome-wide pre-mRNA splicing profile, the HeLa cells were synchronized in G1, G2 and mitotic phase and simultaneously treated with Aurora-A kinase inhibitor. This time Aurora-A was inhibited for 24 hours as 4 hours of inhibition had a modest effect on splicing of cell proliferation

and apoptotic genes. The conditions used for cell synchronization is described in **Figure 37**.

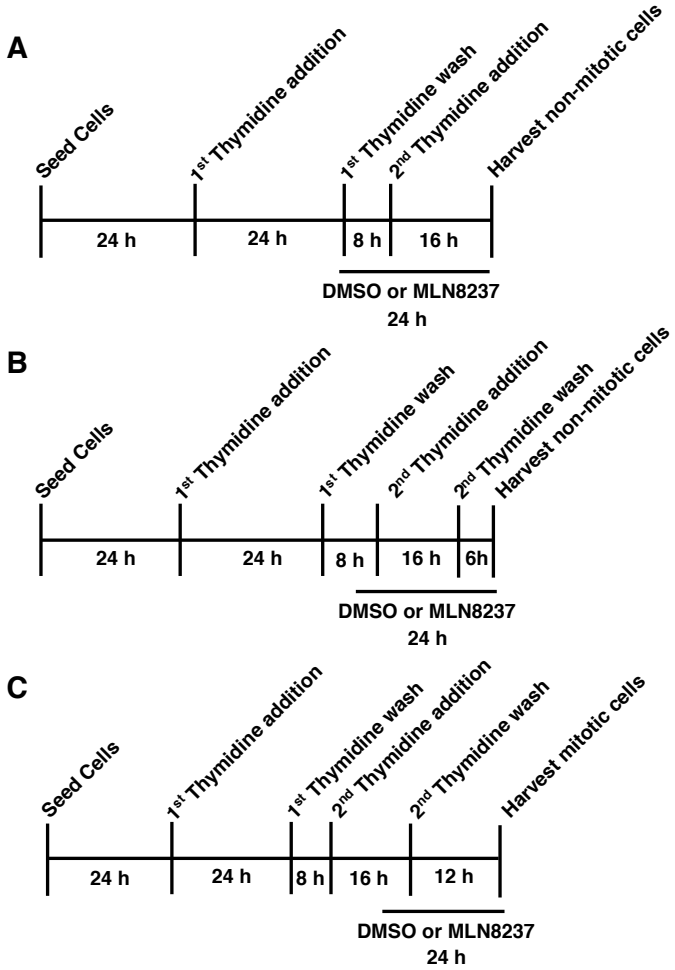


Figure 37 | Cell synchronization conditions of HeLa cells. (A) The scheme shows the condition used for synchronizing HeLa cells at G1 phase using double thymidine block. Mitotic cells were removed by mitotic shake off just before harvesting the cells. **(B)** The scheme shows the condition used for synchronizing HeLa cells at G2 phase after releasing cells from double thymidine block for 6 hours. Mitotic cells were removed by mitotic shake off just before harvesting the cells. **(C)** The scheme shows the condition used for synchronizing HeLa cells at mitotic phase by releasing cells from double thymidine block for 12 hours. Nocodazole was used in the last two hours of synchronization to prevent the release of cells from mitosis. Mitotic cells were collected by mitotic shake-off.

The cell cycle profiling was carried out in order to confirm the proper synchronization of cells. **Figure 38** confirms the synchronization of cells at G1, G2 and mitotic phases respectively without any major difference in cell cycle distribution between control and Aurora-A inhibited condition.

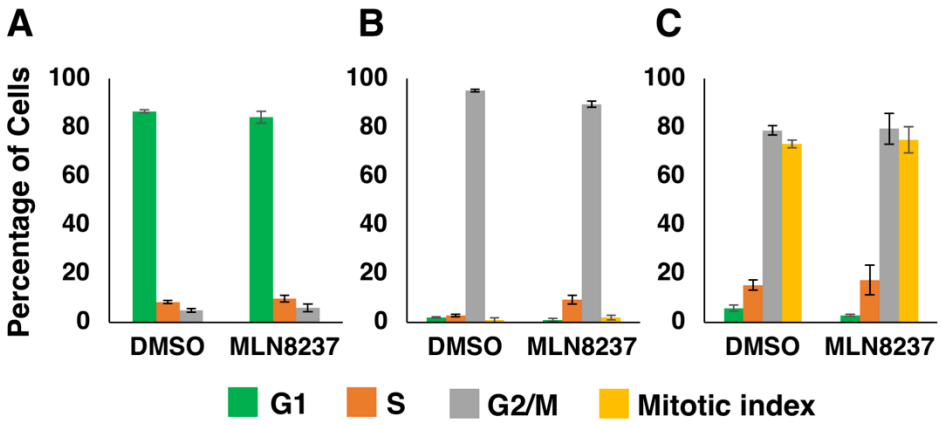


Figure 38 | Cell cycle distribution of HeLa cells. (A) The Graph showing cell cycle profile from FACS indicating the synchronization HeLa cells at G1 phase. **(B)** The Graph showing cell cycle profile from FACS and mitotic index indicating the synchronization HeLa cells at G2 phase. **(C)** The Graph showing cell cycle profile from FACS and mitotic index indicating the synchronization HeLa cells at mitotic phase.

Then western blot analysis was carried out which confirmed the efficient inhibition of Aurora-A kinase activity in all three cell cycle phases by using the antibody that recognizes the auto-phosphorylation of Aurora-A at threonine-288 residue (**Figure 39**). After confirming the inhibition of Aurora-A in all three synchronized cells, the total RNA prepared (see methods for RNA preparation) were subjected to paired end-RNA deep sequencing in order to enable the identification of splicing events that are affected by Aurora-A inhibition.

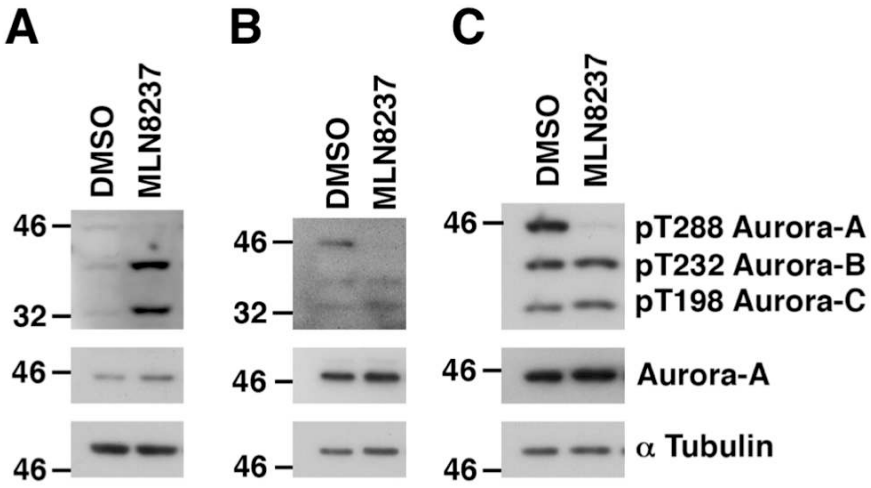
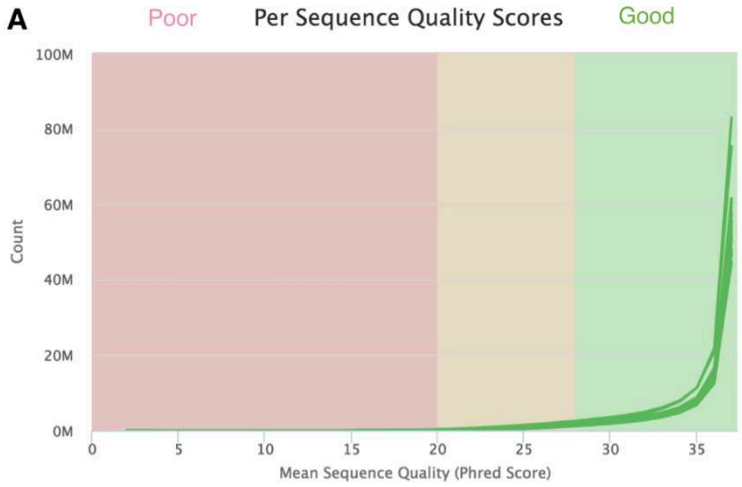


Figure 39 | Inhibition of Aurora-A in HeLa cells synchronized at G1, G2 and mitotic phases. (A) Western blot analysis of HeLa cells synchronized at G1 phase showing the decrease in the levels of T228 phosphorylation of Aurora-A. **(B)** Western blot analysis of HeLa cells synchronized at G2 phase showing the decrease in the levels of T228 phosphorylation of Aurora-A. **(C)** Western blot analysis of HeLa cells synchronized at mitotic phase showing the decrease in the levels of T228 phosphorylation of Aurora-A.

The first step in analyzing RNA-seq data is to evaluate the quality of these reads, as this will greatly affect the downstream analysis. The **Figure 40** shows the quality of the RNA sequence obtained using the MultiQC tool. The per sequence quality score report shows that all the RNA sequences have universally high-quality values without any subset of sequences with low or poor quality values (**Figure 40A**). Sometimes few sequences show universally poor quality as they are poorly imaged. The high-quality value indicates that all the RNA sequences obtained are of good quality. Also, more than 90% of the reads obtained for each sample aligned properly to the human genome (**Figure 40B**). This indicates the overall sequencing accuracy and the absence of contamination. This suggests that results obtained from this RNA sequencing reads are reliable. Overall the quality control analysis using MultiQC demonstrates that all the samples were good and support further analysis of the data.

A



B

Cell Cycle Phase	Sample	% Aligned	Number of mapped or aligned reads (in million)	Total number of reads (in million)
G1 Phase	DMSO-1	91.5	98.5	107.6
	DMSO-2	92.8	93.9	101.2
	MLN8237-1	92.3	85.5	92.6
	MLN8237-2	93.8	95.6	101.9
G2 Phase	DMSO-1	92.7	100.5	108.4
	DMSO-2	93.4	138.8	148.7
	MLN8237-1	93.4	103.5	110.8
	MLN8237-2	93	91.5	98.4
Mitotic Phase	DMSO-1	93	82.3	88.5
	DMSO-2	93.3	84.3	90.4
	MLN8237-1	93.4	95.4	102.1
	MLN8237-2	92.9	100.9	108.7

Figure 40 | Analysis of quality of RNA-sequencing data using MultiQC tool (A) Graph showing the quality score distribution over all sequences **(B)** Table showing the percentage of RNA-seq reads of all samples that have been mapped to the human genome version GRCh37/hg19.

After the quality control, the RNA sequences were subjected to differential gene expression and differential alternative splicing analyzes using edgeR and VAST tools respectively. The RNA-sequencing and analyzes were carried out in collaboration with Dr. Juan Valcarcel's team from Centre for Genomic Regulation, Barcelona, Spain and are detailed in the following sections.

3.6.2.1. Differential gene expression analysis

The differential gene expression analysis was carried out using edgeR tool. The tool identified 75 differentially regulated genes upon Aurora-A inhibition with the threshold of "adjusted P value" < 0.1 across all three cell cycle stages (G1, G2 and Mitosis). In spite of the very less stringent cutoff for the adjusted P value, only 75 genes were identified to be differentially regulated upon inhibition of Aurora-A (**Figure 41A**). The list of differentially expressed genes is mentioned in section 5.3.

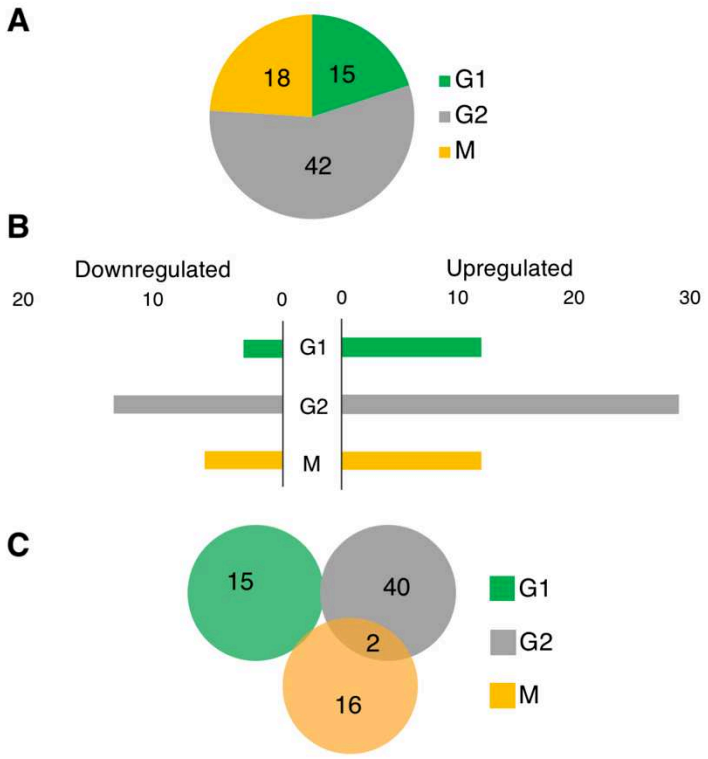


Figure 41 | Gene expression profiles of Aurora-A inhibition reported by edgeR tool (A) Pie chart showing the distribution of differentially expressed genes across cell cycle phases upon inhibition of Aurora-A. **(B)** Bar graph showing the number of genes upregulated or downregulated by Aurora-A inhibition across cell cycle phases. **(C)** The Venn diagram summarising overlap of differentially expressed genes upon inhibition of Aurora-A at G1, G2 and mitotic phases.

15 genes were differentially regulated at G1 phase whereas 42 and 18 genes were differentially expressed at G2 and mitotic phases respectively. At G1 phase, 12 genes were upregulated and 3 genes were downregulated (**Figure 41B**). During G2 phase, 29 genes were upregulated and 13 genes were downregulated whereas 12 genes were upregulated and 6 genes were downregulated upon inhibition of Aurora-A. However, there is no overlap in genes that are altered by Aurora-A inhibition between G1 and G2 phase or G1 and mitotic phase (**Figure 41C**). There is an overlap of just 2 genes between G2 and mitotic phase. Thus transcriptome-wide differential gene expression analysis indicates that Aurora-A inhibition altered the expression of very few genes and in a cell cycle specific manner.

The gene ontology analysis of the genes differentially expressed during G1 phase revealed the enrichment in terms such as "regulation of branching involved in mammary gland duct morphogenesis", "positive regulation of transcription from RNA polymerase II promoter", "cell differentiation", "innate immune response in mucosa" and "antibacterial humoral response" (**Figure 42**). Remarkably, these terms are important for developmental process, cancer cell proliferation and innate immune response against bacteria and virus. Few of these processes have been previously reported to be associated with Aurora-A.

Similarly, gene ontology analysis at G2 phase enriched for terms related to anti-viral responses and innate immunity such as "response to virus", "type I interferon signaling pathway", "defense response to virus", "negative regulation of viral genome replication" and "regulation of toll-like receptor signaling pathway" (**Figure 42**). It also enriched for a few terms related to cancer survival and development such as "regulation of necroptotic process", "regulation of necrotic cell death" and "negative regulation of cilium assembly". It is already known that Aurora-A is important for the dis-assembly of primary cilium (Pugacheva et al., 2007). The gene ontology analysis at mitotic phase enriched for terms related to protein folding and stress responses such as "positive regulation of endoribonuclease activity", "positive regulation of mRNA endonucleolytic cleavage involved in unfolded protein response", "cellular heat acclimation", "protein refolding" and "regulation of protein ubiquitination" (**Figure 42**). Other terms related to mitochondria and energy production such as "negative regulation of mitochondrial outer membrane permeabilization involved in apoptotic signaling pathway" and "ATP metabolic process" were enriched. Also, many terms such as "positive regulation of tumor

necrosis factor-mediated signaling pathway", "negative regulation of cell death", "negative regulation of endoplasmic reticulum stress-induced intrinsic apoptotic signaling pathway", "cell division" and "cell proliferation" which are related to cancer cell proliferation were also enriched.

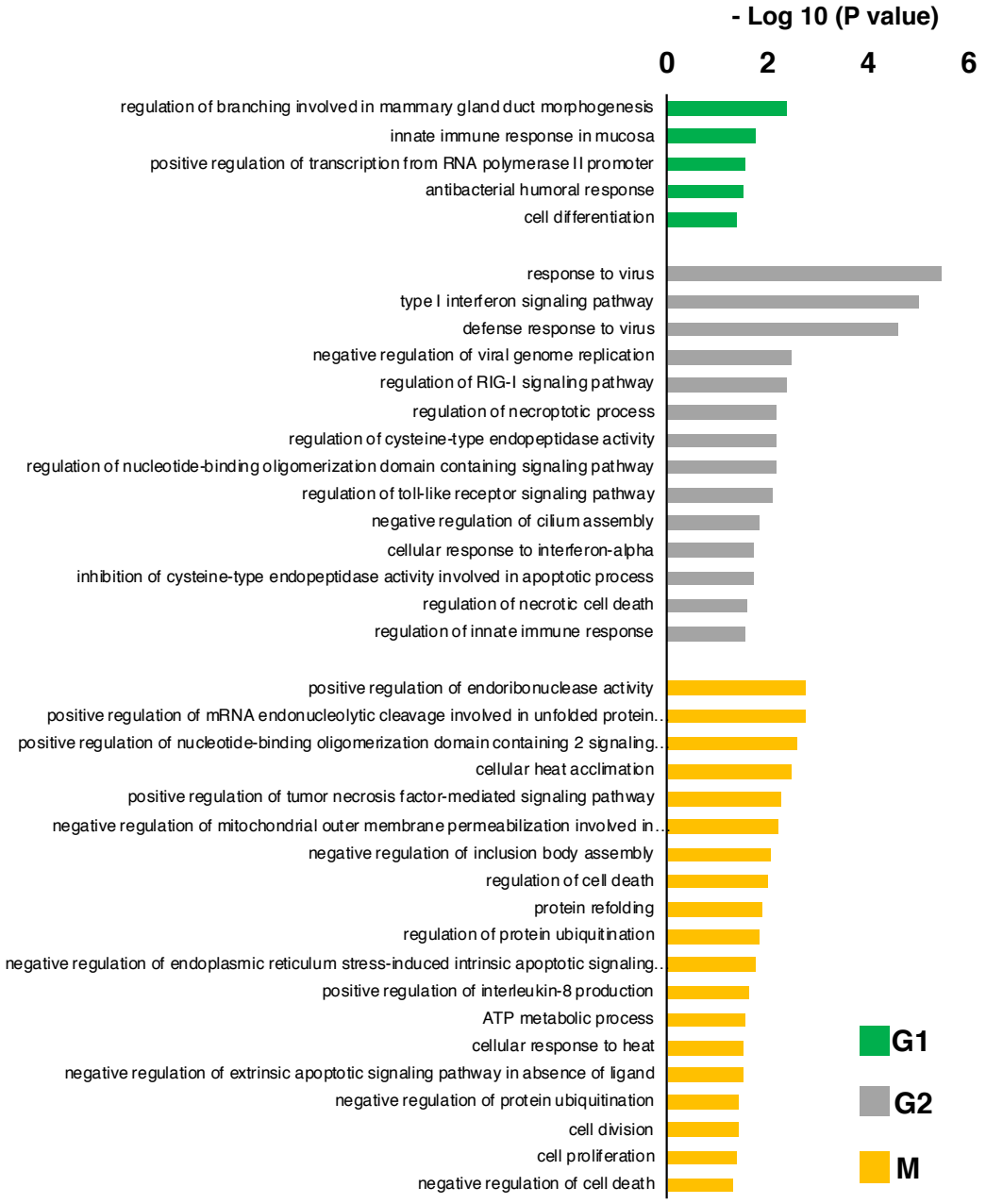


Figure 42 | Gene ontology analysis of differentially expressed genes upon Aurora-A inhibition. The histograms summarize the term enrichment analysis of Gene Ontology (GO) using the differentially expressed genes upon Aurora-A inhibition across G1, G2 and mitotic phases. All the GO terms were plotted relative to their -log10 (P values).

Altogether, the enrichment analysis in these cell cycle stages suggested that the genes differentially regulated by Aurora-A inhibition might be contributing to the

oncogenic functions of Aurora-A. Most surprisingly the inhibition of Aurora-A also enriched for genes related to anti-viral response and innate immunity, which indicates that Aurora-A might also be functioning in innate immunity against viral infections.

3.6.2.2. Differential alternative splicing analysis upon inhibition of Aurora-A

The differential alternative splicing analysis was carried out using the VAST tool. The VAST tool identified 414 differentially regulated AS events upon Aurora-A inhibition with the threshold of ΔPSI > 15 or < -15% (**Figure 43A**). The 414 differentially spliced events span around 372 genes. Notably, the overlap between these alternatively spliced genes and those differentially expressed in Aurora-A inhibited condition is 0%, suggesting that alternative splicing and gene expression are two separate layers of regulation upon inhibition of Aurora-A (**Figure 43B**). This also indicates that the AS changes we detected are not a secondary effect of altered transcription or mRNA stability but instead, a direct consequence of Aurora-A inhibition. The list of differentially spliced events upon Aurora-A inhibition is mentioned in section 5.4.

Out of the 414 AS events, 121 splicing events at G1 phase, 138 splicing events at G2 phase and 155 splicing events at mitotic phases were altered upon Aurora-A inhibition (**Figure 43C**). However, there was no overlap of AS events across cell cycle stage upon Aurora-A inhibition, which suggest that Aurora-A regulates different events at different cell cycle stage. This result fits well with the differential functions of Aurora-A at different cell cycle phases. The 121 events altered in G1 phase spans around 112 genes and majority of these events were classified as cassette exons (77 events) and a few in the alternative 5'splice site (16 events), alternative 3'splice site (12 events) and intron retention (16 events) categories were less common (**Figure 43D**).

The 138 AS events in G2 and 155 AS events in mitosis span around 124 and 136 genes respectively. Aurora-A inhibition predominantly altered the cassette exons in G2 and mitotic phases as well (**Figure 43D**). It is known that regulation of cassette exons is most likely results from the altered activity of RNA binding proteins such as splicing factors (Kanopka et al., 1996; Lavigueur et al., 1993). It is to be noted that Aurora-A could interact with and phosphorylate these splicing factors. Taken together, these results confirm that Aurora-A regulates alternative splicing in a cell cycle specific manner.

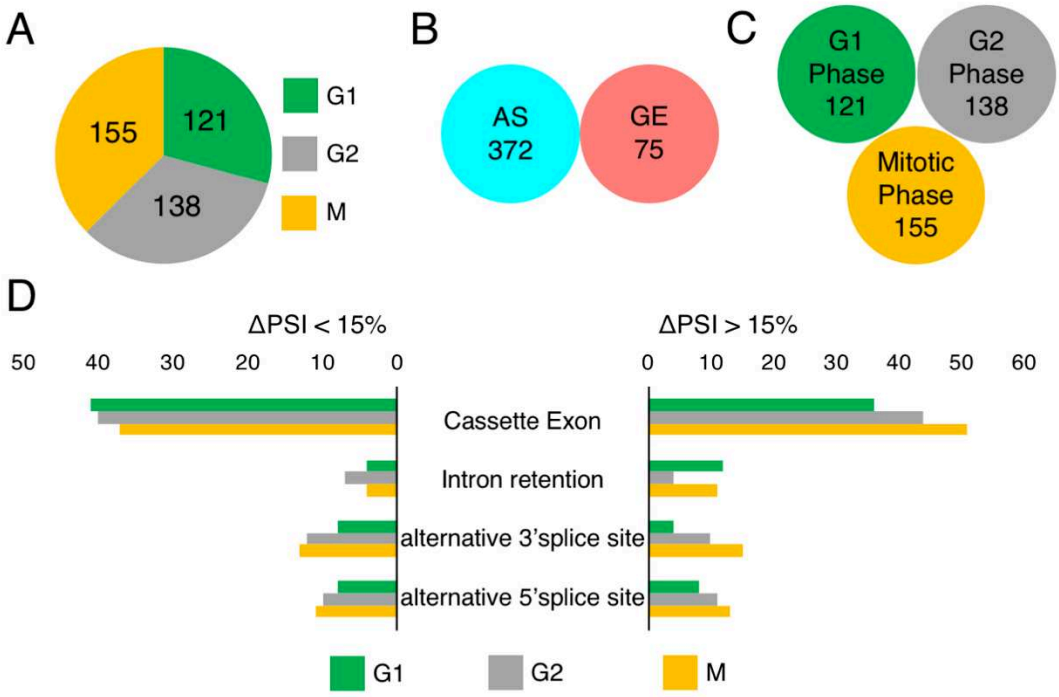


Figure 43 | Alternative splicing profiles of Aurora-A inhibition reported by the VAST tool. (A) Pie chart showing the distribution of differentially spliced events across cell cycle phases upon inhibition of Aurora-A. **(B)** Venn diagram showing the overlap between the differentially spliced events (AS) and differentially expressed genes (GE). **(C)** Venn diagram showing the number of events differentially spliced at each cell cycle stages and the overlap of alternative splicing (AS) events across the cell cycle stage. **(D)** Bar graph showing the different categories of alternative splicing events affected by Aurora-A inhibition across the cell cycle phases. The y-axis indicates the type of events and the x-axis indicates the number of events changing either towards more inclusion (ΔPSI > 15%) or more skipping (ΔPSI < 15%).

3.6.2.3. Gene ontology analysis of AS events altered by Aurora-A

In order to investigate the biological processes related to the genes that are alternatively spliced upon inhibition of Aurora-A, I performed Gene Ontology analysis on the events identified by the VAST tool at each cell cycle phases. The gene ontology was carried out using two tools, DAVID and EnrichR. The DAVID tool has the option to use the custom made background set. In this case, the background generated by the VAST tool was used in the DAVID tool. Since outdated gene annotations can impact the enrichment analysis (Wadi et al., 2016), I also used the EnrichR tool, which is frequently updated. However, EnrichR tool does not allow the use of custom made background set.

3.6.2.3.1. Gene ontology analysis of AS events altered by Aurora-A in G1 phase

The gene enrichment of genes differentially spliced at G1 phase using DAVID tool showed the significant involvement of these genes in "sodium ion transmembrane transport", "G2/M transition of mitotic cell cycle", "regulation of transcription, DNA-templated" and "neuron death" (**Figure 44-top panel**). Some of this biological processes are already known to be the functions of Aurora-A. For instance, Aurora-A is known to help in mitotic entry and normal mitotic progression (Hégarat et al., 2011; Hirota et al., 2003).

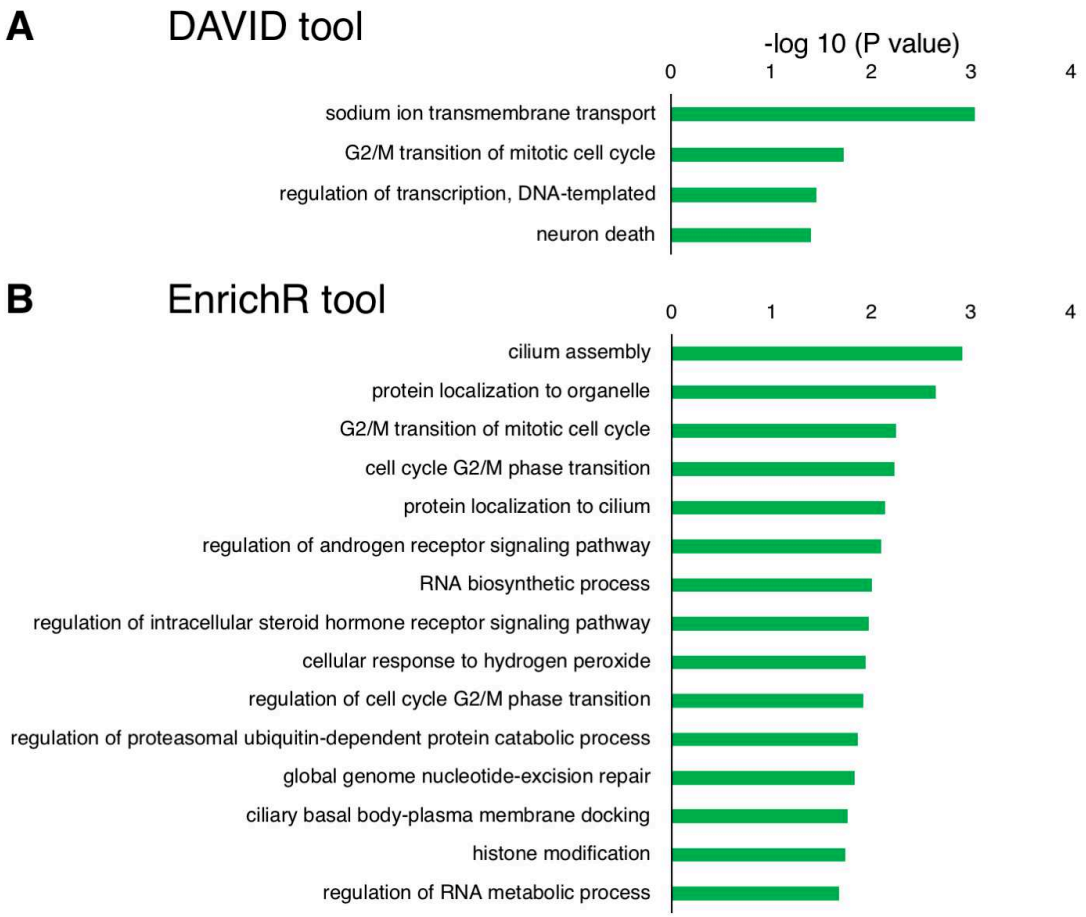


Figure 44 | Gene ontology analysis of differentially spliced events at G1 phase upon Aurora-A inhibition using two different gene ontology tools. (A) The histograms summarizing the term enrichment analysis of Gene Ontology (GO) using the differentially spliced genes upon Aurora-A inhibition using the DAVID tool. All the GO terms were plotted relative to their -log10(P values). **(B)** The histograms summarizing the term enrichment analysis of Gene Ontology (GO) using the differentially spliced genes upon Aurora-A inhibition using the EnrichR tool. Only top 15 enriched terms were shown.

The same analysis using EnrichR tool showed enrichment not only the above mentioned terms by DAVID tool but also enriched for terms related to primary cilia ("cilium assembly", "protein localization to organelle", "protein localization to cilium" and ciliary basal body-plasma membrane docking) and RNA processes ("RNA biosynthetic process" and "regulation of RNA metabolic process") (**Figure 44-bottom panel**). The other terms include "regulation of androgen receptor signaling pathway", "regulation of intracellular steroid hormone receptor signaling pathway", "cellular response to hydrogen peroxide", "regulation of proteasomal ubiquitin-dependent protein catabolic process", "global genome nucleotide-excision repair" and "histone modification". Notably, inhibition of Aurora-A is already known to alter the splicing of AR (Androgen receptor) pre-mRNA to reduce the proliferation and survival in castrate-resistant prostate cancer cells (Jones et al., 2017). These results suggest the involvement of Aurora-A in cancer, RNA processing, protein degradation and epigenetics.

3.6.2.3.2. Gene ontology analysis of AS events altered by Aurora-A in G2 phase

The gene ontology of genes differentially spliced at G2 phase using DAVID tool showed enrichment for the terms, "intracellular mRNA localization", "spindle checkpoint", "monoubiquitinated histone H2A deubiquitination" and "positive regulation of NF-kappaB transcription factor activity" (**Figure 45-top panel**). The gene ontology analysis with EnrichR tool enriched for terms such as "protein heterotetramerization", "positive regulation of glycogen biosynthetic process", "neuron apoptotic process", "focal adhesion assembly", "cell-substrate adherens junction assembly" and "regulation of macrophage activation" (**Figure 45-bottom panel**). Both the tools enriched for different biological process terms. Altogether these tools suggested some of the novel functions of Aurora-A such as mRNA sorting, immune response, neuron apoptosis and glycogen pathway. This analysis also suggests the novel ways by which Aurora-A could take part in some of its well-known functions such as spindle assembly checkpoint and NF-kappaB signaling. It is previously known that Aurora-A is important for both spindle assembly and also to maintain spindle assembly checkpoint (Courtheoux et al., 2018; Roghi et al., 1998). Also, Aurora-A is known to be involved in the NF-kappaB pathway by mediating the phosphorylation and degradation of IκBα (inhibitor of NF-kappaB) (Briassouli et al., 2007).

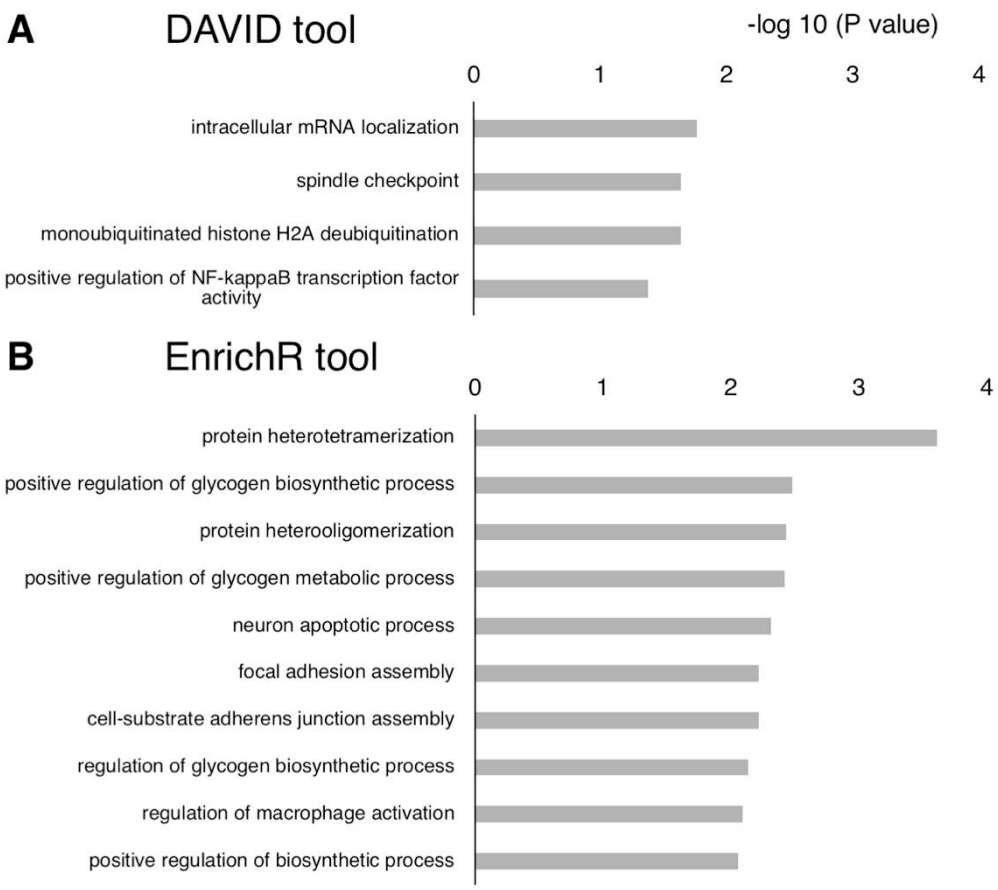


Figure 45 | Gene ontology analysis of differentially spliced events at G2 phase upon Aurora-A inhibition using two different gene ontology tools. (A) The histograms summarizing the term enrichment analysis of Gene Ontology (GO) using the differentially spliced genes upon Aurora-A inhibition using the DAVID tool. All the GO terms were plotted relative to their -log10(P values). **(B)**The histograms summarizing the term enrichment analysis of Gene Ontology (GO) using the differentially spliced genes upon Aurora-A inhibition using the EnrichR tool. Only top 10 enriched terms were shown.

3.6.2.3.3. Gene ontology analysis of AS events altered by Aurora-A in mitotic phase

The gene ontology of genes differentially spliced at mitotic phase using DAVID tool showed enrichment for the terms, "regulation of autophagy", "membrane protein proteolysis involved in retrograde protein transport, ER to cytosol" and "positive regulation of endothelial cell migration" (**Figure 46-top panel**). The gene ontology analysis with EnrichR tool enriched for terms such as "regulation of cellular catabolic process", "regulation of receptor internalization", "multi-ciliated epithelial cell differentiation", "regulation of morphogenesis of an epithelium", "regulation of protein localization", "positive regulation of phosphorylation", "regulation of delayed rectifier potassium channel activity", "negative regulation of TORC1 signaling", "positive

regulation of carbohydrate metabolic process", "retrograde protein transport, ER to cytosol" and "regulation of nucleic acid-templated transcription" (**Figure 46-bottom panel**).

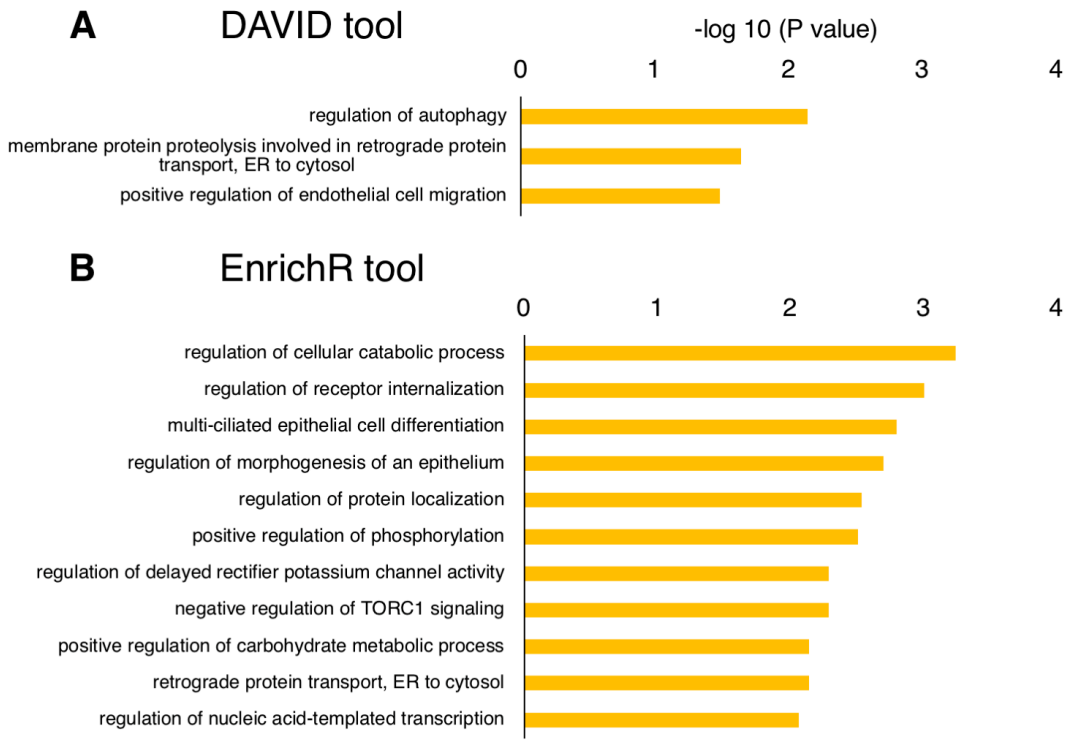


Figure 46 | Gene ontology analysis of differentially spliced events at mitotic phase upon Aurora-A inhibition using two different gene ontology tools. (A) The histograms summarizing the term enrichment analysis of Gene Ontology (GO) using the differentially spliced genes upon Aurora-A inhibition using the DAVID tool. All the GO terms were plotted relative to their -log10(P values). **(B)**The histograms summarizing the term enrichment analysis of Gene Ontology (GO) using the differentially spliced genes upon Aurora-A inhibition using the EnrichR tool.

Previously, Aurora-A is known to suppress the autophagy-mediated cell death by activating mTOR signaling in breast cancer cells (Xu et al., 2014). Consistent with this report, both these tools suggested the role of Aurora-A in autophagy and mTOR signaling. These gene ontology tools also suggested the novel function of Aurora-A in protein transport and proteins quality control pathways. The GO tools also highlighted the function of Aurora-A in gene expression. Previously, Aurora-A is known to transactivate myc promoter by forming a complex with hnRNP-K protein and enhance breast cancer stem cell (BCSC) phenotype. Overall, these GO tools highlights the function of Aurora-A in a wide range of mechanisms which are often mis-regulated in cancer.

3.6.2.4. Impact of inhibition of Aurora-A in alternative splicing of SR protein kinases

The GO analysis of the differentially spliced events by Aurora-A significantly enriched for terms involved in various processes such as G2/M transition, mitotic spindle assembly, cilium assembly and RNA processing. Interestingly, the GO term "regulation of RNA metabolic process" has been enriched (**Figure 44-bottom panel**) upon inhibition of Aurora-A during G1 phase. The term "regulation of RNA metabolic process" included the cassette exon events CLK1 and CLK4. They belong to the family of cdc2-like kinases (Clk). They play an important role in the regulation of RNA splicing by hyper-phosphorylating SR proteins and thereby recruiting the SR proteins from nuclear speckles to the site of transcription (Colwill et al., 1996).

My initial experiments show that Aurora-A interacts with many SRSF (SR) proteins and is important for maintaining the levels of SR proteins. Though CLK1 is known to phosphorylate the SR protein and regulate splicing, nothing is known about the link between CLKs and stability of SR proteins. In order to demonstrate if Aurora-A and CLKs together affect SR proteins levels, I first validated the alteration of cassette exons of CLK1 and CLK4 upon inhibition of Aurora-A. The inhibition of Aurora-A increased the inclusion of exon-4 of the SR protein kinase, CLK1 (**Figure 47**). Similarly, the inhibition of Aurora-A increased the inclusion of exon-4 of another SR protein kinase, CLK4. The ΔPSI calculated by the VAST tool and the semi-quantitative calculation from the gel images (using FIJI tool) for CLK1 are 17.08 and 10.98 respectively. The ΔPSI calculated by the VAST tool and the semi-quantitative calculation from the gel images (using FIJI tool) for CLK4 are 16.41 and 6.5 respectively. Both the methods show the significant increase in inclusion of exon-4 for CLK1 and CLK4 upon inhibition of Aurora-A. Though there was a slight difference in the ΔPSI calculated by the VAST tool and the semi-quantitative method, both the methods showed consistent results. Altogether, these results suggest that the kinase activity of Aurora-A is important for alternative splicing of CLK1 and CLK4.

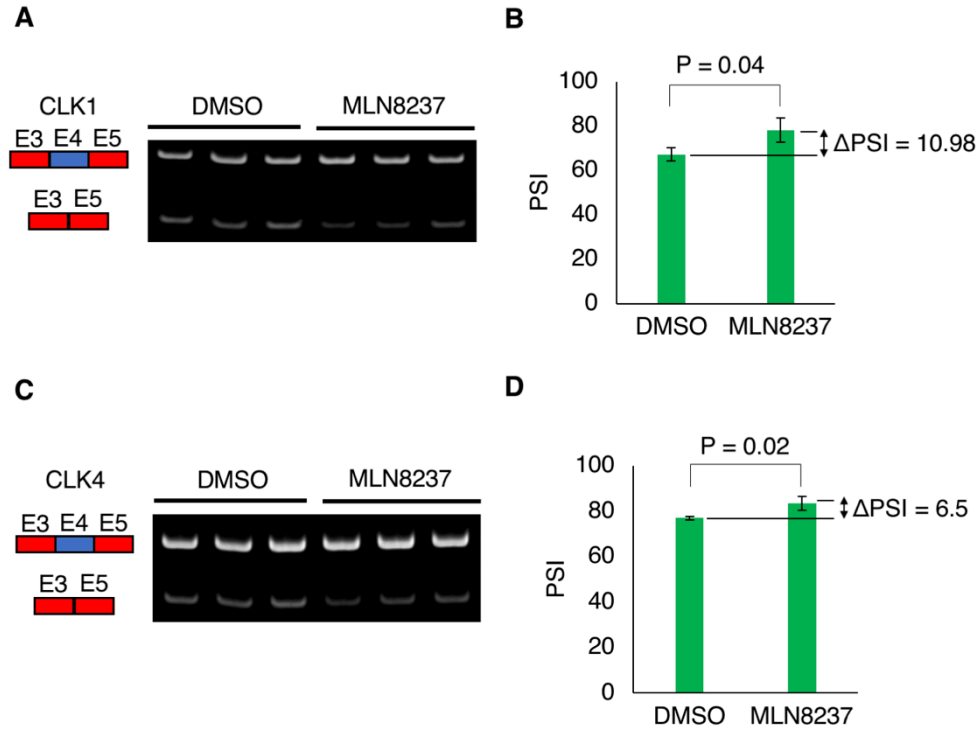


Figure 47 | Inhibition of Aurora-A promotes inclusion of exon-4 of CLK1 and CLK4. (A) RT-PCR validation of alternative splicing of CLK1 upon inhibition of Aurora-A. **(B)** Graph showing the quantification of PSI from **A**. The percentage of inclusion of exon-4 (E4) is calculated as, PSI = (Inclusion / (Inclusion + Skipping)) *100. ΔPSI is calculated as the difference in PSI between MLN8237 and DMSO. **(C)** RT-PCR validation of alternative splicing of CLK4 upon inhibition of Aurora-A. **(D)** Graph showing the quantification of PSI from **C**.

The differential gene expression and splicing analysis suggest the novel functions of Aurora-A and also suggest the mechanistic explanation for some of its known functions such as cilia disassembly, G2/M transition and spindle assembly checkpoint. Taken together, all the results suggest the broader function of Aurora-A throughout the cell cycle by regulating gene expression and pre-mRNA splicing in a cell cycle specific manner.

3.7. Alteration of nuclear speckles by a transfection reagent, JetPRIME

The nature of my PhD project necessitated to carry out several transfection experiments. During one such transfection experiment, I encountered a phenotypical change in the structure of nuclear speckles. I further investigated this interesting observation and serendipitously discovered that the transfection reagent, JetPRIME leads to such artefacts. This study, being the first of its kind, has been submitted to the journal, RNA Biology (Section 3.7.1).

Arun Prasath Damodaran et al., Submitted to RNA Biol (sept 2018)

3.7.1 Manuscript

ALTERATION OF NUCLEAR SPECKLES BY TRANSFECTION REAGENTS

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Running title: JetPRIME alters nuclear speckles

Keywords: transfection, JetPRIME, splicing, speckles, cytoplasmic granules

ABSTRACT

Transfection is a powerful tool that enables the introduction of foreign nucleic acids into the cells in order to study the function of a gene product. Ever since the discovery of transfection many side effects and artifacts caused by transfection reagents have been reported. In this report, we show that the transfection reagent, jetPRIME causes an artifact on a structure called nuclear speckles, which acts as a storehouse for splicing proteins. We demonstrate that transfection of plasmids with jetPRIME leads to the disappearance of speckles from the nucleus and their accumulation in the cytoplasm resulting in cytoplasmic granules. By contrast, transfection of the same plasmid with lipofectamine 3000 does not have any effect on nuclear speckles. Our work indicates that it should be ascertained that the normal dynamics of nuclear speckles is un-altered when studying the function of a gene in gene expression or pre-mRNA splicing through transient transfection system.

INTRODUCTION

Transfection is a method of delivering foreign nucleic acids (DNA and RNA) to the living cells. However, the term "transfection" was initially used to represent the procedure of introducing the λ DNA directly to *E.coli* [1,2]. Ever since the usage of DEAE-dextran to transfer the nucleic acids to mammalian cells [3], many transfection reagents have been developed and commercialized. Transfection methods and transfection reagents have evolved since its outset, from transfection of cell lines to transfection of organs/tissues. Today there are personalized transfection reagents to deliver nucleic acids or proteins to cell lines that differ based on their culturing condition (adherent or suspension), cell type and the level of ease of transfection. There are transfection reagents developed even for *in vivo* delivery to specific organs like liver by exploiting the membrane receptors that these organs carry (like in vivo-jetPEI®-Gal reagent- Polyplus-transfection S.A, Illkirch, France).

Depending on the duration that the transfected nucleic acid exists in cell and the integration status of nucleic acids into the cell's genome, transfection is classified into two types namely transient or stable. In transient transfection, the nucleic acid exists for a limited duration and does not integrate into the genome whereas, in stable transfection, the nucleic acid is integrated to the cell's genome and hence transferred generation after generation and thus persists in the cell for longer duration [4]. The choice between transient or stable transfection depends on the nature of the question to be addressed by taking into account the time required, the degree of expression, the toxicity and above all the side effects.

There are three main methods of transfection: chemical, biological and physical methods. The most widely used chemical methods include the using of carrier reagents such as calcium phosphate, cationic polymers and cationic lipids. These cationic carriers form positively charged complexes along with negatively charged nucleic acid thus enabling the

attraction of this complex to the negatively charged cell membrane. Later the carrier reagent-nucleic acid complex passes through the cell membrane. The biological method involves the virus mediated transfection and is otherwise called transduction. This method integrates the transgene to host genome (stable transfection) and is widely used in clinical research for *in vivo* studies. Physical method involves the direct introduction of the nucleic acids to the cell's cytoplasm or nucleus. The physical methods are electroporation, biolistic particle delivery (particle bombardment), direct microinjection and laser-mediated transfection (phototransfection). The pros and cons of using each method of transfection are described elsewhere [5].

In the recent years several researchers reported the side effects or artifacts caused by various methods of transfection. These off target effects are at the epigenetic, metabolomic, transcriptomic and proteomic levels [6–8]. All these side effects might often be misinterpreted as the function of product expressed by the transfected gene. In this study, we report an artifact created by a transfection reagent, jetPRIME, which alters the normal dynamics of the nuclear speckles when used for transfecting a plasmid DNA. Nuclear speckles also known as splicing speckles or interchromatin granules are dynamic structures that are enriched with proteins important for RNA metabolism and gene expression [9].

RESULTS

DNA Transfection using jetPRIME affects the nuclear speckles

Several off-target effects such as alteration of gene expression and protein levels are attributed to the reagents used for transfection. During a transient transfection experiment using jetPRIME, we serendipitously observed that nuclear speckles were affected, suggesting that their dynamics was somehow disturbed. To investigate further this observation, we transiently transfected pEGFP-C1 (GFP) vector in U2OS cells using jetPRIME and stained the cells for nuclear speckles using a surrogate marker, SC35. We observed foci, that is called cytoplasmic granules in the cytoplasm instead of speckles normally observed in the nucleus of untreated cells. (**Figure 1B and 1F**). However, during the control transfection reactions, when we used the jetPRIME reagent without any plasmid, we did not observe these cytoplasmic foci, instead, we observed speckles in the nucleus, like in the untreated cells (**Figure 1A and 1E**). Also, transfection reactions performed only with plasmid without jetPRIME did not affect the normal speckled pattern of SC35 in nucleus. This result suggests that the use of jetPRIME to transfect plasmids could be the prime cause of the observed artifact: an alteration of the nuclear speckles.

Transfecting using Lipofectamine 3000 did not alter nuclear speckles

We further investigated whether transfection of the same pEGFP-C1 plasmid in the same U2OS cells using another transfection reagent, lipofectamine 3000, would also affect the nuclear speckles. Remarkably, transient transfection with lipofectamine 3000 reagent did not alter SC35 localization as previously seen with jetPRIME (**Figure 1C and 1D**). The number of cells with cytoplasmic granules due to transfection using jetPRIME very well correlates with the number of GFP positive cells (**Figure 1G and 1H**). Although the number

of cells expressing GFP is lower after transfection with Lipofectamine 3000 than after transfection with jetPRIME, we observed practically no cells containing cytoplasmic granules. This strongly suggests that the change in the morphology of nuclear speckles seen in case of transfection using jetPRIME is actually due to the transfection reagent.

Cell type specific effect of jetPRIME on nuclear speckles

Since nuclear speckles are dynamic structures that differ depending on the cell type [10], we asked whether the effect of jetPRIME mediated transfection on nuclear speckles could be observed in a different cell line. We selected HeLa cells and transfected the same plasmid pEGFP-C1. After transfection with jetPRIME, unlike U2OS cells, HeLa cells showed two different phenotypes. Firstly, some cells contained bigger and rounded nuclear speckles that we called enlarged nuclear speckles (ENS) (**Figure 2B and 2G – second panel**). Secondly some cells contained cytoplasmic granules (CG) as well as enlarged nuclear speckles (ENS) (**Figure 2G – third panel**). The formation of enlarged nuclear speckles have already been reported to be due to the accumulation of splicing factors inside the nuclear speckles when transcription is hampered by treatment with transcription inhibitors or due to heat shock [11–13]. Similar to U2OS cells, transfection of GFP plasmid in HeLa cells with Lipofectamine 3000 did not alter the normal dynamics of nuclear speckles (**Figure 2E and 2F**). Although the nuclear speckles are differentially affected in HeLa cells and U2OS when transfected with jetPRIME, we did not observe any perturbation of the speckles after transfection with Lipofectamine 3000.

Effect of jetPRIME on nuclear speckles is independent of gene expression by the vector transfected

The efficiency of transfection estimated by the number of GFP positive cells was higher with jetPRIME than with Lipofectamine 3000 (**Figure 1G and 2E**). The low transfection efficiency observed with Lipofectamine is mainly due to the fact that we refrained from using the Enhancer P3000TM reagent during transfection because its use resulted in massive cell death. However we did not observe any difference in nuclear speckle dynamics with or without the P3000TM Enhancer Reagent (not shown). This alteration of nuclear speckles is also independent of the level of the protein expressed by the transfected vector since the Lipofectamine 3000 transfected cells which showed higher GFP expression levels than the jetPRIME transfected cells did not show any abnormal nuclear speckles. This confirmed that the phenotype caused by jetPRIME is independent of the expression level of the plasmid DNA (**Figure 1D and 1B**). Next, we asked whether the nuclear speckles perturbation caused by jetPRIME was due to the exogenous expression of a protein. We then chose to transfect the empty vector pcDNA3 in U2OS cells (**Figure 3B**). We did observe alteration of the nuclear speckles similar to the one observed after transfection with the pGFP-C1 plasmid. Our result indicates that jetPRIME-mediated transfection alters nuclear speckles independently of any expression by the plasmids.

DISCUSSION

Transfection is a powerful method used to decipher the function of a gene. In spite of the extensive use of transfection techniques, very little is known about their side effects. In this study we reported that the treatment of HeLa or U2OS cells with both jetPRIME and plasmid together drastically altered the normal dynamics of nuclear speckles in both cell lines, whereas treatment with only plasmid or only jetPRIME did not have any impact on the dynamics of nuclear speckles. On the contrary, the use of Lipofectamine 3000 in place of jetPRIME did not provoke such alteration. JetPRIME is a cationic polymer-based molecule whereas Lipofectamine 3000 is a cationic lipid based transfection reagent. It is possible that the cationic polymer based methods are inducing such side effect in nuclear speckles.

In U2OS cells, jetPRIME-mediated transfection induces the appearance of cytoplasmic granules of SC35, a phenotype similar to that observed upon depletion of Ran-binding protein 2 (RanBP2), a nucleoporin involved in RAN nucleocytoplasmic transport system [14]. In the same study, the authors further observed a similar morphology of nuclear speckles upon depletion of other proteins involved in the RAN nucleocytoplasmic transport system (RAN, RCC1 or TNPO3). The RAN nucleocytoplamic transport system is necessary for the sequential entry of a set of splicing components such as snRNA, hnRNP-A1 and SC35 and thus is important for maintaining normal dynamics of the nuclear speckles. The presence of cytoplasmic granules containing cells signed the presence of an altered alternative splicing pattern [14]. In Hela cells, jetPRIME-mediated transfection triggered two distinct and prominent phenotypes. The first phenotype corresponds to the appearance of cytoplasmic granules, similar to the one seen in U2OS cells, but also displayed enlarged and rounded nuclear speckles. The second phenotype, not seen in U2OS cells, corresponds to enlarged

rounded nuclear speckles, a phenotype similar to the one observed in cells compromised for transcription and RNA splicing [10,15]. This collection of phenotypes in HeLa cells could be as a result of defective RAN dependent nucleocytoplamic transport and defective transcription or RNA splicing. The effect of JetPRIME on nuclear speckle is thus cell specific.

Tight interdependency exists in regulation of chromatin, transcription and RNA splicing. For instance, RNA splicing participates in gene expression through AS-NMD (Alternative splicing resulting in nonsense-mediated mRNA decay) [16]. It is known that approximately 80 % of splicing in HeLa cells is co-transcriptional [15]. The RNA splicing is known to be modulated by transcription machinery by the recruitment of the splicing factors to the transcription site by the Pol II CTD (carboxy-terminal domain) [17]. Reciprocally, chromatin structure and histone signatures have been implicated in regulating gene expression and alternative splicing [18,19]. Consistently, treatment with transcription inhibitor or histone deacetylases inhibitor (trichostatin A) also trigger accumulation of the splicing factors in splicing speckles and enlarges it [10,20]. Since nuclear speckles contain factors not only involved in RNA processing but also virtually all aspects of cell biology (in cytoskeletal organization, epigenetic regulation, cellular signaling, cellular transport, proteasome subunits, heat shock, genome integrity and DNA replication) [21], this side effect on nuclear speckles might impact cellular processes beyond RNA processing. Such effects on nuclear speckles should be carefully considered when choosing the transfection reagents to study the function of a gene in RNA splicing, gene expression and chromatin organization.

In our work, the different cell lines showed different phenotypes upon jetPRIME-mediated transfection. Similarly, different transfection reagents have been shown to induce different side effects in different cells (cell type specific side effects). For instance, the two lipid based reagents, lipofectamine and FuGENE® HD mediated plasmid transfection

activates interferon signalling pathway at mRNA and protein level in HeLa cells but not in HEK293T cells [7]. Similarly lipofectamine is reported to trigger type I interferon response in macrophages whereas such side effect is unseen with another transfection reagent, Xfect (polymer based transfection reagent). Also the side effect is seen only with macrophage-like cells but not in 293T or L929 cells [22]. These reports gives a novel example where the lipid based transfection systems are unsuitable in studies related to innate immunity where the lipid signalling plays an important role. These works also suggest the use of the right combination of cell line and transfection reagents to study immune function of a gene.

Various transfection reagents have been reported to be the source of various side effects. In most cases, these artifacts can often be mis-judged as a phenotype induced by the overexpression or knockdown of the gene of interest. These mis-interpretations can be avoided by using proper negative control to the transfection experiments. These side effects caused by transfection reagents are cell-type specific. Hence these types of studies reporting artifacts strongly recommend the use of right transfection reagent for the right cell lines for answering the right question of interest. Such studies also put forth the scope of improvement and innovation for the companies that manufacture transfection reagents, by taking into account such artifacts while developing the new transfection reagents.

MATERIALS AND METHODS

Cell culture and transient transfection

U2OS cells and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM)- GlutaMAX ™ -I (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS)(GE Healthcare) and 1% penicillin–streptomycin (GE Healthcare). For transfection of pEGFP-C1 plasmids, cells were grown till 70 percent confluence and were transfected using jetPRIME® (Polyplus-transfection S.A, Illkirch, France) or Lipofectamine-3000 (ThermoFisher scientific) transfection reagents according to the manufacturer's instructions.

Immunofluorescence staining

The cells grown on coverslips were fixed after 24 hours of transfection with 4% paraformaldehyde (Electron Microscopy Sciences) and permeabilized with 0.5% Triton-X100 (Sigma-Aldrich) in 1X PBS (Sigma-Aldrich). The cells were then blocked with 5% FBS in PBS and incubated with primary antibody (SC35- S4045 Sigma) at 1:2000 dilution in blocking solution for 1 hour at room temperature. Cells were washed thrice in PBS and incubated with secondary antibody (anti-mouse antibody conjugated to Alexa 647 or Alexa 546, Thermo Fisher Scientific) at 1:4000 dilution in blocking buffer for one hour at room temperature. The cells were then washed and the DNA was stained using Hoechst 33342 (Thermo Fisher Scientific). Finally, the coverslips were mounted using ProLong® Gold antifade reagent (Thermo Fisher Scientific).

Confocal spinning disk and deltavision microscopy

The images were obtained using Eclipse Ti-E microscope (Nikon) equipped with 60X oil immersion objective, spinning disk (CSU-X1; Yokogawa) and a charge-coupled device camera (CoolSNAP HQ2; Roper Scientific) and driven by Metamorph Software (Universal imaging). Alternatively, images were taken using a deltavision (Olympus IX7) microsocpe equipped with 60X oil immersion objective, CoolSnapHQ Princeton BW camera and image acquisition software MetaVue (Molecular Devices, Inc.). All the images were processed using the FIJI (imageJ) software.

Statistical analysis

All statistical analyses were performed using two-tailed pairwise student's t-tests. The number of cells quantified under each condition is detailed on each figure.

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FIGURE LEGENDS

Figure 1: Transfection of GFP plasmid using jetPRIME affects nuclear speckles in U2OS cells.

U2OS cells were transfected using jetPRIME or Lipofectamine 3000 with (B and D) or without (A and C) GFP-plasmid and then grown for 24 hours at 37°C. Nuclear speckles and DNA were stained with anti-SC35 antibody and DAPI respectively. The last panel in A, B, C and D shows the merged image excluding the GFP channel. (E) and (F) Magnification of nuclear speckles corresponding to the white boxed region in A and B. (G) Graph showing the quantification of cells expressing GFP in A, B, C and D. (H) Graph showing the quantification of cells with cytoplasmic granules of SC35 in A, B, C and D. At least 100 cells were counted for each condition. [Scale bar = 10 μm]

Figure 2: Transfection of GFP plasmid using jetPRIME affects nuclear speckles in HeLa cells.

HeLa cells were transfected using jetPRIME or Lipofectamine 3000 with (B and D) or without (A and C) GFP-plasmid and then grown for 24 hours at 37°C. Nuclear speckles and DNA were stained with anti-SC35 antibody and DAPI respectively. The last panel in A, B, C and D shows the merged image excluding the GFP channel. (E) Graph showing the quantification of cells expressing GFP in A, B, C and D. (F) Graph showing the quantification of cells with artifact in nuclear speckles in A, B, C and D. At least 100 cells were counted for each condition. [Scale bar = 10 μm]. (G) Examples of cells with normal NS and affected NS: ENS (Enlarged Nuclear Speckles) and CG (Cytoplasmic Granules) quantified in F.

Figure 3: jetPRIME affects nuclear speckles independently of the exogenous expression of protein.

U2OS cells were transfected using jetPRIME without any plasmid (A) or with the empty plasmid pcDNA3 (B) or pEGFP-C1 expressing GFP (C) and were grown for 24 hours at 37°C. Nuclear speckles were stained with anti-SC35 antibody and DNA visualized with DAPI respectively. The last panel in A, B, C and D shows the merged image excluding the GFP channel. The Panel on the extreme right with numbers 1, 2 and 3 show the magnification of nuclear speckles corresponding to the white boxed region in A, B and C. [Scale bar = 10 μm].

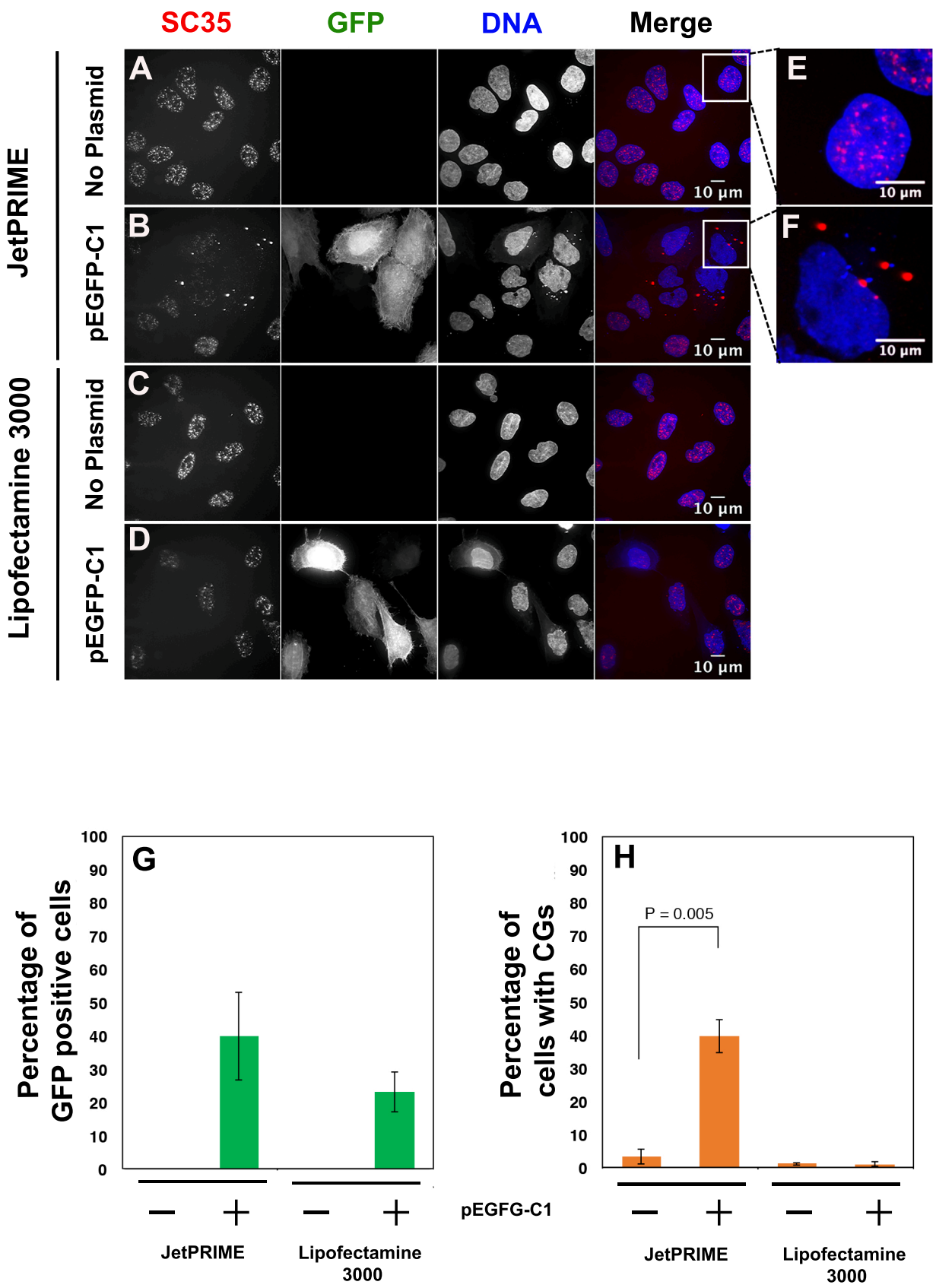

Figure 1 : Arun Prasath Damodaran et al., 2018

Figure 2

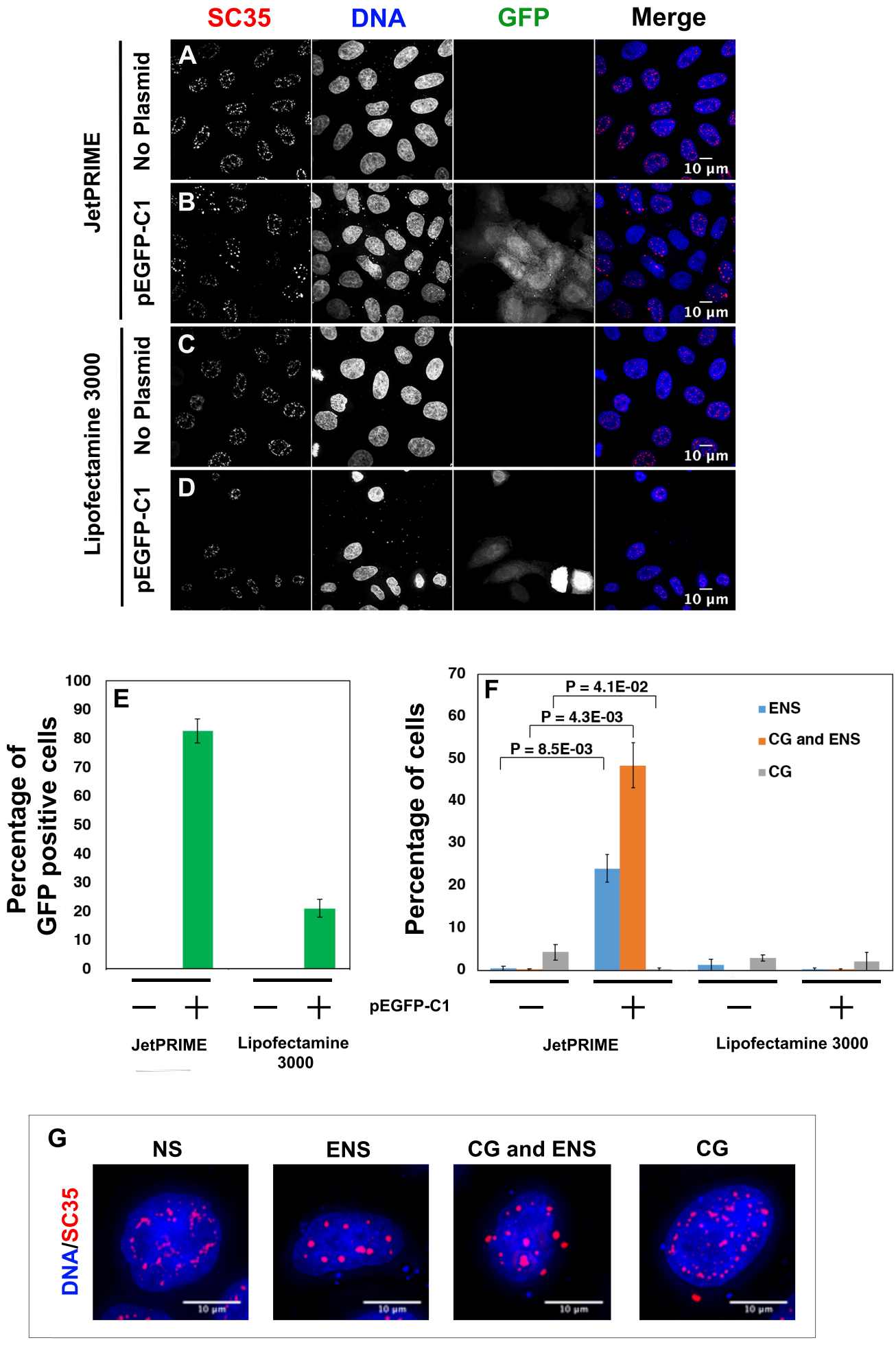

Figure 2 : Arun Prasath Damodaran et al., 2018

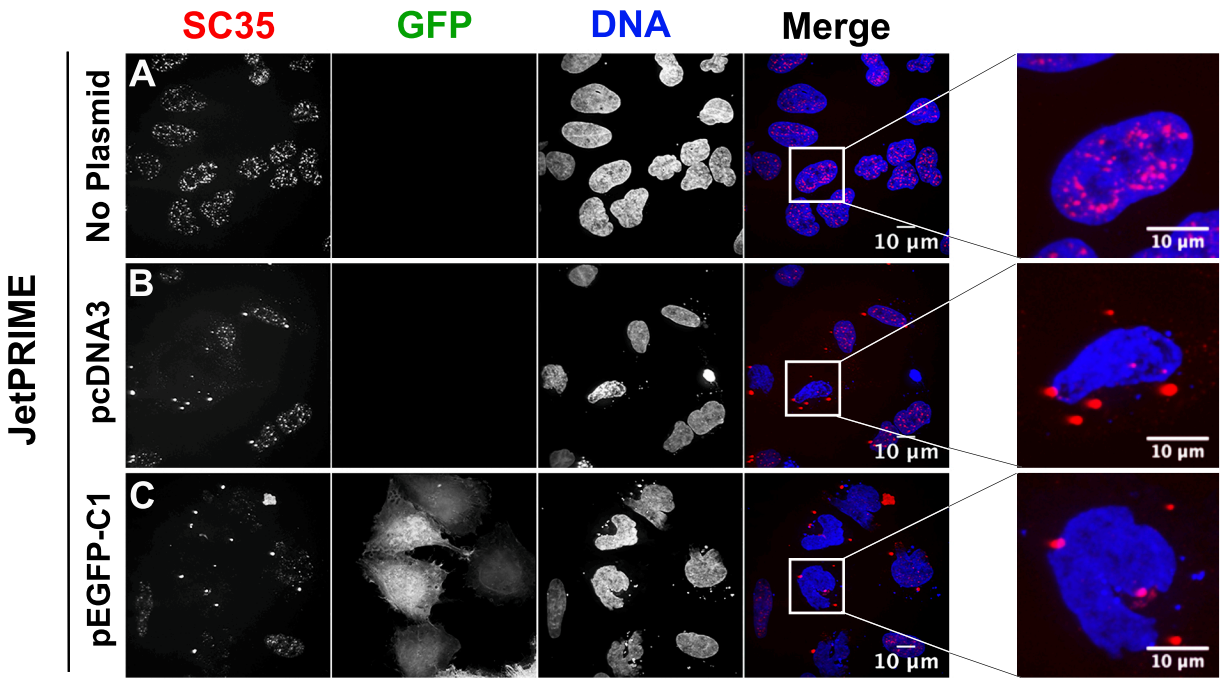

Figure 3 : Arun Prasath Damodaran et al., 2018

4. DISCUSSION

4.1. Aurora-A, a new member in the family of splicing regulating kinases

So far, many kinases have been identified to be involved in the regulation of pre-mRNA splicing. Some of these kinases are important for the normal kinetics and dynamics of the splicing reaction and structures associated with splicing. Sometimes the kinases required for normal splicing are misregulated and in turn cause pathologies. For instance, SRPKs, CLKs and PRP4 are the three classes of kinases that are known to phosphorylate the splicing factors and spliceosomal proteins but when misregulated, they help in tumorigenesis (Corkery et al., 2015).

Apart from these canonical splicing kinases many other kinases whose normal functions involve signal transduction, are interfering with splicing mechanisms. They link the splicing mechanism with other cellular processes. These signal transduction kinases help the cells to modulate the splicing mechanism in response to the physiological and pathological stimulus. These kinases include the AKT, Mitogen-Activated Protein Kinases (MAPKs), Protein Tyrosine Kinases (PTKs) and cAMP-Dependent Protein Kinase (PKA) (Naro and Sette, 2013). In recent days, many such signal transduction kinases have been identified to be involved in pre-mRNA splicing. NEK2 is a centrosomal kinase and was found to be one of the novel splicing kinase (Naro et al., 2014). Our laboratory possesses an expertise in the mitotic kinase Aurora-A and a former PhD has been working on an interactome of Aurora-A that revealed interactions with many proteins involved in the splicing of pre-mRNA. Thus the project of my PhD was to determine whether the Aurora-A kinase could be involved in the regulation of pre-mRNA splicing

During my PhD, I demonstrate that Aurora-A interacts with proteins not only in the core of the spliceosome but also with proteins that regulates the activity of the spliceosome. *In vitro*, I showed that the kinase alters the splicing efficiency of β-globin pre-mRNA incubated in HeLa nuclear extracts. In parallel, *in vivo*, I showed that the kinase alters the stability of SR proteins. Eventually, I showed that inhibition of Aurora-A in HeLa cells altered the alternative splicing of many genes. Overall, these results

demonstrate that Aurora-A is indeed a modulator of pre-mRNA splicing and is a novel member in the family of splicing regulating kinases.

4.2. Aurora-A interacts with the splicing proteins that contain specific domains

The function of a protein and its interaction with other macromolecules are determined by the domains they possess (Vogel et al., 2004). For instance, zinc finger domain containing proteins are involved in translation, mRNA trafficking and gene transcription (Laity et al., 2001). The HRDC (Helicase and RNaseD C-terminal) domain is involved in unwinding of DNA in an ATP-dependent manner and function in DNA replication and recombination (Liu et al., 1999). The interactome of Aurora-A was enriched with proteins involved in various functions (**Figure 24**) such as RNA splicing and processing, mitochondrial dynamics, gene expression and so on. Consistent with this enrichment for RNA splicing and processing, the GO analysis against InterPro database enriched for "RNA Recognition Motif domain" (RRM) and "DNA/RNA helicase, DEAD/DEAH box type, N-terminal" **(Figure 28)**.

The DEAD and DEAH box helicases are involved in unwinding of nucleic acids and function in many RNA metabolisms such as gene expression, pre-mRNA splicing, nucleocytoplasmic export of RNA, ribosome biogenesis, protein translation and RNA degradation (Aubourg et al., 1999; Cruz et al., 1999). The spliceosome is known to be a complex and dynamic machinery that undergoes structural and compositional rearrangements throughout the splicing reaction (Will and Lührmann, 2011). These structural rearrangements and remodelling between the snRNAs of the spliceosome are carried out by the RNA helicases that contain DEAD or DEAH box domain (Cordin and Beggs, 2013; De et al., 2016; Gilman et al., 2017; Liu and Cheng, 2015). The *in vitro* interaction assays show the direct interaction of Aurora-A with the eIF4A3 **(Figure 27A).** eIF4A3 is an RNA helicase domain (DEAD box) containing protein and is a component of the spliceosome (Ballut et al., 2005; Bertram et al., 2017; Jurica et al., 2002; Zhang et al., 2017). Though eIF4A3 is not present in the interactome of Aurora-A, when tested in an *in vitro* assay, showed its interaction with Aurora-A. This implies that Aurora-A has selectivity towards the RNA helicase domains. However further experiments with the helicase domain deletion mutants of these splicing proteins are needed in order to prove the specificity of Aurora-A towards RNA helicase domain.

The other domain that is enriched and implied the function of Aurora-A in splicing is the RRM (RNA Recognition Motif) domain **(Figure 28)**. The hnRNPs and SR proteins are the two families of splicing factors that regulate the spliceosome. Majority of these splicing factors are known to contain this RRM domain (Busch and Hertel, 2012). Few splicing proteins have other domains that recognize RNA in place of RRM domains. These include some of the hnRNP proteins that contain KH (K Homology) and/or RGG (repeats of Arg-Gly-Gly tripeptides) domains (Birney et al., 1993; Geuens et al., 2016; Han et al., 2010). For long this RRM domain was believed to be involved in the interaction with RNA (Kenan et al., 1991). But some studies also suggest that this RRM domain can also take part in Protein-Protein Interaction (PPI) (Maris et al., 2005). In agreement with this function of RRM, the interactome data (**Figure 26**) and *in vitro* interaction assay (**Figure 27A**) showed the interaction of Aurora-A with many splicing factors that contain RRM. The SR (SRSF) proteins are known to contain one or two RRM and an RS domain (Manley and Krainer, 2010). The interaction of Aurora-A with all the SR proteins (**Figure 27B**) suggests that Aurora-A can specifically bind to RRM or RS domains. On the other hand, most of the hnRNP proteins are also known to contain RRM domain. Hence the interaction of Aurora-A with both the SR and hnRNP proteins suggest that Aurora-A might have high specificity towards the RRM domain.

However, as an exception the hnRNP proteins (hnRNP-U, hnRNP-K, PCBP1 and PCBP2) that don't have RRM domain were also putative interactors of Aurora-A. These four hnRNP proteins have different sets of domains. At least in the case of hnRNP-K protein, the domain that is responsible for interaction with Aurora-A was found to be KI (K Interaction) domain (Zheng et al., 2016). Though this domain is conserved across species, it is specific to hnRNP-K. So it needs to be further investigated to understand how Aurora-A interacts with hnRNP-U, PCBP1 and PCBP2.

4.3. Splicing proteins as substrates of Aurora-A and its implication in pre-mRNA splicing

Post translational modifications (PTMs) such as phosphorylation, ubiquitination, SUMOylation, acetylation and glycosylation are some of the important signals that drive the splicing mechanism (McKay and Johnson, 2010). These modifications influence splicing catalysis either by interfering directly with the splicing machinery (spliceosome) or with the trans-acting splicing factors that regulate the spliceosome. The

phosphorylation and dephosphorylation cycle is important for the spliceosome to form an active complex and in turn perform the splicing reaction. On the other hand, the phosphorylation of splicing factors such as SR proteins is important for the recruitment of spliceosome components, snRNPs to the pre-mRNA. Again these splicing factors are dephosphorylated in order to proceed with the late steps of splicing reaction (Misteli, 1999). The core spliceosomal protein, SF3B1 (SAP155) undergoes phosphorylation and dephosphorylation cycle in order to complete the splicing reaction (Shi et al., 2006; Wang et al., 1998). The *in vitro* kinase assay showed that some of the splicing factors such as RALY, hnRNP-C2, PCBP2 and PTBP1 are substrates of Aurora-A *in vitro* **(Figure 29)**. In the future, the phosphorylation sites of these substrates by Aurora-A *in vivo* has to be determined in order to explore in detail the functional aspects of these phosphorylated substrates in the cells.

There are a few phospho-proteomics studies that identify the *in vivo* substrates of Aurora-A. Sardon and his colleagues employed computational approach and incorporated various biological information and came up with 90 potential substrates of Aurora-A with 80% prediction accuracy (Sardon et al., 2010). However, their final list (90 proteins) was confined only to the proteins that are localizing to spindles and centrosomes. Performing similar studies and filtering for proteins associated with splicing will help us predict the splicing proteins that would be substrates for Aurora-A. Kettenbach et al identified substrates of the Aurora-A,-B and PLK-1 in HeLa cells using the inhibitors (Kettenbach et al., 2011). However, their identification was confined to the synchronized mitotic cells. They identified 127 proteins as potential substrates for Aurora-A. Interestingly their work also suggested the possibility of Aurora-A playing a role in RNA processing and splicing. They have identified the splicing proteins, hnRNPA1, PRPF40A, hnRNPK, CD2BP2, RBMX to be the substrates of Aurora-A. Out of the five proteins, hnRNPA1, hnRNPK and RBMX are present in our interactome data. The very less number of splicing proteins in the phospho-proteomic analysis by Kettenbach could be attributed to the difficulty in identifying the very transient and less abundant phosphorylation of the splicing proteins. Phospho-proteomics using affinity purified snRNPs or spliceosomal proteins (with anti-m3G antibody) will further help in efficiently identifying the splicing proteins that are substrates of Aurora-A.

The heterogeneous nuclear ribonucleoprotein-K (hnRNP-K) is a well-known interactor and substrate of Aurora-A. Aurora-A phosphorylates hnRNP-K at S379 which

reduces the affinity between hnRNP-K and p53 and thereby abolishes the transcriptional activity of p53 (Hsueh et al., 2011). Another study reports that Aurora-A interacts with the KI domain of hnRNP-K in the nucleus and thereby facilitates breast cancer stemness through *c-MYC* expression in a kinase activity independent manner (Zheng et al., 2016). Though these two studies talk about the kinase-dependent and independent functions of Aurora-A and its functional interaction with hnRNP-K in cancer, there is no study focussing on the post-transcriptional functions (RNA splicing, mRNA export and translation) of the Aurora-A-hnRNP-K complex. Another such study also describes the non-splicing function of a splicing protein, hnRNP-U (SAF-A) in mitotic spindle regulation. hnRNP-U protein is present in TPX2-Aurora-A complex and is important for the localization of Aurora-A to mitotic spindle (Ma et al., 2011).

The phosphorylation of the splicing factors can also have an impact on the splicing by altering their localization, activity and stability. Further studies are required to understand the link between Aurora-A kinase and the phosphorylation-mediated regulation of the splicing proteins. Though the studies of the phosphorylation-mediated regulation of the splicing factors date back to two or three decades, there remain hundreds and thousands of questions to be answered in order to completely understand the impact of phosphorylation on splicing and to exploit it as a potential therapeutic target.

4.4. The possible regulatory mechanisms of Aurora-A in maintaining the level of SR proteins

Moore et al showed for the first time the inhibition or depletion of Aurora-A reduced the ASF/SF2 protein levels (Moore et al., 2010). Another study also reported the same effect in order to alter the splicing of AR (Androgen Receptor) pre-mRNA in castrate-resistant prostate cancer (CRPC) (Jones et al., 2017). During the *in vitro* interaction assay, I observed that Aurora-A interacted with all the 12 canonical SR proteins including ASF/SF2 (SRSF1) (**Figure 27B**). So I assessed whether depletion of Aurora-A could also have an impact on the levels of other SR proteins. Indeed, we observed that depletion of Aurora-A in HeLa cells greatly decreased the protein levels of many SR proteins including ASF/SF2 (SRSF1) (**Figure 30**). The decrease in the level of SR proteins are evident from both anti-SR and anti-phospho-SR blots. Moore et al postulated that Aurora-A inhibition or depletion decreases the ASF/SF2 levels through post-translational modifications since they did not observe a change in its mRNA levels.

They also observed the same effect even in the exogenously expressed ASF/SF2 protein and hence ruled out the possibility that this effect is a consequence of transcriptional downregulation or effects on mRNA stability. So, it has to be ascertained in our study that the decrease in the SR protein levels is not due to the change in their mRNA levels. However, in our differential gene expression analysis of the RNA-seq data, we did not observe a significant difference in the expression of all the 12 SR. Overall these results suggest that the decrease in SR proteins levels that we observed is in protein level and not in RNA level. Further investigations are needed to confirm whether Aurora-A regulates SR protein levels through post-translational modifications.

The post-translation modification mediated destabilization has been reported for some of the SR proteins. For instance, ASF/SF2 is shown to be ubiquitinated and degraded by the proteasome in T cells from Systemic Lupus Erythematosus (SLE) patients or in stimulated normal T cells (Moulton et al., 2014).

Another interesting correlative study describes a cascade of events that are responsible for the degradation of ASF/SF2 protein in colorectal cells. Firstly, the pharmacological inhibition of GSK3β leads to a decrease in the levels of the SR proteins kinase, SRPK1. Secondly, the decrease in SRPK1 protein levels leads to cytoplasmic accumulation of ASF/SF2 protein. Thirdly, the cytoplasmic accumulation of ASF/SF2 protein leads to its proteasome mediated degradation. Finally, the degradation of ASF/SF2 protein leads to splicing mediated increase in Rac1b transcript (Gonçalves et al., 2014). Overall this study highlighted an important role of SRPK1 in facilitating the proteasome mediated degradation of ASF/SF2. Though we have not observed the SRPK1 protein to be an interactor of Aurora-A (from our Aurora-A interactome data), another interactome study reports the interaction of SRPK1 with Aurora-A (Varjosalo et al., 2013). It is plausible that depletion of Aurora-A can impact the protein level of SRPK1 in a GSK3β dependent or independent manner and thus can decrease the SR protein levels. Alternatively, it is also possible that Aurora-A directly facilitates the cytoplasmic accumulation of SR proteins. This would help to describe the mechanism of regulation of SR protein by Aurora-A.

In our study, depletion of Aurora-A had an impact on more than one SR proteins and this result hints that Aurora-A depletion might also affect the splicing-related structures such as nuclear speckles which act as a store house of the splicing factors. It is to be noted that Aurora-A localizes to nuclear speckles (**Figure 31**).

Overall, the detailed study of the regulation of SR proteins by Aurora-A would contribute greatly to the cancer field as Aurora-A is important for the cell proliferation and there is a positive correlation between the expression of SR proteins and cell proliferation (Fay et al., 2009).

4.5. Localization of Aurora-A to nuclear speckles and its function in RNA splicing regulation

I have shown that Aurora-A interacts with many splicing proteins and is important for maintaining the SR protein levels. Since nuclear speckles are enriched with splicing proteins, I addressed the question of whether Aurora-A localizes to nuclear speckles. Indeed Aurora-A localized to nuclear speckles and its periphery (**Figure 31**). It is known that co-transcriptional splicing occurs at the periphery of nuclear speckles and post-transcriptional splicing occurs inside the nuclear speckles (Girard et al., 2012). Since Aurora-A shows localization to both inside and periphery of nuclear speckles, it can be speculated that Aurora-A can regulate both co- and post-transcriptional splicing. In agreement with this hypothesis Aurora-A enhanced the splicing efficiency of β-globin pre-mRNA *in vitro* (**Figure 32**). Also inhibition of Aurora-A altered the splicing pattern of 414 events in HeLa cells (**Figure 43**). Overall, the localization of Aurora-A and its interaction with splicing proteins are in agreement with its function in splicing.

A number of splicing specific kinases such as SRPKs, CrkRS, DYRK1A and Clks, possess specific nuclear speckle localization domain that direct their localization to nuclear speckles. For instance, RS domain in CrkRS and histidine repeats in DYRK1A are responsible for their localization to nuclear speckles (Ko et al., 2001; Álvarez et al., 2003). Aurora-A lacks any of these nuclear speckles localization domains. Alternatively, Aurora-A could also be targeted to nuclear speckles through interaction with any of the splicing proteins that it interacts with. Therefore, identifying the domain that favors the localization of Aurora-A to nuclear speckles and using it as a drug target would be a potent strategy to inhibit the splicing function of Aurora-A.

4.6. Kinase-independent and dependent action of Aurora-A in regulating gene expression and alternative splicing respectively

In order to identify the function of Aurora-A in splicing, RNA seq analysis was carried out upon inhibition of Aurora-A. The differential gene expression and alternative splicing were identified using edgeR and VAST tools. Inhibition of Aurora-A showed a modest effect on the gene expression (**Figure 41**). Zheng et al showed that Aurora-A forms a complex with hnRNP-K and acts as a transcription factor. This function of Aurora-A is independent of its kinase activity (Zheng et al., 2016). In agreement with Zheng et al., inhibition of Aurora-A kinase activity did not greatly alter gene expression. However, Aurora-A inhibition modulated significantly the alternative splicing pattern (**Figure 43**). These results suggest that modulation of gene expression by Aurora-A is independent of its kinase activity whereas modulation of alternative splicing by Aurora-A is dependent on its kinase activity. In coherence with this hypothesis, we did not observe any overlap in genes that are both differentially expressed and spliced (**Figure 43B**).

The nuclear Aurora-A has been reported to have more oncogenic transforming activity than cytoplasmic Aurora-A (Tatsuka et al., 2009). This nuclear function of Aurora-A might be attributed to its function in regulating gene expression and alternative splicing in cancer cells. In fact, Zheng et al showed that nuclear Aurora-A is responsible for its transactivation function and is responsible for enhancing the breast cancer stem cell phenotype (Zheng et al., 2016). Hence targeting the mechanism of nuclear localization of Aurora-A would be a novel and potential therapeutic strategy - hitting two birds with one stone approach (gene expression and alternative splicing function of Aurora-A).

4.7. Differential gene expression and alternative splicing analysis suggested the novel functions of Aurora-A

The interactome of Aurora-A revealed a novel function of Aurora-A in pre-mRNA splicing. Further, exploring the functional aspect of Aurora-A through RNA-seq analysis suggested novel functions of Aurora-A which have not been previously reported. The differential gene expression analysis upon inhibition of Aurora-A showed modest effect of Aurora-A on gene expression but still enriched for the terms related to immune

response against the viruses (**Figure 42**). The enriched genes (DDX58, IFIT3, IFIT2, IFIT1, OASL, IFI44, ISG15, PMAIP1) encode for antiviral proteins that interfere with different stages of viral life cycle (Shim et al., 2017). However, it has to be further studied if Aurora-A helps in the viral life cycle or is acting against it by inhibiting or inducing anti-viral response respectively.

The gene ontology analysis of differentially spliced genes upon inhibition of Aurora-A, suggested the novel functions of Aurora-A in a cell cycle specific manner. The gene ontology analysis also suggested the role of Aurora-A in ciliogenesis, transcriptional regulation, RNA processing at G1 phase (**Figure 44**); mRNA sorting, immune response, neuron apoptosis, spindle assembly checkpoint and glycogen pathway at G2 phase; autophagy and mTOR signaling, protein transport and proteins quality control, and gene expression at mitotic phase. In spite of altering the splicing pattern of many zinc finger proteins, which are implicated in transcriptional regulation, we did not observe a great alteration in gene expression.

Previously many studies have reported Aurora-A as an important factor in ciliogenesis. For instance, Aurora-A mediated phosphorylation of HDAC6 is shown to be important for cilia resorption/disassembly (Pugacheva et al., 2007). In our study, we have identified that the Aurora-A inhibition altered the splicing of HDAC6 and other proteins involved in ciliogenesis such as CEP250, BBS9, CEP192, WDR35, SFI1. This shows the new mode of action of Aurora-A in regulating ciliogenesis through modulation of alternative splicing pattern of genes involved in ciliogenesis. Genome wide RNAi screening identified spliceosome (mRNA processing) and proteasome components as novel regulators of cilia biogenesis (Kim et al., 2016). They also showed that mRNA processing factors regulate the expression of cilia disassembly regulators such as Aurora-A and PLK1. On the basis of Kim et al and my work, I postulate a working model for the regulation of ciliogenesis (**Figure 48**)

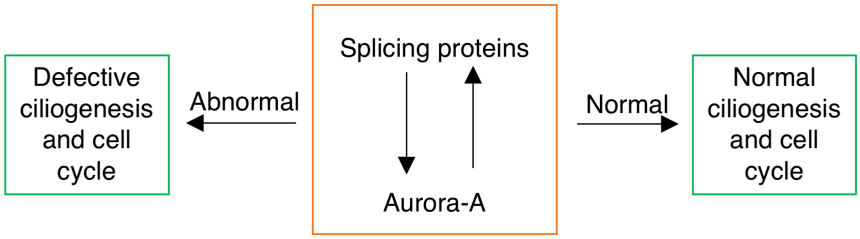

Figure 48 | A working model for the regulation of ciliogenesis by Aurora-A. There is a crosstalk between Aurora-A and splicing proteins, where Aurora-A by interacting with splicing proteins can

modulate the alternative splicing of ciliogenesis proteins and on the other hand, splicing proteins modulate the expression of ciliogenesis regulators. The misregulation of this crosstalk might cause defective ciliogenesis and cell cycle.

Interestingly Aurora-A also altered the alternative splicing of genes involved in RNA processing such as CLK1 and CLK4 (**Figure 47**). CLK protein kinases are known to be involved in recruiting the SR proteins to the site of transcription/splicing (Colwill et al., 1996). Dominguez et al identified the periodic splicing changes across cell cycle which don't show a significant overlap (Dominguez et al., 2016). Our results are also in coherence with Dominguez et al, who also found that the periodic splicing of these genes is controlled by the SR protein kinase CLK1. In our study, we found that Aurora-A indeed altered the alternative splicing of CLK1 and CLK4, which suggests that Aurora-A can be a master regulator in controlling the cell cycle specific alternative splicing. Inhibition of Aurora-A promotes more inclusion of exon 4 of CLK1 and CLK4 (**Figure 47**). It is worth noting that inclusion of exon 4 of CLK1 codes for an active form of CLK1 protein (Duncan et al., 1997). The VAST tool also predicted that inclusion of exon 4 of CLK1 and CLK4 to have an impact on their protein levels. The inclusion of exon 4 of CLK1 and CLK4 upon inhibition of Aurora-A is a serious matter to be taken into account as overexpression of CLK1 and CLK4 is associated with many cancers. Since it requires the right amount of Aurora-A for the normal cycling of cells and too much or too less of Aurora-A might result in tumorigenesis (Aurora-A is both oncogene and tumour suppressor), targeting Aurora-A might cause the risk of developing secondary tumours (Damodaran et al., 2017). The inclusion of exon 4 and the subsequent increase in protein levels of CLK1 and CLK4 might be attributed to this secondary effect caused by inhibitors of Aurora-A. Hence, combinatorial therapy with low doses of drugs targeting both Aurora-A and CLKs might be beneficial to overcome the adverse side effects.

Overall the RNA-seq analysis suggested novel functions of Aurora-A and also suggested the new mode of action for some of its well-known functions. Though the first inhibitor of Aurora-A was identified a decade ago and followed by many more in the later years, none of them has gone beyond phase III clinical trials because of the adverse effects. A better understanding of the new functions of Aurora-A would help us to develop better and potent drugs with minimal side effects.

4.8. The role of Aurora-A in pre-mRNA splicing

Based on the results obtained and published results, I propose a working hypothesis to describe the role of Aurora-A in pre-mRNA splicing (**Figure 49**). Aurora-A is seen to localize to nuclear speckles and also to its periphery. When in nuclear speckles, Aurora-A could interact with and phosphorylate the splicing proteins and thereby recruit the splicing proteins (non-core splicing factors) to the periphery of nuclear speckles (the site of transcription and splicing). When in periphery of nuclear speckles, Aurora-A could regulate the splicing proteins (core spliceosomal proteins) and participate in splicing reaction. When Aurora-A is inhibited or depleted, it might affect the recruitment of splicing factors to the site of splicing and hence would alter the alternative splicing pattern. Subsequently, the unphosphorylated splicing factors might be re-localized to cytoplasm and thereby degraded by either proteasome or autophagy.

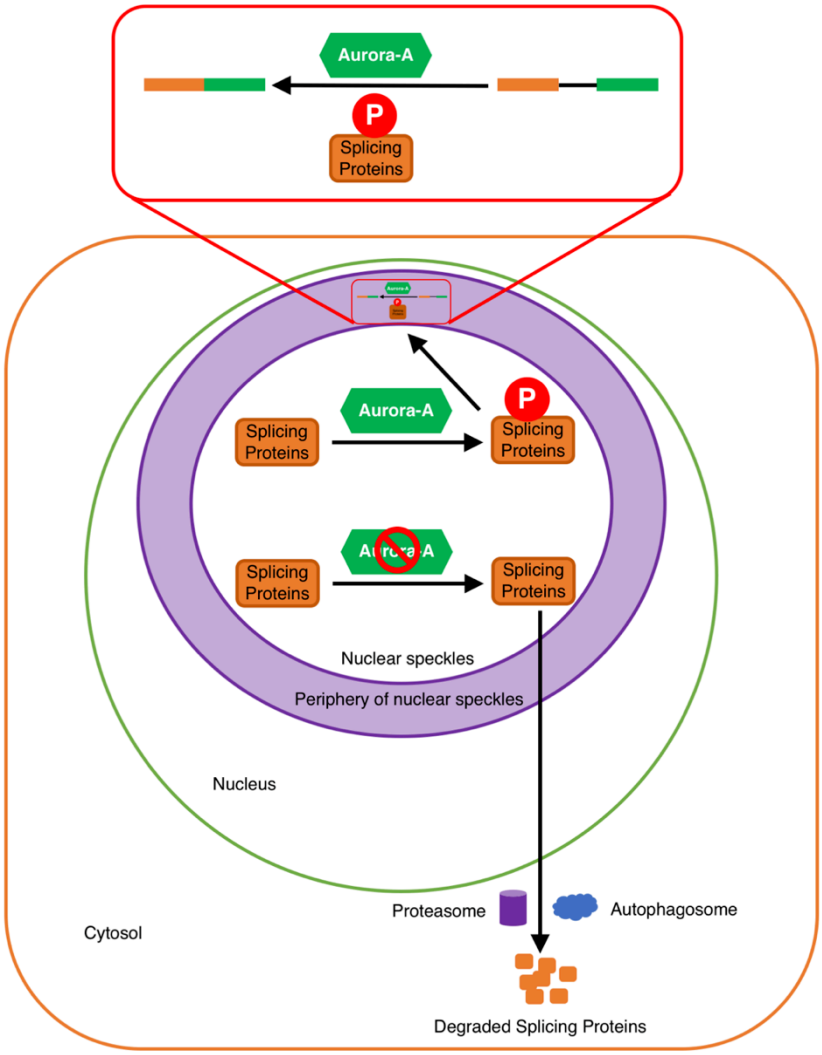

Figure 49 | A working model to explain the role of Aurora-A in pre-mRNA splicing. Aurora-A phosphorylates the splicing proteins in nuclear speckles and recruits them to the site of splicing, where they

alter the fate of pre-mRNA. Aurora-A can also take part directly in the splicing reaction by modulating the core spliceosomal proteins on the periphery of nuclear speckles. On the other hand, Aurora-A stabilizes the splicing proteins by inhibiting their re-localization and degradation.

4.9. Future prospective

In order to go further with this working model, the following questions have to be answered in the future,

1. Splicing proteins as substrates of Aurora-A
    a. Are SR proteins the bonafide substrates of Aurora-A *in vitro*?
    b. Does Aurora-A phosphorylate the splicing proteins *in vivo*?
2. Determine if Aurora-A promotes the localization of splicing proteins to the site of transcription/splicing. This question can be addressed by over-expression or knocking down of Aurora-A in *pem*-HeLa cells (stably expressing the rat homeobox gene *pem*) and analyse the *in vivo* targeting of splicing factors (SC35) to the *pem* reporter gene. This can be assayed by simultaneous visualization of SC35 and the pem transcription site using indirect immunofluorescence and fluorescence in situ hybridization, respectively. (Note: *pem* HeLa cells system was used by Misteli and Spector, 1999 to prove CTD of RNA pol-II is required for the targeting of pre-mRNA splicing factors to transcription sites *in vivo*)
3. Determine the mechanism of Aurora-A in stabilizing SR proteins.
    a. Analyse the level of SR proteins upon overexpression of Aurora-A.
    b. Determine if the destabilization of SR proteins upon Aurora-A depletion is due to cytoplasmic accumulation of SR proteins (Note: cytoplasmic accumulation of SR proteins is known to de-stabilize ASF/SF2 upon inhibition of GSK3β).
    c. Determine if the de-stabilization of SR proteins upon Aurora-A depletion is through proteasome or autophagy-mediated degradation.
4. Determine the splicing role of Aurora-A in ciliogenesis.
    a. Validate the splicing pattern of ciliogenesis related events found by VAST-tools upon inhibition of Aurora-A.
    b. Determine if the modified splicing pattern of these events affects the ciliogenesis through immunofluorescence by staining for cilia markers (such as smoothened protein or acetylated α-tubulin).

5. Determine if nuclear Aurora-A is important for its function in splicing. It is very important to address this question since the nuclear Aurora-A is reported to be responsible for its tumorigenic property and breast cancer stemness.
6. High throughput drug screening to identify the inhibitors that prevent the nuclear accumulation of Aurora-A. Zheng et al identified the NLS in Aurora-A (amino acids 333–383) (Zheng et al., 2016) and this region can be used for *in silico* drug screening as a first step. This will be a new class of Aurora-A inhibitor that does not target its kinase activity. This can potentially eliminate the tumorigenic property of Aurora-A leaving behind its normal cell cycle related functions untouched.

5. Appendix

5.1. Manuscript

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Aurora A and cancer

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Abstract

Aurora-A is a serine-threonine kinase found over expressed in cancers and demonstrated to be an oncogene. Since the kinase regulates cell cycle progression several pharmaceutical companies were involved in the identification of inhibitors to be used in cancer treatments. Many of such inhibitors are now commercially available but none of them succeeded any phase III clinical trials, yet. The concern here is, whether Aurora-A is still a good target or biomarker?

Introduction

Aurora-A was discovered in 1995 as a protein kinase involved in centrosome separation in Drosophila [1]. In 1997 a human cDNA related to Aurora-A and named BTAK (Breast Tumour Activated Kinase) was identified to be over expressed in breast cancers [2]. One year later the kinase was demonstrated to behave like an oncogene whose over expression was sufficient to induce tumour formation [3,4]. The first function associated with the kinase was bipolar spindle assembly and the first localisation was centrosomal [5]. So the idea was that over expression of Aurora-A would disturb bipolar spindle assembly leading to mitotic defects at the origin of genome instability that would, in turn, lead to cancer. This was indeed nicely demonstrated in mice where over expression of Aurora-A in mammary epithelia is sufficient to induce tumour formation but only after a prolonged period of genomic instability [6]. This kind of tumours derived from Aurora-A over expression in mice even showed the same gains and losses of genes expression like the ones observed in human breast cancers [7].

The prolonged genomic instability necessary for cells over expressing Aurora-A to form tumours, clearly suggests that an accumulation of genetic events is responsible for the origin of cancer. This has been also observed in mouse through an accumulation of gene mutations [7]. Having said that, is Aurora-A a good target for cancer treatment? The number of failures in various phase III clinical trials clearly indicate that inhibiting Aurora-A is not sufficient. However, Aurora-A is a good biomarker. The simple fact that tumours have conserved the over expression of the kinase, strongly indicate that it should be advantageous to them. And indeed, high level of Aurora-A has been demonstrated to help cancer cells to become resistant to chemotherapeutic drugs. Etoposide or cisplatin, for instance, are is inducing DNA damages that are detected by a cell cycle checkpoint which in turn arrests cell cycle progression either to repair the damages or to induce cell death. Cells over expressing Aurora-A have been shown to override this checkpoint as they keep dividing even in the presence of DNA damages [8-11]. The same is true for taxanes that are microtubule poisons used in chemotherapies. Taxanes induce cell cycle arrest in mitosis due to the fact that the spindle assembly checkpoint remains active, and this arrest eventually leads to cell death. Again cells over expressing Aurora-A override the mitotic checkpoint and keep dividing [12,13].

Detecting the over expression of Aurora-A in cancer cells can then be useful to adapt treatments like supplementing or complementing Aurora-A inhibitors to DNA damaging agents or to microtubule poisons in the cancer cells over expressing Aurora-A to counteract chemo resistances [14-16]. For these reasons even if many phase III clinical trials failed, the search for Aurora-A inhibitors should be pursued. The last comment to be done on Aurora-A and cancer is that basic research on Aurora-A should also be reinforced since numerous non-canonical functions of the kinase have been discovered in these past few years that have a crucial impact on cancer research [17]. One does not completely understand yet, how Aurora-A triggers cancers.

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5.2. Manuscript

Aurora A kinase activity is required to maintain the spindle assembly checkpoint active during pro-metaphase.

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Abstract

During the prometaphase stage of mitosis, the cell builds a bipolar spindle of microtubules that mechanically segregates sister chromatids for two daughter cells in anaphase. The spindle assembly checkpoint (SAC) is a quality control mechanism that monitors proper attachment of microtubules to chromosome kinetochores during prometaphase. Segregation occurs only when each chromosome is bi-oriented with each kinetochore pair attached to microtubules emanating from opposite spindle poles. Overexpression of the protein kinase Aurora A is a feature of various cancers and is thought to enable tumour cells to bypass the SAC leading to aneuploidy. Here, we took advantage of a chemical and chemical-genetic approach to specifically inhibit Aurora A kinase activity in late prometaphase. We observed that a loss of Aurora A activity directly affects SAC function, that Aurora A is essential for maintaining the checkpoint protein Mad2 on unattached kinetochores, and that inhibition of Aurora A leads to SAC extinction, even in the presence of nocodazole or taxol. This is a new finding that should affect the way Aurora A inhibitors are used in cancer treatments.

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Introduction

Aurora kinases are key regulators of mitosis that fulfil complementary functions, as suggested by their localisation on mitotic structures; Aurora A localises at the centrosome and spindle poles, whereas Aurora-B and Aurora-C localise on chromosomes and at the midbody (Giet et al., 2005). Aurora-B and Aurora-C belong to the chromosome passenger complex (CPC) (Carmena et al., 2012b); their kinase activities are required for chromosome condensation by phosphorylating histones (Hsu et al., 2000; Wilkins et al., 2014), for the spindle assembly checkpoint (SAC) by phosphorylating and regulating Zwint-1 (Andrews et al., 2004; Kasuboski et al., 2011), for cytokinesis by phosphorylating the central spindlin component (Guse et al., 2005), and for the abscission checkpoint (Mathieu et al., 2013; Norden et al., 2006). Aurora A is involved in G2/M transition (Dutertre et al., 2004; Seki et al., 2008), centrosome maturation (Hannak et al., 2001), and spindle assembly during prometaphase (Roghi et al., 1998). The SAC is a surveillance mechanism that monitors the attachment of kinetochores to microtubules during the process of bipolar spindle assembly (Rieder and Maiato, 2004). Every mitotic chromosome made of two sister chromatids possesses one kinetochore per chromatid. Once every chromosome kinetochores are attached to microtubules, the SAC is switched off, chromosome segregation occurs and the cell enters anaphase (Foley and Kapoor, 2013). The SAC components are localised at the kinetochores, a macromolecular structure organised in different layers of protein complexes. Starting at the level of the centromeric chromatin, the histone H3 variant centromere protein A (CENP-A) defines the localisation of the constitutive centromere-associated network (CCAN), which is composed of 16 CENPs. This complex provides a platform on which to build an interface between the kinetochore, the microtubules and the KMN network, which is made up of Knl1, Mis12, and Ndc80.

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At the molecular level, the SAC maintains the ubiquitin ligase Anaphase Promoting Complex/Cyclosome (APC/C) in an inactive state through sequestration of its activator Cdc20 by the protein Mad2 (Nilsson et al., 2008). The MCC (Mitotic Checkpoint Complex) responsible for the SAC signal in human cells is composed of the four proteins Mad2, BubR1, Bub3, Cdc20. Mad2 localises to unattached kinetochores and is released upon attachment by microtubules (Buffin et al., 2005; Chen et al., 1996). When all kinetochores are attached to microtubules, the inhibition of Cdc20 by Mad2 is relieved, allowing Cdc20 to bind to and activate the APC/C, which in turn ubiquitinates cylin B and securin for their degradation by the proteasome. Without securin, the separase becomes active and cleaves the cohesin subunit Scc1/Rad21/Mcd1, triggering sister chromatid separation (Uhlmann et al., 1999). APC/C also targets cyclin B1 for degradation, which induces the inhibition of CDK1 activity and allows the cell to exit mitosis (Thornton and Toczyski, 2003). BubR1 and Mad2 are present on unattached kinetochores and participates in SAC activity (Fang, 2002). Although the regulation of the SAC has been attributed to Aurora-B rather than Aurora A, data suggest that Aurora A might also participate in SAC regulation. For example, phosphorylation of the centromere histone H3 variant CENP-A on serine 7 by Aurora A is required to localise Aurora-B at the kinetochore (Kunitoku et al., 2003). CENP-A is considered as a platform on which to build the kinetochore where the mitotic checkpoint complex (MCC) is assembled. Unfortunately, inhibition of Aurora A kinase leads to defects in spindle assembly, which hinders any study of the role of the kinase in regulating the SAC (Hoar et al., 2007). To overcome this problem, we used two independent approaches that allow Aurora A inhibition in a timely and precise manner (analog-sensitive inhibition and specific inhibitor). Using analog-sensitive inhibition method, we previously showed that the kinase activity of Aurora A is required for central spindle assembly through the phosphorylation of dynactin subunit p150Glued (Reboutier et al., 2013). Here, we report that, in the absence of Aurora A activity, cells exhibit defective chromosome

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congression, premature entry into anaphase and delocalisation of Mad2 from kinetochores to centrosomes, demonstrating for the first time that Aurora A activity is required to maintain an active SAC during prometaphase.

Method

Cell culture

For analog-sensitive inhibition, three U20S cell lines were used: a normal U2OS cell line as control and two stable cell lines expressing a wild-type version of Aurora A tagged with GFP (wt-Aurora A) and an allele-sensitive version of Aurora A tagged with GFP (as-Aurora A). Both versions of Aurora A were expressed under the control of the Aurora A endogenous promoter (Reboutier et al., 2013). These cell lines were maintained in McCoy's 5A medium containing GluthaMAX (Gibco, Invitrogen) supplemented with 10% FBS (PAA) and 1% penicillin-streptomycin (Gibco, Invitrogen). In addition, the wt-Aurora A and as-Aurora A cells were grown in the presence of 1.25 mg/ml geneticin (G418 sulphate, PAA).

Hela tubulin-GFP and U2OS Aurora A-GFP under endogenous promotor were grown in a humidified incubator at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium (Gibco, Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum (PAA Laboratories) and 1% penicillin/streptomycin (Gibco).

Small interfering RNA and transient transfections

The oligonucleotide sequence of the siRNA targeting Aurora A (Qiagen) was 5'-AAATGCCCTGTCTTACTGTCA - 3' (Reboutier et al., 2013). Transfection was performed using JetPRIME (Polyplus transfection). Depletion of endogenous Aurora A kinase was controlled by Western blot in every experiment as described by Reboutier et al. (2013).

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Western blot

Cells were harvested by treatment with trypsin-EDTA (Gibco, Invitrogen) and resuspended in McCoy's 5A medium containing GluthaMAX, centrifuged at 1200 rpm at 4°C for 3 min, and washed three times in PBS. The cell pellet was resuspended in Laemmli buffer, sonicated, and incubated for 5 min at 95°C. Proteins in the cell extracts were separated on 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. After transfer, the membrane was blocked with 3% non-fat milk in TBST for 1 h at room temperature followed by an overnight incubation in TBST containing 3% non-fat milk and primary antibodies. The primary antibodies were mouse anti-Aurora A 5C3 (1/100) (Cremet et al. 2003); rabbit anti-Mad2 (1/1000, Covance); rabbit anti-HCAP-D2 (1/10000) (Collas et al. 1999); mouse anti-Cyclin B1 (1/1000, Santa Cruz); mouse anti-HA (1/1000, Covance); mouse anti- β - tubulin (1/2000, Sigma-Aldrich); rabbit anti Phospho-Aurora A/B/C (1/1000, Cell Signaling); rabbit antiphospho-histone H3 (H3S10, 1/1000, Euromedex). The secondary antibodies were anti-mouse or anti-rabbit IgG coupled to peroxidase (1/5000 and 1/110,000, Jackson Laboratories). Finally, the membranes were processed for chemiluminescent enhancement with Dura or Pico (Thermo Fisher Scientific) before film exposure.

Cell synchronisation

MLN8237 approach: The cells were grown on coverslips in 12-well plates. Cells were then synchronized in late G2 (G2/M transition) by treatment with 100 nM nocodazole for 12 hours at 37°C.

Analog sensitive approach: The cells were grown on coverslips in 12-well plates. They were depleted of endogenous Aurora A using siRNA as described by (Reboutier et al., 2012). Cells were then synchronised in late G2 (G2/M transition) by treatment with 2 μM CDK1 inhibitor RO3306 (Calbiochem) at 37°C for 5 h. Next, they were released in late prometaphase by

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washing out RO3306 three times for 4 min. Finally, the cells were treated with 10 µM 1-Na-PP1 to inhibit as-Aurora A, fixed for 10 min at either -20°C in 100% cold methanol or at room temperature in 4% paraformaldehyde, and processed for immuno-fluorescence microscopy.

Immunofluorescence

MLN8237 approach: Cells fixed in –20°C methanol for 5 min and treated as described in (Courthéoux et al., 2016). For GFP stable cell line, GFP nano-booster (chromotek) was used at the secondary antibody incubation step. Images were taken with the new LSM800 Airyscan (Zeiss Inc. and processed with FIJI (imageJ). For figures 3, 4 and 5, the Fast-Airyscan mode was used with optimum parameter settings. Lasers power and acquisition settings are the same for all conditions within an experiment. Enlarged pictures in Figures 3C and 5A are z-projection of 5 z stacks.

Analog sensitive approach: Cells fixed in methanol or paraformaldehyde as described above were washed with PBST buffer (PBS containing 0.1% Triton X100) and permeabilised by incubation in MBS buffer (100 mM PIPES pH 6.8, 1 mM MgCl2, 0.1 mM CaCl2, 0.1% Triton X100). The cells were then incubated in PBST containing 1% BSA for 1 h at room temperature, followed by 1 h incubation at room temperature in PBST containing 1% BSA and primary antibodies. After several washes in PBST containing 1% BSA, the cells were incubated with secondary antibodies. Images were taken using a Coolsnap ES (Photometrics) equipped Leica DMRXA2 microscope and image acquisition software MetaVue (Molecular Devices, Inc.). The images were deconvolved by the Metamorph software (Molecular Devices, Inc.) and edited using Adobe Photoshop CS3.

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Antibodies

We used antibodies directed against: mouse anti-α-tubulin B-5-1-2 (1/2000, Sigma); rat anti-β-tubulin (YL1/2 1/500, Millipore), rabbit anti-Mad2 (1/500, Covance), human anti-CREST centromere protein (1/3000, Antibodies Incorporated); mouse anti-Aurora B (1/1000, BD). Secondary antibodies: rabbit and mouse CF^{TM} (1/1000, Biotium). Alexa Fluor 488 donkey anti-rat IgG (1/1000), Alexa Fluor 555 donkey anti-mouse IgG (1/1000), or Alexa Fluor 555 goat anti-rabbit IgG (1/1000) (Invitrogen). Finally, samples were mounted with Vectashield or Vectashield-DAPI (1/1000, Vector Laboratories) for DNA staining.

Quantification of fluorescence signals

MLN8237 approach: For quantitative analysis in Fig. 3C and Fig. 4C of Aurora A and Mad2 intensities at kinetochore, kinetochore volume was isolated using "3D object counter" from FIJI on thresholded CREST z-stack images. Kinetochore volumes were imported in "3D ROI manager". Average intensity per kinetochore were collected and normalized to the maximum mean from NOCO (for Mad2) or MLN condition (respectively showing the highest Mad2 or Aurora A mean intensity per kinetochore). Aurora A and Mad2 kinetochore values were pooled per condition and showed as box plots.

Analog sensitive approach: The integrated densities of Mad2 (equatorial plates and bipolar spindle poles) were determined from deconvolved images taken with the Leica DMRXA2 (63°-objective) using the FIJI (ImageJ) 1.46i software (NIH).

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Live cell imaging

MLN8237 approach: HeLa cell line expressing Tubulin-GFP (Reboutier et al., 2013) was grown on Zeiss High resolution cover sleeps for 24h then synchronized as describe in Fig. 1A. Video microscopy was performed on Spinning disk CSU-X1 on Nikon Ti-E. Seven z-stack (3 µm step) were collected every 3 min using 63X magnification objective. Z-stack were z-projected and movie were analysed using FIJI. Anaphase was detected manually (as 00:30 in control condition Fig. 1B). Timing was collected for experiments, pooled and presented as box plot (Fig. 1C).

Analog sensitive approach: Endogenous Aurora A was depleted from both stable U2OS cell lines expressing wt-Aurora A and as-Aurora A using siRNA as described by (Reboutier et al., 2013). The cells were then incubated in 100 ng/ml (0.2 µM) nocodazole or 5 µM taxol until the end of the time lapse (16 h). After 13 h incubation in nocodazole, the cells were treated with 10 µM 1-Na-PP1 and filmed for 3 h. The addition of 1-Na-PP1 marks T0 for each movie. Video microscopy was performed on a Leica DM IRB microscope equipped with a 63x/1.4 oil-objective and a Coolsnap HQ camera (Photometrics) driven by Metamorph software (Molecular Devices, Inc.). Images were acquired every 5 min and analysed using the same software.

Statistical analysis

All experiments have been performed at least three independent times and T-test on the mean was performed using Igor (Wavemetrics).

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Other statistical analyses were performed using Tukey's Honestly Significant Difference (HSD) test in R 2.13.0 software (R Core Group). Values were represented as histograms or box plots.

Results

Aurora A inhibition causes premature exit from mitosis of cells arrested in pro-metaphase due to SAC activation.

We previously developed an allele-sensitive Aurora A (as-Aurora A), of which the activity can be inhibited by an ATP analogue (Reboutier et al., 2013), allowing specific inhibition of Aurora A in a very narrow window of time during cell cycle progression. We took advantage of this system to determine whether Aurora A activity plays a role in the SAC. We used a cell line expressing a GFP-tagged wild-type version of Aurora A (WT-U2OS) and another cell line expressing a GFP-tagged allele-sensitive Aurora A (AS-U2OS). Only as-Aurora A is sensitive to the ATP analogue 1-Na-PP1 (Reboutier et al., 2013). Importantly the ectopic kinase was expressed under the control of its own minimum promoter (Reboutier et al., 2013; Tanaka et al., 2002). We depleted the endogenous Aurora A via RNA interference, controlled the efficiency of the depletion (**Fig. 1A and B**) and left the cells with ectopic Aurora A. The cells were then treated with the microtubule poisons nocodazole at 100 nM for 13-hours. This compound affects microtubule dynamics and arrests the cells in prometaphase of mitosis for several hours by maintaining the SAC active (Rieder and Maiato, 2004). To directly test whether Aurora A kinase activity was required in this cell cycle arrested cells, each cell line AS-U2OS and WT-U2OS was treated with 10 μM 1-Na-PP1 for 30 min in the presence of nocodazole and filmed during 3 hrs. In the presence of the inhibitor only the WT-U2OS cells containing an active Aurora A kinase remain arrested in prometaphase, while the cells containing an inactive Aurora A kinase exit from mitosis as soon as we added the as-Aurora A

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inhibitor (**Fig. 1A** and **1C)**. To control that Aurora A inhibition did not stabilize kinetochore-microtubule attachments we repeated the experiment with 3.3 µM nocodazole that not only maintains the SAC active but also depolymerizes all mitotic microtubules (De Brabander et al., 1981; Jordan et al., 1992). We obtained the same result. When treated with 3.3 µM nocodazole or with 3.3 µM nocodazole plus 10 µM 1NaPP1 the two cell lines AS-U2OS and WT-U2OS behave the same way, they arrest in prometaphase (**Fig. 1D)**. Only when cells treated with nocodazole and 1-Na-PP1 were depleted of endogenous Aurora A, cells expressing wt-Aurora A (insensitive to 1-Na-PP1) remained in mitosis, whereas cells expressing as-Aurora A (inhibited by 1-Na-PP1) exited mitosis in less than 1 h **Fig. 1C and D**).

We also counted the number of mitotic cells upon depletion of endogenous Aurora A and treated with nocodazole and 1-Na-PP1. The number of cells in mitosis was much higher in cells expressing wt-Aurora A (78.29%, +/- 4.65, n=46) than in cells expressing as-Aurora A and treated with 1-Na-PP1 (9.41%, +/-1.99, n=62): 7-times higher in the presence of nocodazole (**Fig. 1E**). 21.71% of the cells expressing active wt-Aurora A escape the nocodazole block and exit mitosis within 1 h, whereas the percentage of cells escaping the block reached 91.59 % upon inhibition of Aurora A (**Fig. 1E**). Similar observations were made when cells were treated with taxol; 40% of cells escaped mitosis in the presence of active Aurora A, whereas 85% escaped mitosis in the absence of active Aurora A (data not shown).

These finding demonstrated that inhibition of Aurora A induces cells to exit mitosis in condition where the SAC should prevent it.

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Premature exit and abortive cytokinesis

To confirm and further characterized observations above, we simplified the approach and used the Aurora A inhibitor MLN8237 at a concentration (50 nM) that affects only Aurora A and not B and C (Asteriti et al., 2014). We also decided to used low concentration of nocodazole to avoid a complete depolymerisation of microtubules, but a concentration that remains sufficient to keep the SAC active (Wang and Burke, 1995)**.** HeLa cells expressing tubulin-GFP were treated with 100 nM nocodazole for ten hours, released in prometaphase for 15 min, then incubated with 50 nM MLN8237, 20 nM nocodazole or both and filmed (**Fig.2A**).

As already reported, Aurora A inhibition affects spindle morphology in particular spindle length (Bird and Hyman, 2008)**, Fig. 2B compare green lines 1 & 2)** but does not affect mitotic timing (**Fig. 2C and D)**. Low dose of nocodazole (20 nM), however, affects drastically spindle formation (**Fig. 2B yellow line**) and as it maintains the SAC active, induces a mitotic arrest (**Fig. 2C and D**). By adding MLN on these mitotic arrested cells (20 nM nocodazole and 50 nM MLN8237), we confirm our first observation (**Fig. 1**), almost 75 percent of mitotic cells exit from mitosis (**Fig. 2D**) without having been able to assemble a proper bipolar spindle structure. Nonetheless, these cells that prematurely exit mitosis do not divide (**Fig2B red line**). Instead, we observed a deformation of the cell cortex that might reveal cell attempt to complete mitosis and to divide. Cytokinesis eventually aborted and the tetraploid cell re-adhered to the cover slip (**Fig. 2B red line**).

Because these phenotypes were reminiscent to that Aurora-B inhibition, we decided to control whether Aurora-B could be affected during Aurora A inhibition. Cells were synchronized in prophase and release in presence on MG132 (50 minutes, a proteasome inhibitor) to inhibit anaphase execution. Then cells were treated with DMSO, MLN, Nocodazole, MLN and nocodazole (**Fig. 3A and B**). In control cells Aurora B signal was detected in between CREST (a marker of centromere) signals indicating that the kinase is localised in between kinetochore

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pairs as previously reported (**Fig. 2B** and **Fig. 3C right graphics,** line scans). We did not detect any overlapping area between Aurora A and Aurora-B. Nor did we detect any modification of Aurora-B localisation under MLN8237 treatment (line scans, **Fig. 3C**) or using the as-Aurora A/1NaPP1 approach (not shown).

To control the specificity of MLN8732 towards Aurora A kinase activity, we compared the auto-phosphorylation state of each Aurora kinases (A, B and C) in the absence or in the presence of 50 nM MLN8237 using anti-phosphoserine antibodies in immunoblots (**Fig. 3D**). Aurora A auto-phosphorylation was almost completely lost under MLN8237 treatment, demonstrating that Aurora A was inactivated. In the same condition, we did not observe any change in Aurora B or C auto-phosphorylation. Additionally, the phosphorylation level of serine10 on histone H3, an *in vivo* substrate of Aurora B and C was not affected by MLN8732 (**Fig. 3D**).

These data indicate that the condition we used to inhibit Aurora A kinase does not affect Aurora B localisation or activity.

Inactivation of Aurora A disrupts the localisation of Mad2.

To obtain molecular insight into the role of Aurora A in mitotic exit in the presence of abnormal spindle we investigated the behaviour of Mad2, one major MCC component that localises on unattached kinetochores during prometaphase and that signals the presence of an active SAC.

Using the as-Aurora A approach, we observed that without any treatment, 90% of the control cells had Mad2 at the kinetochores in prometaphase. In the presence of the as-Aurora A inhibitor 1-Na-PP1, 87% of cells expressing wt-Aurora A had Mad2 at the kinetochores, whereas only 10% of cells expressing as-Aurora A did **(Fig. Sup 1A and B).** Western blot analysis revealed that Mad2 protein levels were not affected by Aurora A inhibition, indicating

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that the absence of the protein at the kinetochores was not due to a reduced amount of protein (**Fig. 1B**). Interestingly, in these last cells Mad2 was accumulating at the centrosomes **(Fig. Sup 1A)** as revealed by a quantification of Mad2 signal at the kinetochore and centrosome **(Fig. Sup 1C).**

We then used MLN8237 treatment to confirm these data by following the previously described experimental approaches (**Fig. 3A**). Then in the presence of MG132 the cells were treated with either 50 nM MLN8237 or 20 nM nocodazole or both and images were acquired using super resolution microscopy (Zeiss Airyscan) to evaluate the localisation of Mad2 in late prometaphase (**Fig. 4A**). Like previously reported by others (Bird and Hyman, 2008), inhibition of Aurora A leads to shorter spindles indicative of a good efficiency of MLN8237. By using super resolution images and quantitative analysis, mean Mad2 intensity per kinetochore (stained with CREST) was calculated, pooled and compared. As expected, we observed a recruitment of Mad2 at kinetochores under nocodazole treatment (**Fig. 4B)**. Interestingly, MLN8237 treatment reduces Mad2 signal levels at kinetochore in nocodazole treated as well as in untreated cells (**Fig. 4B**). Like observed in the as-Aurora A approach, this decrease of Mad2 at kinetochores is concomitant with a massive recruitment of Mad2 at centrosomes (**Fig. 4A** red arrows, **Fig. 4B and C**).

This data indicates that Aurora A activity is required to maintain Mad2 on unattached kinetochores thereby possibly contributing to maintain the SAC active.

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Kinetochore localisation of Aurora A

Because we observed Mad2 localisation at kinetochores is disrupted upon Aurora A inhibition, we investigated whether the Aurora A itself could be detected at or near the kinetochores. Although it is thought that Aurora A localises at the centrosome while Aurora-B at the kinetochores during prometaphase, a presence of the Aurora A at kinetochores has been previously reported (Katayama et al., 2008). Using super resolution, we investigated Aurora A localisation around kinetochores during kinetochores-microtubules attachment process. Like above, we used cells synchronized with nocodazole and blocked in metaphase by 50 nM of the proteasome inhibitor MG132 (**Fig. 3A**). We investigated the localisation of Mad2 and Aurora A along the axis of microtubules attached to kinetochores (red discontinued lines in **c** panels of **Fig. 5A**). Microtubules are visualised by tubulin staining and kinetochores by CREST staining. In control cells, we observed Aurora A signal following that of microtubules with an intensity (reflecting protein levels) regularly decreasing until reaching the kinetochore. Two kinetochores are shown in **figure 5A** in the first row. Mad2 is absent on the first kinetochore on the left but present in the one on the right signalling the presence of an active SAC (**Fig. 5A panel 1b**). The drawing scheme representing the area occupied by the CREST signal (kinetochore) in purple and by Aurora A signal in red reveals an area occupied by both signals at the external surface of the kinetochore where the microtubules attach (**Fig. 5A panel 1e).**

After MLN8237 treatment, as observed above, Mad2 was not found at kinetochores anymore (**Fig. 5A panel 2b**). Interestingly, MLN8237 treatment also affects Aurora A localisation, the kinase signal accumulated at kinetochores (**Fig. 5A panel 2d and 2e**, **Fig. 5B second row)**.

Nocodazole treatment at low dose on the other hand, induced a massive recruitment of Mad2 at kinetochores (**Fig. 4A panel 3b and 3e**) but also a recruitment of Aurora A (**Fig. 5A panel 3d and 3e**, **Fig. 5B third row**).

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In the presence of both nocodazole and MLN8237, only kinetochores still linked to microtubules showed Aurora A accumulation (**Fig. 5A panel 4c and 4d, Fig. 5B fourth row**), suggesting Aurora A accumulation at kinetochores relies on the presence of microtubules.

We tested this hypothesis by investigating the level of Aurora A signal at kinetochores using the same methodology we used to quantify Mad2 (**Fig. 3C**). We observed an increase of Aurora A signal at kinetochores when cells were treated with either MLN8237 or nocodazole. This indicate that inhibiting Aurora A or maintaining the checkpoint active triggers Aurora A localisation at kinetochores. In cells treated with MLN8237 and nocodazole together, the level of Aurora A to kinetochore further increased again (**Fig. 5A panel 4d and 4e, Fig. 5B fourth row, Fig. 5C**). This data strongly suggests that Aurora A uses microtubules to localize at kinetochores.

Discussion

Until very recently, all of the studies describing the function of Aurora A have used loss of function by RNA interference, mutations in animal models, expression of a dominant negative version of the kinase, or gain of function by over-expressing wild-type Aurora A or a mutated version that become hyperactive or non-degradable (Glover et al., 1995; Littlepage et al., 2002; Sasai et al., 2008; Schumacher et al., 1998; Zhou et al., 1998). Because Aurora A is essential for centrosome maturation, the phenotype observed in all of these cases corresponds to a defect in spindle assembly and the cell never reaches metaphase (Berdnik and Knoblich, 2002; Hannak et al., 2001). We investigated whether Aurora A is involved in the SAC by specifically inhibiting Aurora A activity after the centrosomes have matured and the cell reaches metaphase. We succeeded by taking advantage of the as-Aurora A isoform already described (Reboutier et

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al., 2013). And we confirmed our results using the Aurora A specific inhibitor MLN8237. Although Aurora A has never been directly involved in kinetochore functions, it phosphorylates CENP-A in prophase to allow Aurora-B localisation at kinetochores (Kunitoku et al., 2003). Aurora A also phosphorylates Haspin in late G2, participating indirectly to the phosphorylation of Threonine 3 of Histone H3 and to the recruitment of Aurora-B to kinetochores and more largely to the localisation of CPC and SAC proteins at kinetochores (Yu et al., 2017). Aurora A was also recently reported to phosphorylate Hec1 and to associate with the inner centromere Aurora-B partner INCENP, when overexpressed, to localise at the mitotic chromosome kinetochore (DeLuca et al., 2018).

Eventually, Aurora A and Aurora-B share substrates with phosphorylation events occurring where the kinases are located. Aurora A phosphorylates MCAK and PLK1 at the centrosome (Macurek et al., 2008; Seki et al., 2008; Zhang et al., 2008), whereas Aurora-B phosphorylates the same two proteins on the same residues but at the kinetochore (Andrews et al., 2004; Carmena et al., 2012a; Lan et al., 2004). Therefore, both Aurora A and Aurora-B act in concert on the same substrates but at different locations to coordinate mitotic progression. This coordination was demonstrated further in DT40 cells in which Aurora A was depleted (KO) and Aurora-B inhibited. The authors found cooperation between both proteins in the coordination of chromosome segregation in metaphase and microtubule depolymerisation in anaphase (Hégarat et al., 2011).

In the present study and in contrast to Aurora A KO, Aurora A was present and active until very late prometaphase. Its inhibition in that precise window of time led to a premature exit from mitosis with reduced Mad2 at kinetochores. In the presence of nocodazole or paclitaxel, which maintains the SAC active due to Mad2 recruitment at the kinetochores (Rieder and Maiato, 2004), inhibition of Aurora A led to the removal of Mad2 from the kinetochores, illustrating the extinction of the checkpoint, and a relocalisation of Mad2 to the centrosomes. This indicates

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that Aurora A activity is required to maintain Mad2 at non-attached kinetochores during prometaphase, ensuring that the SAC remains active. Whether this is a direct or indirect effect of Aurora A remains unknown.

Consistent with such role of Aurora A, we also observed the kinase inhibition (MLN8237) leads to its retention at kinetochores. Consistently with a recent report by DeLuca and collaborators (DeLuca et al., 2018) who identified HEC1 (Ndc80 component) as Aurora A kinase substrate, we detected Aurora A at the outer kinetochores. This localisation of Aurora A seems to be very dynamic and the kinase might in fact shuttle at this localisation to phosphorylate substrates. Indeed, we observed that an inhibition of Aurora A increases its localization to kinetochores as if the inactive kinase remained blocked on its a substrate (Widmann et al., 2012). This localisation of Aurora A under MLN8237 shed light on a possible cooperation between both Aurora A and B in regulating kinetochores bi-orientation leading to SAC silencing. Aurora A localises at the outer kinetochore while Aurora-B at the inner kinetochore **(Fig. 6)**

Aurora A has been observed at kinetochores where it participates in kinetochore/chromatin microtubule nucleation (Katayama et al., 2001). Consistent with a potential function in the SAC, overexpression of Aurora A has been shown to be sufficient to override the SAC in the presence of taxol (Anand et al., 2003; Dutertre et al., 2004). Increase taxol sensitivity was also observed in pancreatic cancer cell lines upon Aurora A depletion (Hata et al., 2005). More recently, it has been reported that the phosphorylation of p73 by Aurora A contributes to the breakdown of the Mad2-Cdc20 complex releasing Cdc20 to degrade cyclin B and to securin, providing a molecular explanation for the SAC override (Katayama et al., 2012). Although resistance to taxol treatment has been observed in breast cancer cells over-expressing Aurora A, this is only true in estrogen receptor (ER)-positive tumours and not in ER-negative tumours (Noguchi, 2006). The reason for this difference remains to be elucidated, but the

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phosphorylation by Aurora A of Ser167 and Ser305 in ERα might be involved (Zheng et al., 2014).

Reports have also indicated a bypass of the SAC upon Aurora inhibition, but the inhibitors used were not specific to one Aurora kinase and the bypass was eventually attributed to Aurora-B inhibition. For example, the use of MLN8054, a more specific inhibitor of Aurora A than Aurora-B, revealed that inhibition of Aurora A accelerates mitosis exit in the presence of nocodazole or taxol (Wysong et al., 2009). The concentration of MLN8054 used in the study was optimised for Aurora A inhibition, but the observed phenotype mimicked Aurora-B inhibition (Tyler et al., 2007).

Here, we used two strategies to specifically inhibit Aurora A to clearly demonstrate for the first time that inhibition of Aurora A is sufficient to inhibit SAC activity and exit from mitosis. We demonstrated that the mechanism involved is not SAC override, but SAC inactivation. The molecular mechanism underlying the involvement of Aurora A in maintaining an active SAC during pro-metaphase remains to be elucidated, and a search for Aurora A substrates has begun. This report is the first to demonstrate a role of Aurora A kinase activity at the kinetochore for the SAC maintenance. Aurora A is a major target in cancer therapy with several inhibitors currently in clinical trials (Kollareddy et al., 2012). A precise understanding of the multiple functions of Aurora A during mitosis will undoubtedly help in designing drug associations and increase the efficiency of chemotherapeutic strategies to eliminate cancer cells (Bush et al., 2013; Schmidt et al., 2010).

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Figures

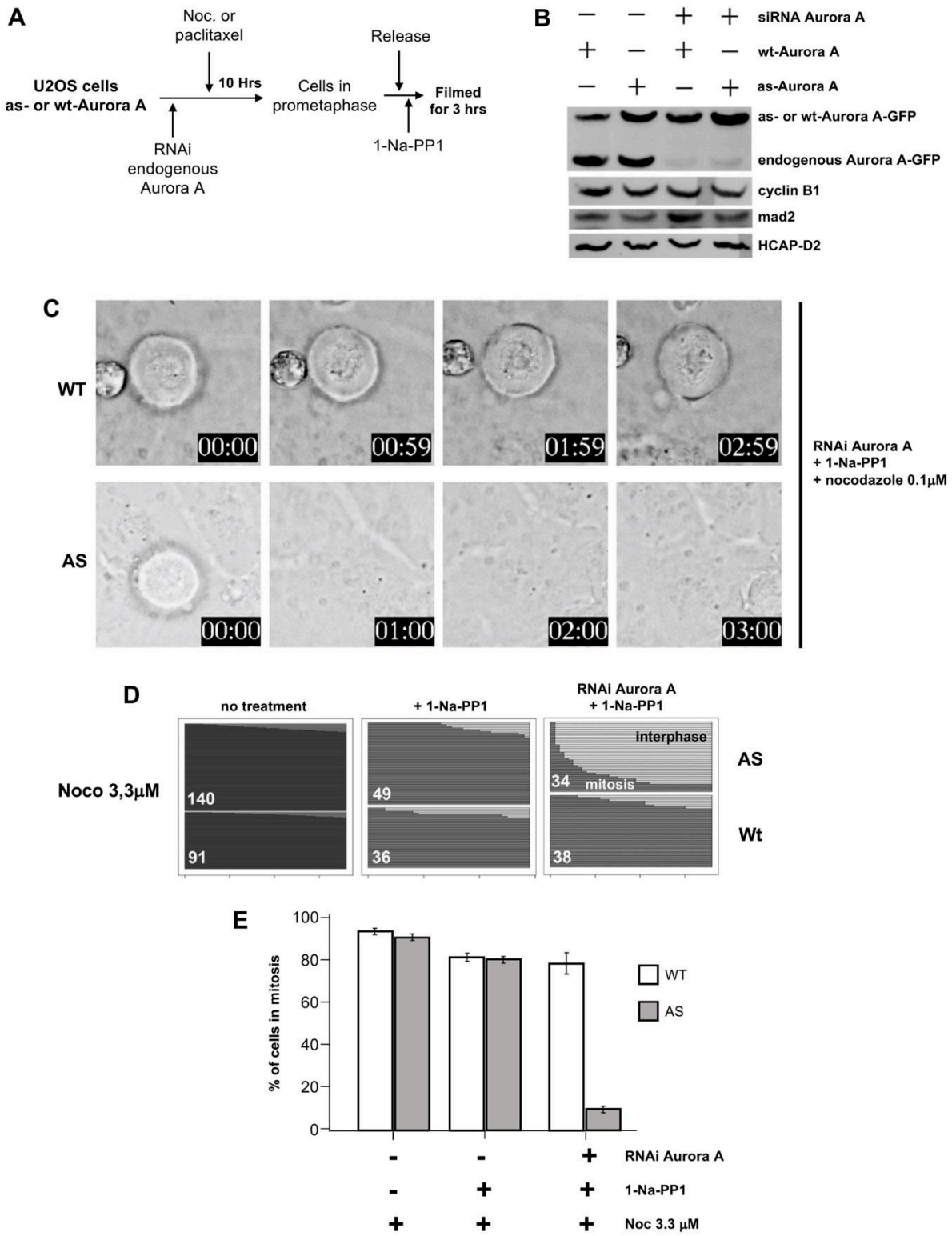

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Figure 1: Aurora A inhibition during mitosis causes SAC override. **(A)** Methods used to analyse the effect of Aurora A inhibition on the SAC. Cells were first depleted of endogenous Aurora A by RNAi and then treated with nocodazole for 16 h to hold them in mitosis. 13 h later, the cells were treated with 1-Na-PP1 to inhibit as-Aurora A and filmed for 3 hours. **(B)** Western blots showing the efficiency of endogenous Aurora A depletion and exogenous WT and AS expression in both stable cell lines. Mad2 levels was controlled in Aurora A depleted cells treated with 1-Na-PP1. Cyclin B1 was used as mitotic marker. HCAP-D2 was used as a loading control. **(C)** Snapshots of movies corresponding to nocodazole-treated stable cell lines depleted of endogenous Aurora A and expressing wt- Aurora A (WT) and as-Aurora A (AS). **(D)** The graph represents the kinetics of mitosis exit in the presence of 3.3 μM nocodazole of stable cell lines expressing either WT or AS. The dark grey lines correspond to mitosis and light grey to interphase (exit from mitosis). The addition of 1-Na-PP1 marks the T0 for each film. *Left*: 1-Na-PP1 treatment of cells depleted of endogenous Aurora A by siRNA. *Middle*: no treatment. *Right*: 1-Na-PP1 treatments. Numbers in the panels indicate the number of cells observed. **(E)** The percentage of cells in mitosis in both stable cell lines treated as described above (Noc: nWT=199, nAS=250, Noc+RNAi: nWT=66, nAS=70, Noc+ RNAi+1Napp1: nWT=46, nAS=62, error bars: standard deviation).

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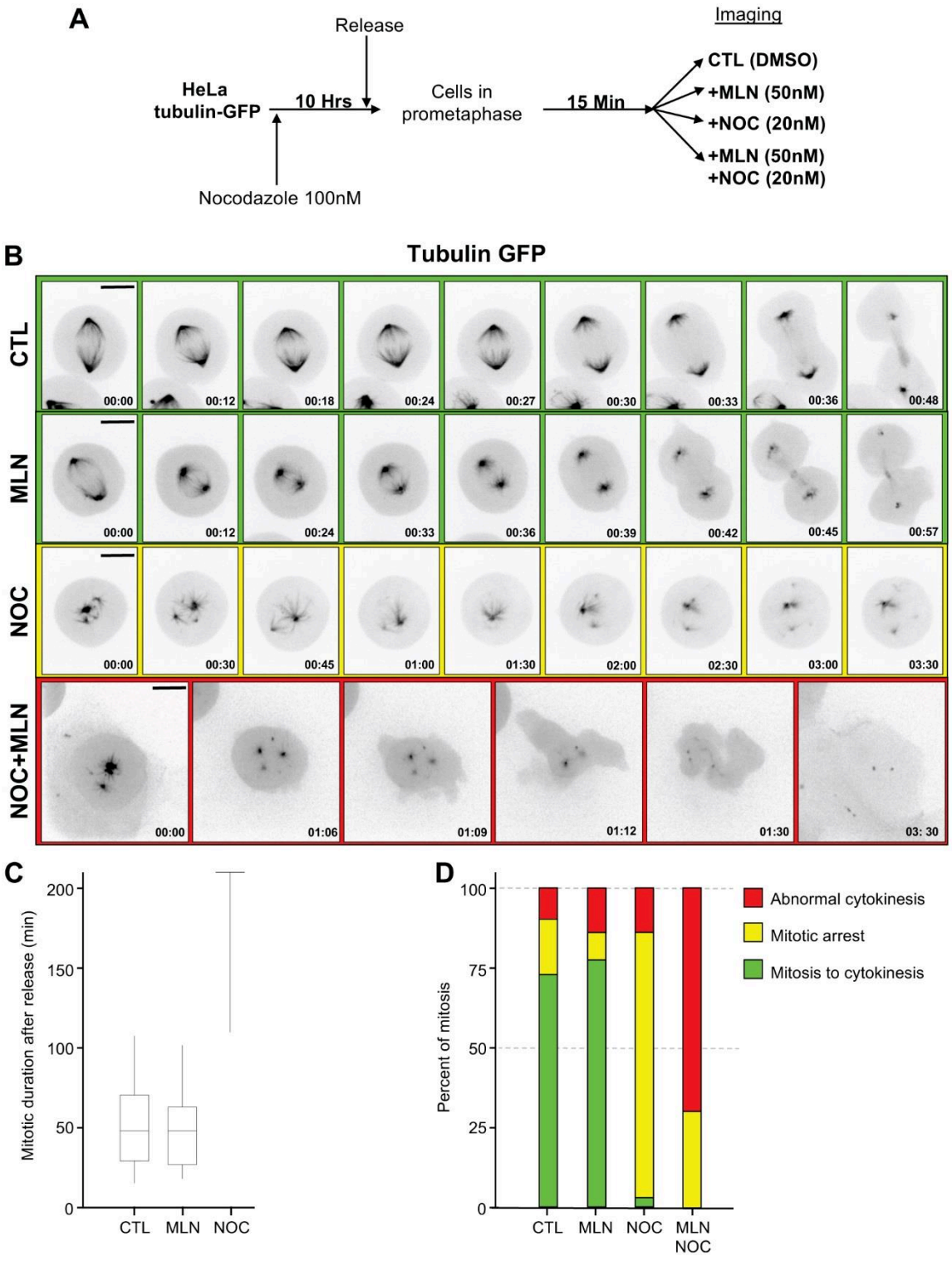

Figure 2: Aurora A inhibition during mitosis causes SAC override. (A) Methods used for cell synchronisation, Aurora A is inhibited in late prometaphase using Aurora A specific inhibitor MLN8237. **(B)** Live cell imaging of synchronized HeLa Tubulin-GFP. Image panel

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showing representative case of mitotic progression under CTL, MLN, Nocodazole or Nocodazole and MLN treatment. Scale bar: 5μm. **(C)** and **(D)** Quantification of mitotic duration (C) and mitotic behaviour (D) after release (CTL n=49, MLN8237 n=49, NOC=49), green: mitosis to cytokinesis, yellow: mitotic delay and red: abnormal cytokinesis.

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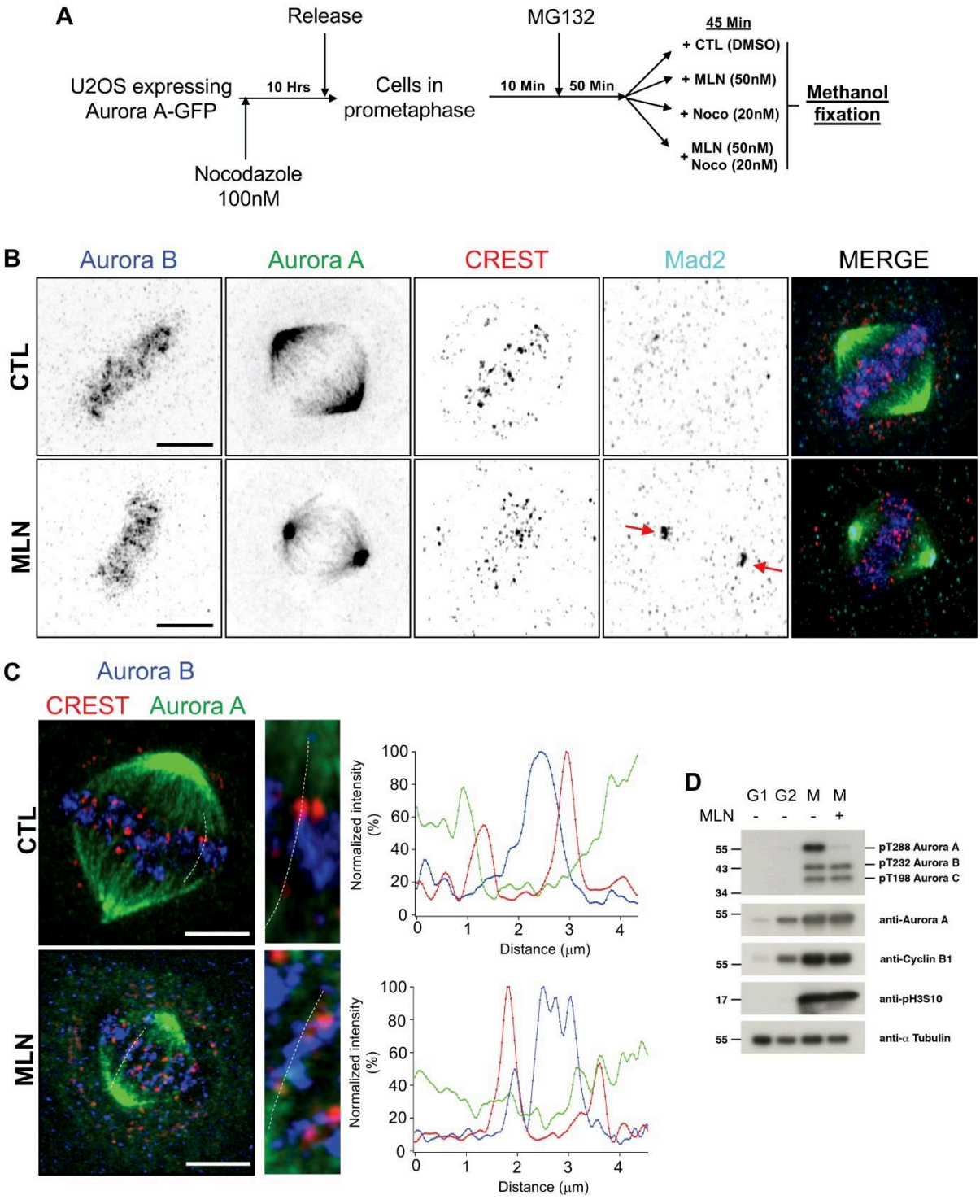

Figure 3: Aurora A inhibition does not affect Aurora B localisation. (A) Methods used for cell synchronisation, Aurora A is inhibited in late prometaphase using Aurora A specific inhibitor MLN8237. **(B)** and **(C)** Immunostaining of U2OS stable cell line expressing Aurora A-GFP under endogenous promoter. Cells were synchronized as in fig1A and imaged using

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Airyscan confocal super-resolution microscope. Red arrows showed Mad2 centrosomal aggregation. Scale bar: 5μm. **(C)** Three z-stacks have been projected to select a kinetochore pair. Graphical representation of fluorescence signal intensity for kinetochore (CREST, red), Aurora B (blue) and Aurora A (green). Scale bar: 5μm **(D)** Western blot of HeLa cells lysis synchronized at different cell cycle stages (G1, G2 and M). Cells were treated with Aurora A inhibitor (MLN8237, 50 nM) during mitosis.

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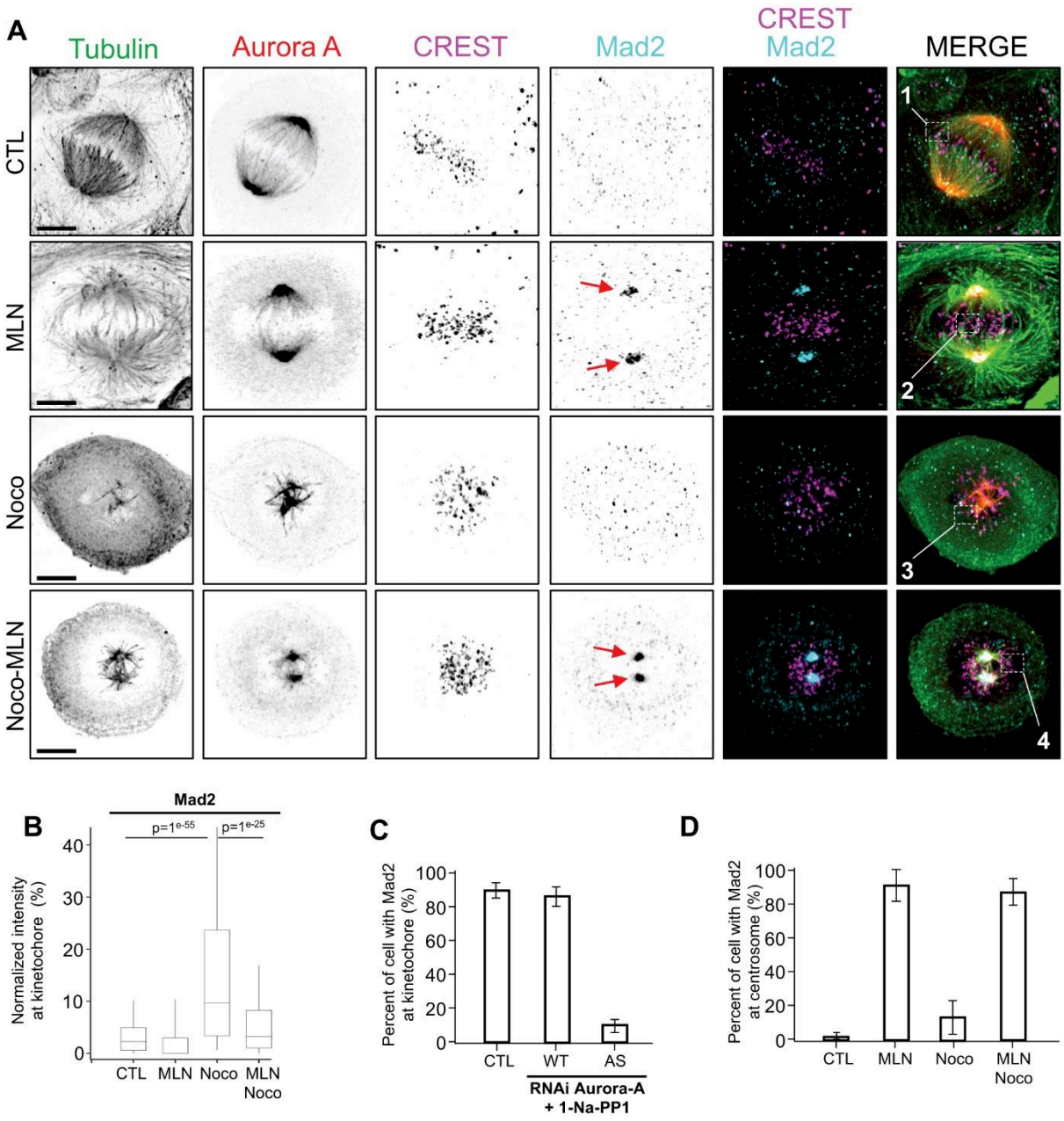

Figure 4: Aurora A Inhibition during mitosis causes Mad2 mis-localisation (A) Immunostaining of U2OS stable cell line expressing Aurora A-GFP under endogenous promotor. Cells were synchronized as in fig2A and imaged using Airyscan confocal super-resolution microscope. Numbered white boxes on right column are enlarged and analysed in fig 4A. Red arrows showed Mad2 centrosomal aggregation. Scale bar: 5µm. **(B)** Quantitative analysis of Mad2 intensity at kinetochore. Average intensity per kinetochore were collected and

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normalized to the maximum mean from nocodazole condition (representative of SAC activation, n=914 kinetochores per condition, error bars = s.d). Mad2 kinetochore values were pooled per condition and showed as box plots. T-test on the means were performed and p-values are presented. **(C)** Percentage of cells with normal localisation of Mad2 in (**Fig. sup1A**) during late prometaphase (CTL=14, WT+Na-PP1= 20, AS+Na-PP1= 26). **(D)** Quantification of cells showing mad2 centrosomal aggregation in percent of cell (CTL n=90 , Noc n=128 , MLN n=139 and Noc with MLN n=94, error bars=s.d)**.**

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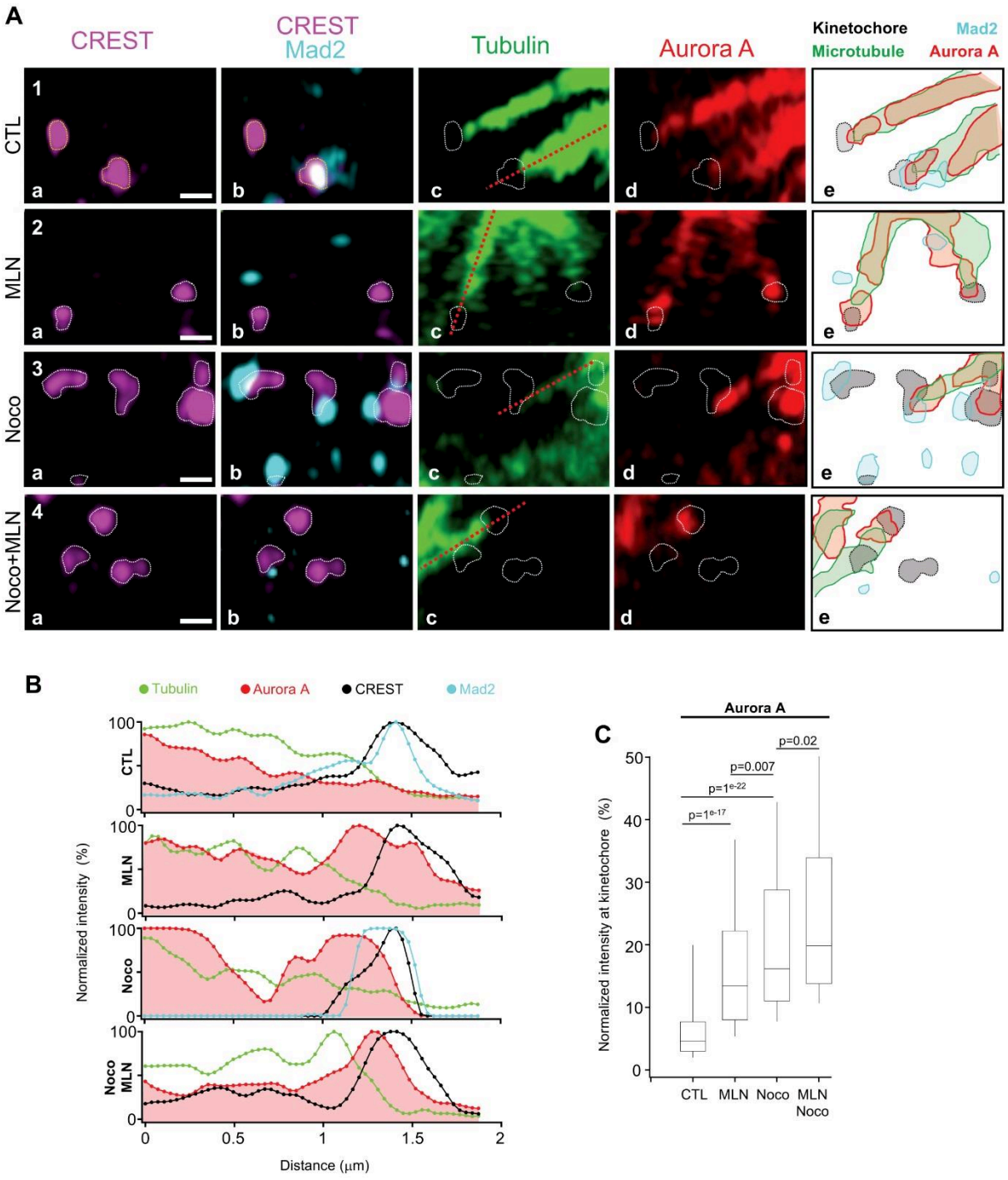

Figure 5: Mad2 and Aurora A localisation at kinetochore (A) Enlarged pictures from Fig 4A**.** White circles represent kinetochore positions. Right column, cartoon of different localisations showing overlap. Scale bar: 500 nm. **(B)** Line scans from red discontinued lines in A. Aurora A signal overlaps with Mad2 signal at kinetochores **(C)** Quantitative analysis of Aurora A intensity at kinetochore. Average intensities per kinetochore were collected and

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normalized to the maximum mean from MLN condition (condition with highest values). Aurora A kinetochore values were pooled per condition and showed as box plot (n=914 kinetochores per condition), T-test on the means were performed and p-values are presented.

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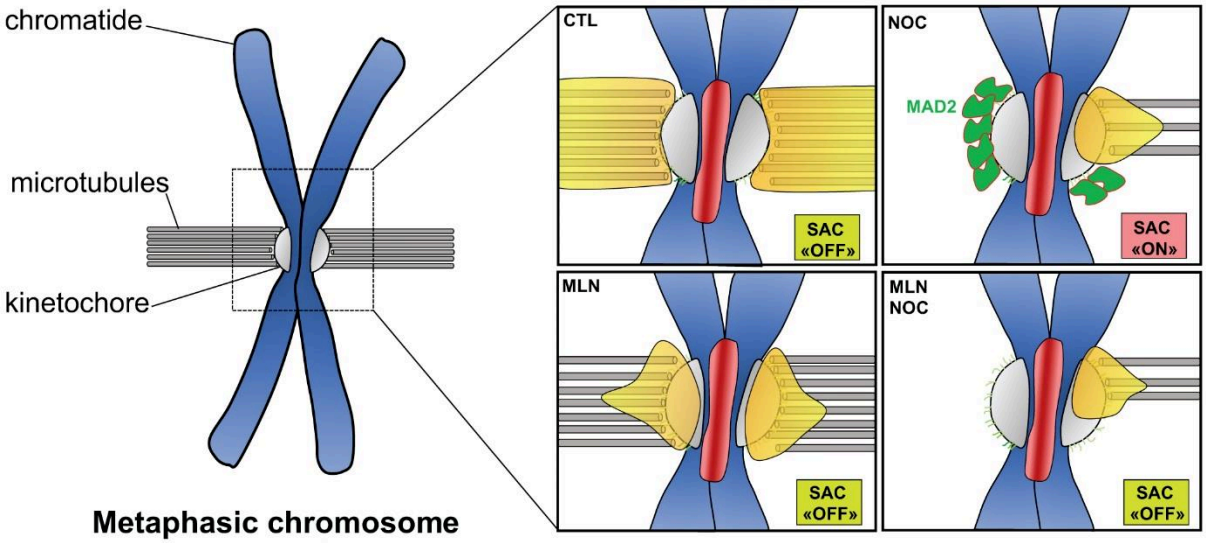

Figure 6: Schematic representation of Aurora A and Mad2 localization at mitotic chromosome. Aurora A localizes to kinetochore microtubules and outer kinetochore in cells arrested at metaphase (**CTL**- MG132), the Spindle Assembly Checkpoint is inactivated (SAC "OFF"). Under Nocodazole (NOC): Mad2 accumulates at kinetochore, the Spindle Assembly Checkpoint is activated (SAC "ON"), Aurora A accumulates at partially attached kinetochore. Under MLN and/or Nocodazole (MLN and MLN+NOC), Aurora A accumulates at outer kinetochore and Mad2 is no longer at kinetochore. The SAC is "OFF", cells are escaping from mitotic arrest.

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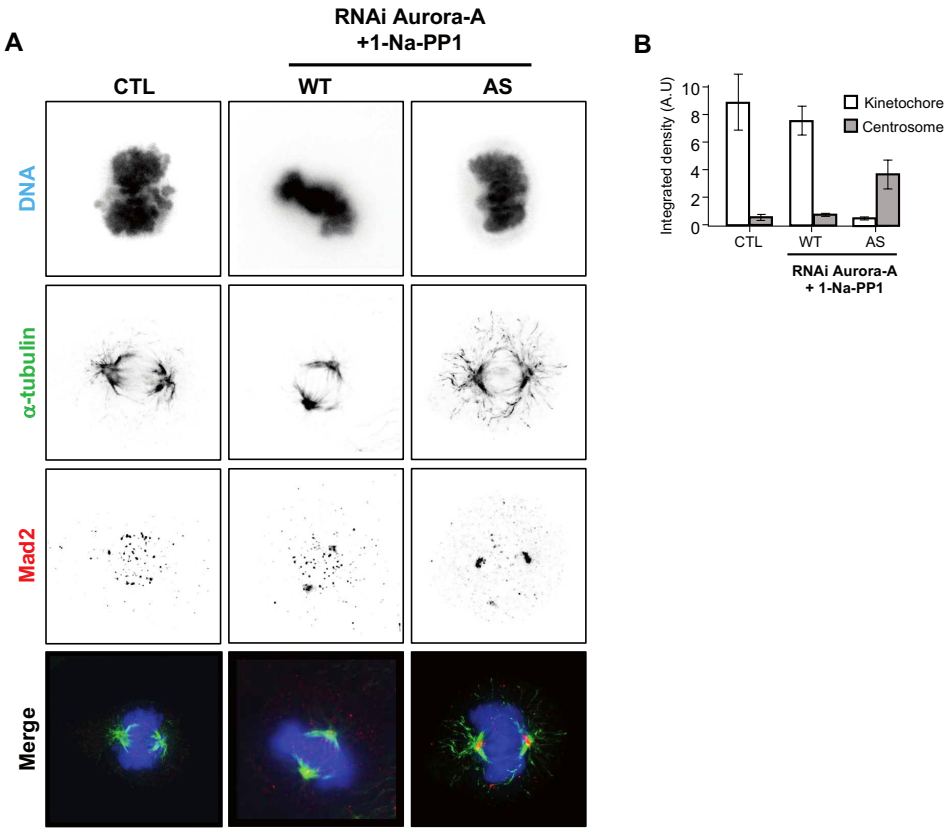

Figure Sup 1: (A) Stable control, wt-Aurora A (WT), and as-Aurora A (AS) cell lines (as indicated) immunostained to detect α-tubulin (Green and Kaplan, 2003) and Mad2 (Kollareddy et al., 2012) and counterstained for DNA with Vectashield containing DAPI during late prometaphase under Aurora A inhibition. Scale bar= 10 μ m. **(B)** The integrated density of Mad2 at the kinetochores and centrosomes in (A) during late prometaphase (CTL=14, WT+Na-PP1= 20, AS+Na-PP1= 26, error bar = s.d).

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5.3. List of genes differentially expressed upon inhibition of Aurora-A

G1 Phase						
S.NO	**Ensembl ID**	**logFC**	**logCPM**	**PValue**	**padjust**	**Gene Name**
1	ENSG00000219451	-0.781	1.479	5.50E-06	6.65E-02	RPL23P8
2	ENSG00000005102	-0.636	4.676	3.28E-12	3.98E-08	MEOX1
3	ENSG00000186487	-0.565	1.506	6.03E-06	7.29E-02	MYT1L
4	ENSG00000140451	0.528	5.409	6.43E-06	7.77E-02	PIF1
5	ENSG00000175832	0.561	5.665	7.07E-09	8.58E-05	ETV4
6	ENSG00000135318	0.575	5.189	1.23E-06	1.48E-02	NT5E
7	ENSG00000168685	0.635	6.140	3.71E-10	4.50E-06	IL7R
8	ENSG00000134061	0.640	3.163	3.03E-06	3.66E-02	CD180
9	ENSG00000244405	0.640	6.041	7.16E-08	8.67E-04	ETV5
10	ENSG00000080573	0.728	1.309	5.77E-07	6.98E-03	COL5A3
11	ENSG00000158373	0.750	3.112	6.36E-15	7.71E-11	HIST1H2BD
12	ENSG00000148677	0.769	2.053	5.02E-06	6.07E-02	ANKRD1
13	ENSG00000180573	0.921	3.896	4.41E-18	5.35E-14	HIST1H2AC
14	ENSG00000172927	0.958	2.085	1.68E-08	2.04E-04	MYEOV
15	ENSG00000184678	1.730	1.824	2.45E-19	2.97E-15	HIST2H2BE

Mitosis						
S.No	**Ensembl ID**	**logFC**	**logCPM**	**PValue**	**padjust**	**Gene name**
1	ENSG00000129173	0.347	4.003	4.14E-06	5.02E-02	E2F8
2	ENSG00000165434	0.296	4.634	3.00E-06	3.64E-02	PGM2L1
3	ENSG00000004799	0.381	5.916	2.01E-06	2.43E-02	PDK4
4	ENSG00000106459	-0.261	4.283	7.39E-06	8.96E-02	NRF1
5	ENSG00000163535	0.272	5.959	2.54E-07	3.09E-03	SGO2
6	ENSG00000123473	0.249	6.499	1.20E-07	1.45E-03	STIL
7	ENSG00000117289	0.319	6.250	2.86E-08	3.47E-04	TXNIP
8	ENSG00000143476	0.317	6.603	9.66E-10	1.17E-05	DTL
9	ENSG00000136122	0.313	5.529	4.12E-09	5.00E-05	BORA
10	ENSG00000204389	-0.265	7.624	9.26E-07	1.12E-02	HSPA1A
11	ENSG00000204388	-0.273	6.925	2.26E-06	2.74E-02	HSPA1B
12	ENSG00000137193	0.309	4.131	7.39E-06	8.95E-02	PIM1
13	ENSG00000114107	0.289	4.755	1.29E-06	1.57E-02	CEP70
14	ENSG00000199916	-0.809	2.638	6.83E-06	8.28E-02	RMRP
15	ENSG00000166532	-0.262	6.484	6.23E-06	7.55E-02	RIMKLB
16	ENSG00000167767	-0.334	7.680	2.79E-06	3.39E-02	KRT80
17	ENSG00000175063	0.278	7.263	3.11E-08	3.77E-04	UBE2C
18	ENSG00000113369	0.720	4.168	8.73E-16	1.06E-11	ARRDC3

G2 Phase						
S.NO	**Ensembl ID**	**logFC**	**logCPM**	**PValue**	**padjust**	**Gene Name**
1	ENSG00000023445	0.382	5.355	1.82E-07	2.20E-03	BIRC3
2	ENSG00000110330	0.245	5.900	9.19E-07	1.11E-02	BIRC2
3	ENSG00000188660	-0.421	4.882	1.52E-07	1.85E-03	LINC00319
4	ENSG00000136205	-0.222	6.982	4.94E-06	5.98E-02	TNS3
5	ENSG00000100297	0.247	6.679	1.18E-06	1.43E-02	MCM5
6	ENSG00000187608	0.276	4.892	7.11E-06	8.60E-02	ISG15
7	ENSG00000069424	-0.257	5.544	3.49E-06	4.22E-02	KCNAB2
8	ENSG00000186603	-0.278	5.771	2.21E-08	2.67E-04	HPDL
9	ENSG00000162616	0.272	6.431	7.24E-06	8.76E-02	DNAJB4
10	ENSG00000137965	0.400	3.400	7.40E-08	8.97E-04	IFI44
11	ENSG00000117289	0.329	6.250	2.08E-09	2.52E-05	TXNIP
12	ENSG00000134508	-0.248	5.734	3.30E-06	3.99E-02	CABLES1
13	ENSG00000141682	0.426	4.355	7.30E-08	8.85E-04	PMAIP1
14	ENSG00000122042	0.282	5.056	1.84E-06	2.23E-02	UBL3
15	ENSG00000103540	0.324	5.282	3.50E-07	4.24E-03	CCP110
16	ENSG00000005189	0.311	3.900	2.10E-06	2.54E-02	REXO5
17	ENSG00000137310	0.272	5.502	4.63E-06	5.60E-02	TCF19
18	ENSG00000174010	0.318	5.633	1.78E-08	2.16E-04	KLHL15
19	ENSG00000144711	-0.227	5.773	5.94E-06	7.19E-02	IQSEC1
20	ENSG00000182504	0.282	5.177	1.00E-06	1.21E-02	CEP97
21	ENSG00000240891	0.578	2.776	5.86E-06	7.09E-02	PLCXD2
22	ENSG00000107201	0.347	4.763	1.20E-07	1.45E-03	DDX58
23	ENSG00000107130	-0.216	6.730	5.64E-06	6.83E-02	NCS1
24	ENSG00000126882	-0.486	3.873	6.54E-09	7.93E-05	FAM78A
25	ENSG00000160360	-0.335	4.627	1.32E-06	1.60E-02	GPSM1
26	ENSG00000126838	-0.464	3.704	3.78E-06	4.57E-02	PZP
27	ENSG00000135114	0.384	5.401	1.27E-10	1.54E-06	OASL
28	ENSG00000205189	0.336	4.287	1.02E-07	1.23E-03	ZBTB10
29	ENSG00000022567	-0.285	4.675	6.42E-06	7.77E-02	SLC45A4
30	ENSG00000137449	0.385	3.400	5.50E-08	6.67E-04	CPEB2
31	ENSG00000151012	0.650	4.238	2.59E-11	3.14E-07	SLC7A11
32	ENSG00000067082	0.394	4.750	4.54E-09	5.51E-05	KLF6
33	ENSG00000196372	-0.242	5.458	5.19E-06	6.29E-02	ASB13
34	ENSG00000119922	0.515	6.067	4.47E-19	5.42E-15	IFIT2
35	ENSG00000119917	0.388	5.535	9.58E-09	1.16E-04	IFIT3
36	ENSG00000185745	0.312	5.823	3.04E-08	3.69E-04	IFIT1
37	ENSG00000155254	-0.332	6.127	1.95E-10	2.36E-06	MARVELD1
38	ENSG00000087074	0.384	4.555	3.43E-09	4.16E-05	PPP1R15A
39	ENSG00000171522	0.417	3.755	3.58E-07	4.33E-03	PTGER4
40	ENSG00000113638	0.384	4.886	3.49E-08	4.23E-04	TTC33
41	ENSG00000113369	0.289	4.168	5.00E-06	6.05E-02	ARRDC3
42	ENSG00000164463	0.571	1.672	7.99E-06	9.67E-02	CREBRF

5.4. List of alternatively spliced events upon inhibition of Aurora-A

G1 Phase			
GENE	**Ensembl id**	**EVENT-VAST ID**	**delta PSI**
CDK7	ENSG00000134058	HsaEX0014341	-32.6
KIAA0240	ENSG00000112624	HsaEX0033530	-29.37
RPS3A	ENSG00000145425	HsaALTD0005582-2/4	-28.88
ZNF311	ENSG00000197935	HsaALTA0009788-2/2	-27.84
ZNF434	ENSG00000140987	HsaINT0187232	-27.78
GREB1L	ENSG00000141449	HsaEX0028595	-25.94
PXK	ENSG00000168297	HsaEX0051387	-25.07
ZBTB48	ENSG00000204859	HsaALTD0007296-2/2	-23.08
ATG2A	ENSG00000110046	HsaALTD0000544-2/2	-22.69
RASA4B	ENSG00000170667	HsaEX0052381	-22.52
ATRX	ENSG00000085224	HsaALTA0000828-2/2	-21.92
CEP250	ENSG00000126001	HsaALTA0001799-2/2	-21.72
LL0XNC01-250H12.3	ENSG00000234405	HsaEX0035952	-21.71
ZNF160	ENSG00000170949	HsaEX0073090	-21.65
CASP10	ENSG00000003400	HsaEX0012560	-21.08
CEP192	ENSG00000101639	HsaEX0014753	-21.05
PRMT5	ENSG00000100462	HsaALTD0005064-2/2	-20.85
C9orf93	ENSG00000164989	HsaEX0011769	-20.19
SPINK5L3	ENSG00000214510	HsaALTA0008179-2/2	-19.98
RASA4B	ENSG00000170667	HsaEX0052380	-19.63
PAQR6	ENSG00000160781	HsaEX0045492	-19.53
GPR133	ENSG00000111452	HsaEX0028292	-19.24
NHSL1	ENSG00000135540	HsaEX0042887	-19.16
IQCK	ENSG00000174628	HsaEX0032131	-19.11
ANKRD13D	ENSG00000172932	HsaINT0008461	-18.79
C17orf57	ENSG00000178852	HsaEX0009779	-18.67
HDAC6	ENSG00000094631	HsaALTD0002982-2/2	-18.59
SLC20A2	ENSG00000168575	HsaEX0058833	-17.98
ZNF438	ENSG00000183621	HsaEX0073689	-17.78
GUCY1A2	ENSG00000152402	HsaEX0029064	-17.7
WDR27	ENSG00000184465	HsaEX0071045	-17.56
SENP6	ENSG00000112701	HsaALTA0007612-2/2	-17.15
RP11-326F20.5	ENSG00000233554	HsaEX0054657	-16.82
AFMID	ENSG00000183077	HsaALTA0000271-2/2	-16.77
MAGI1	ENSG00000151276	HsaALTA0004920-3/3	-16.74
PRKCD	ENSG00000163932	HsaEX0050144	-16.63
C8orf73	ENSG00000204839	HsaEX0011557	-16.5
ACCS	ENSG00000110455	HsaEX0001873	-16.38
RP11-543D5.3	ENSG00000204018	HsaEX0055000	-16.38
KREMEN1	ENSG00000183762	HsaALTD0003517-2/2	-16.37
BBS9	ENSG00000122507	HsaEX0007783	-16.24
IQCK	ENSG00000174628	HsaEX0032130	-16.22
KIF27	ENSG00000165115	HsaEX0034568	-16.21
WDR35	ENSG00000118965	HsaEX0071077	-16.16
C6orf168	ENSG00000146267	HsaEX0011283	-16.08
SLC24A1	ENSG00000074621	HsaEX0058943	-15.85
TAF6	ENSG00000106290	HsaALTD0006360-2/2	-15.82
KLHL17	ENSG00000187961	HsaEX0034771	-15.8
C4orf21	ENSG00000138658	HsaEX0011002	-15.68
AC023055.2	ENSG00000182796	HsaEX0001183	-15.59
RP1-199H16.5	ENSG00000184949	HsaEX0054292	-15.56
HES2	ENSG00000069812	HsaINT0075760	-15.55
DET1	ENSG00000140543	HsaEX0019309	-15.53
GPR133	ENSG00000111452	HsaEX0028291	-15.52
LL0XNC01-250H12.3	ENSG00000234405	HsaEX0035953	-15.49
KLHL17	ENSG00000187961	HsaALTD0003500-2/2	-15.39
MYH10	ENSG00000133026	HsaEX0040951	-15.38
PQLC2	ENSG00000040487	HsaEX6028944	-15.18
MECR	ENSG00000116353	HsaALTA0005096-2/2	-15.17
SFI1	ENSG00000198089	HsaINT0148621	-15.07
NEBL	ENSG00000078114	HsaEX0042377	-15.03
TTLL11	ENSG00000175764	HsaEX0068062	15.02

G1 Phase			
GENE	**Ensembl id**	**EVENT-VAST ID**	**delta PSI**
KLHL17	ENSG00000187961	HsaINT0088643	15.04
KIAA1958	ENSG00000165185	HsaEX0034320	15.09
RP4-694B14.5	ENSG00000213742	HsaEX0055284	15.1
ACCN3	ENSG00000213199	HsaINT0002374	15.13
WDR13	ENSG00000101940	HsaALTD0007170-2/2	15.17
CCDC24	ENSG00000159214	HsaEX0013182	15.26
BAG5	ENSG00000166170	HsaINT0016360	15.3
FDPS	ENSG00000160752	HsaALTD0002539-2/2	15.34
CTD-3107M8.4	ENSG00000254109	HsaEX0017789	15.37
MEGF6	ENSG00000162591	HsaINT0101848	15.37
ZNF343	ENSG00000088876	HsaALTD0007430-2/2	15.38
ARHGAP19	ENSG00000213390	HsaEX0005435	15.41
N4BP2	ENSG00000078177	HsaEX0041422	15.44
SLC12A3	ENSG00000070915	HsaALTA0007834-2/2	15.45
AZI1	ENSG00000141577	HsaINT0016058	15.47
ADRBK2	ENSG00000100077	HsaEX0002878	15.51
OBSCN	ENSG00000154358	HsaINT0117615	15.55
PHF20L1	ENSG00000129292	HsaALTA0006241-2/2	15.64
SYNRG	ENSG00000006114	HsaEX0063237	15.76
RGS3	ENSG00000138835	HsaEX0053396	16.05
RPS3A	ENSG00000145425	HsaALTD0005582-4/4	16.06
RBPMS	ENSG00000157110	HsaINT0138794	16.13
RP11-314N13.4	ENSG00000234684	HsaEX0054623	16.17
TPCN2	ENSG00000162341	HsaEX0066654	16.19
CLK4	ENSG00000113240	HsaEX0015705	16.41
AIG1	ENSG00000146416	HsaEX0003305	16.51
ATP9B	ENSG00000166377	HsaEX0007140	16.71
LIME1	ENSG00000203896	HsaINT0091981	16.79
POLM	ENSG00000122678	HsaEX0048986	16.89
TMEM59L	ENSG00000105696	HsaINT0169251	16.99
C4orf21	ENSG00000138658	HsaALTD0001008-2/2	17.03
FAM193B	ENSG00000146067	HsaEX0024337	17.05
CLK1	ENSG00000013441	HsaEX0015695	17.08
NCRNA00171	ENSG00000204623	HsaEX0042156	17.2
EYA4	ENSG00000112319	HsaINT0058985	17.33
FAM59A	ENSG00000141441	HsaALTA0003195-2/2	17.48
LSM12	ENSG00000161654	HsaEX0036840	17.66
SORBS2	ENSG00000154556	HsaEX0060987	17.66
RP11-206L10.11	ENSG00000228794	HsaEX0054529	17.77
FAM76A	ENSG00000009780	HsaINT0061476	17.92
PDE4DIP	ENSG00000178104	HsaEX0046247	18.73
AC110491.1	ENSG00000233280	HsaEX0001569	18.81
ATHL1	ENSG00000142102	HsaINT0014145	18.98
USP45	ENSG00000123552	HsaEX0069928	20.28
AC016683.6	ENSG00000189223	HsaEX0001115	20.39
CCDC163P	ENSG00000236624	HsaEX0013140	20.49
TRIM68	ENSG00000167333	HsaEX0067187	20.67
SUMO1	ENSG00000116030	HsaALTA0008356-2/2	20.89
STOML1	ENSG00000067221	HsaEX0062580	21.16
ZNF320	ENSG00000182986	HsaEX0073408	21.6
GLB1L	ENSG00000163521	HsaEX0027562	21.68
OSBPL6	ENSG00000079156	HsaEX0044929	22.53
C5orf33	ENSG00000152620	HsaALTD0001025-2/2	22.85
CCDC58	ENSG00000160124	HsaEX0013339	26.06
CCDC163P	ENSG00000236624	HsaEX0013139	29.74
KCMF1	ENSG00000176407	HsaALTD0003388-2/2	32.08
ZNF81	ENSG00000197779	HsaALTD0007521-2/2	33.07
LMBR1L	ENSG00000139636	HsaEX0036009	35.12
RGS12	ENSG00000159788	HsaEX0053361	36.77

G2 Phase			
GENE	**Ensemble id**	**EVENT- VAST ID**	**dPSI**
TRIM5	ENSG00000132256	HsaEX0067152	-31.87
NADK	ENSG00000008130	HsaALTD0004158-2/2	-29.22
KIAA0319	ENSG00000137261	HsaEX0033555	-28.82
EMR2	ENSG00000127507	HsaEX0022414	-25.52
UBE2D3	ENSG00000109332	HsaALTD0006976-3/3	-23.2
ZNF783	ENSG00000204946	HsaALTD0007514-2/2	-22.08
AP2A1	ENSG00000196961	HsaALTA0000521-2/2	-22.07
EMR2	ENSG00000127507	HsaEX0022413	-22.04
ASXL1	ENSG00000171456	HsaALTA0000733-3/3	-21.84
KBTBD3	ENSG00000182359	HsaEX0032926	-21.16
FDPS	ENSG00000160752	HsaALTD0002539-2/2	-20.4
RPS6KL1	ENSG00000198208	HsaEX0055664	-20.38
C12orf26	ENSG00000127720	HsaEX0009144	-20.04
EME2	ENSG00000197774	HsaINT0055870	-19.73
TLR4	ENSG00000136869	HsaEX0065159	-19.7
SLC22A4	ENSG00000197208	HsaEX0058898	-19.67
PFDN5	ENSG00000123349	HsaALTA0006201-2/2	-19.59
POLR2H	ENSG00000163882	HsaALTA0006527-3/3	-19.54
POLRMT	ENSG00000099821	HsaEX0049135	-19.53
NINL	ENSG00000101004	HsaEX0042943	-19.52
SNX21	ENSG00000124104	HsaALTD0006125-2/2	-19.5
RP11-323F5.2 (or) STX18-AS1	ENSG00000247708	HsaEX0054650	-19.39
RABGAP1	ENSG00000011454	HsaALTD0005246-2/2	-19
MAGI1	ENSG00000151276	HsaALTA0004920-2/3	-18.98
WDR27	ENSG00000184465	HsaEX0071034	-18.95
ZCWPW1	ENSG00000078487	HsaEX0072398	-18.93
C13orf38 or CCDC169	ENSG00000242715	HsaEX6039958	-18.79
KLHL5	ENSG00000109790	HsaEX0034865	-18.64
POLM	ENSG00000122678	HsaEX0048984	-18.63
ZNF839	ENSG00000022976	HsaEX0074439	-18.54
ZNF619	ENSG00000177873	HsaEX6055273	-18.31
GUK1	ENSG00000143774	HsaINT0073767	-18.15
ZNF12	ENSG00000164631	HsaEX0073008	-17.95
HCST	ENSG00000126264	HsaALTA0003916-2/2	-17.76
INSR	ENSG00000171105	HsaEX0031843	-17.54
IL6ST	ENSG00000134352	HsaALTD0003241-2/2	-17.33
KIAA0895L	ENSG00000196123	HsaINT0086232	-17.25
EIF4G2	ENSG00000110321	HsaALTA0002854-2/2	-17.19
CCDC111	ENSG00000164306	HsaALTA0001543-2/2	-17.12
NVL	ENSG00000143748	HsaALTD0004465-2/2	-17.08
NINL	ENSG00000101004	HsaEX0042944	-17.01
COL16A1	ENSG00000084636	HsaEX0016350	-16.99
SDCCAG8	ENSG00000054282	HsaEX0056726	-16.92
TRERF1	ENSG00000124496	HsaEX0067005	-16.64
OPLAH	ENSG00000178814	HsaINT0118303	-16.59
TRAF2	ENSG00000127191	HsaEX0066794	-16.58

G2 Phase			
GENE	**Ensemble id**	**EVENT - VAST ID**	**dPSI**
KIAA1529	ENSG00000197816	HsaEX0034152	-16.51
STK36	ENSG00000163482	HsaINT0160888	-16.46
ZNF91	ENSG00000167232	HsaEX0074482	-16.45
SORBS1	ENSG00000095637	HsaEX0060963	-16.42
SLC12A3	ENSG00000070915	HsaEX0058585	-16.42
VPS13B	ENSG00000132549	HsaEX0070476	-16.32
SP140L	ENSG00000185404	HsaEX0061115	-16.31
SLC12A3	ENSG00000070915	HsaEX0058584	-16.22
RHOC	ENSG00000155366	HsaALTD0005401-2/2	-16.11
DPF1	ENSG00000011332	HsaALTA0002622-2/2	-16.08
ZMYM5	ENSG00000132950	HsaEX0072921	-15.96
SMCR7L	ENSG00000100335	HsaALTA0008039-2/2	-15.88
CCDC99	ENSG00000040275	HsaALTA0001600-2/2	-15.75
WDR27	ENSG00000184465	HsaEX0071053	-15.74
KIF13B	ENSG00000197892	HsaEX0034403	-15.73
AC107883.1	ENSG00000173517	HsaEX0001547	-15.6
LIN7B	ENSG00000104863	HsaINT0092103	-15.42
NUDT18	ENSG00000173566	HsaINT0116641	-15.38
CTC-564N23.3	ENSG00000250802	HsaEX0017719	-15.35
BICD1	ENSG00000151746	HsaEX0008082	-15.31
SMUG1	ENSG00000123415	HsaALTD0006090-2/5	-15.3
MYSM1	ENSG00000162601	HsaEX0041369	-15.21
CPSF7	ENSG00000149532	HsaALTA0002142-2/2	-15.05
C8orf38	ENSG00000156170	HsaEX0011532	15.07
TASP1	ENSG00000089123	HsaEX0063698	15.1
ERBB3	ENSG00000065361	HsaEX0022952	15.12
ANKS6	ENSG00000165138	HsaALTA0000476-2/2	15.13
SLC25A35	ENSG00000125434	HsaEX0059102	15.34
TBC1D3	ENSG00000197681	HsaEX0063877	15.37
SFXN2	ENSG00000156398	HsaEX0057731	15.43
JMJD5	ENSG00000155666	HsaEX0032784	15.45
GAPDH	ENSG00000111640	HsaALTA0003560-3/3	15.46
ZNF354B	ENSG00000178338	HsaEX0073509	15.47
FYTTD1	ENSG00000122068	HsaINT0066840	15.52
ELL3	ENSG00000128886	HsaINT0055586	15.55
ZNF618	ENSG00000157657	HsaEX0074057	15.64
C4orf21	ENSG00000138658	HsaEX0010988	15.67
SLC47A1	ENSG00000142494	HsaEX0059763	15.69
C19orf54	ENSG00000188493	HsaALTD0000877-2/2	15.72
FKRP	ENSG00000181027	HsaEX6095795	15.77
PGAP3	ENSG00000161395	HsaEX0046796	15.79
CXorf23	ENSG00000173681	HsaALTD0001754-2/2	15.98
RP4-694B14.5	ENSG00000213742	HsaEX0055282	16.01
AC016683.6	ENSG00000189223	HsaEX0001115	16.36
GANAB	ENSG00000089597	HsaALTA0003553-2/3	16.38
C9orf25	ENSG00000164970	HsaALTD0001084-2/2	16.39
HCN3	ENSG00000143630	HsaINT0074496	16.41

G2 Phase			
GENE	**Ensemble id**	**EVENT - VAST ID**	**dPSI**
RCC1	ENSG00000180198	HsaEX0052881	16.42
ZNF140	ENSG00000196387	HsaEX0073057	16.42
IL17RB	ENSG00000056736	HsaEX0031359	16.52
WHAMM	ENSG00000156232	HsaEX0071476	16.54
CORO6	ENSG00000167549	HsaALTA0002099-2/2	16.54
BAG4	ENSG00000156735	HsaEX0007545	16.74
NFYA	ENSG00000001167	HsaALTA0005715-2/2	16.92
HDAC11	ENSG00000163517	HsaEX0029376	16.99
KATNAL2	ENSG00000167216	HsaEX0032908	16.99
C9orf126	ENSG00000173611	HsaEX0011617	17.04
RAB24	ENSG00000169228	HsaALTD0005228-2/2	17.3
BTBD16	ENSG00000138152	HsaEX6090913	17.5
MTHFSD	ENSG00000103248	HsaALTA0005416-2/2	17.82
BTBD16	ENSG00000138152	HsaEX6090915	17.84
TRIM66	ENSG00000166436	HsaEX0067183	18.03
LRDD	ENSG00000177595	HsaALTD0003646-3/3	18.06
AC034193.5	ENSG00000180385	HsaEX0001238	18.13
TPCN2	ENSG00000162341	HsaEX0066636	18.15
SETD4	ENSG00000185917	HsaEX0057473	18.22
SETD4	ENSG00000185917	HsaEX0057472	18.25
ACOX3	ENSG00000087008	HsaEX0001994	18.72
AURKB	ENSG00000178999	HsaALTA0000843-2/2	18.81
FAM89B	ENSG00000176973	HsaALTA0003221-2/2	19.04
C21orf51	ENSG00000205670	HsaALTD0000945-2/2	19.24
DEPDC5	ENSG00000100150	HsaEX0019268	19.29
COL4A4	ENSG00000081052	HsaEX0016557	19.37
RHBDL1	ENSG00000103269	HsaINT0140387	19.46
AC016683.6	ENSG00000189223	HsaEX0001118	19.53
MYH10	ENSG00000133026	HsaEX0040946	19.92
TASP1	ENSG00000089123	HsaEX0063699	20
MFSD2A	ENSG00000168389	HsaALTD0003898-2/2	20.02
AC087650.2	ENSG00000188825	HsaEX0001348	20.1
LYPLA1	ENSG00000120992	HsaALTA0004895-2/2	20.37
CLCN6	ENSG00000011021	HsaEX0015560	20.63
UBE2D3	ENSG00000109332	HsaALTD0006976-2/3	20.81
ASXL1	ENSG00000171456	HsaALTA0000733-2/3	21.19
ASXL1	ENSG00000171456	HsaALTD0000527-2/2	21.44
TPCN2	ENSG00000162341	HsaEX0066637	21.64
C17orf57	ENSG00000178852	HsaEX0009776	22.07
AP2A1	ENSG00000196961	HsaALTD0000374-2/2	22.07
MYO18A	ENSG00000196535	HsaEX0041047	22.61
NPHP3	ENSG00000113971	HsaEX0043533	22.66
PCNXL2	ENSG00000135749	HsaEX0046001	22.78
FAM111A	ENSG00000166801	HsaALTD0002367-2/2	23.07
DEPDC5	ENSG00000100150	HsaEX0019274	24.64

Mitosis			
GENE	**Ensemble id**	**EVENT-VAST ID**	**delta PSI**
ZNF678	ENSG00000181450	HsaEX0074168	-40.89
KIAA1529	ENSG00000197816	HsaEX0034151	-36.2
BCAS3	ENSG00000141376	HsaEX0007843	-35.8
THBS3	ENSG00000169231	HsaEX0064798	-26.52
DYRK1A	ENSG00000157540	HsaEX0021335	-26.22
GALK2	ENSG00000156958	HsaALTA0003539-2/3	-26.11
NPL	ENSG00000135838	HsaEX0043581	-25.83
GAPDH	ENSG00000111640	HsaALTA0003559-3/3	-25.8
GAPDH	ENSG00000111640	HsaALTD0002708-2/2	-23.97
NVL	ENSG00000143748	HsaALTA0005916-2/2	-23.71
ZNF691	ENSG00000164011	HsaEX0074200	-22.74
DEPDC5	ENSG00000100150	HsaALTA0002449-3/3	-22.34
DLG4	ENSG00000132535	HsaALTA0002523-3/3	-22.18
HAGHL	ENSG00000103253	HsaALTD0002961-2/2	-22.04
VEPH1	ENSG00000197415	HsaEX0070314	-21.82
WDR27	ENSG00000184465	HsaEX0071046	-21.73
WDR27	ENSG00000184465	HsaEX0071050	-21.49
SLMO1	ENSG00000141391	HsaALTD0006061-2/2	-20.95
LRRC23	ENSG00000010626	HsaEX0036545	-20.66
PSMA3-AS1	ENSG00000257621	HsaEX0000048	-20.22
SEMA4D	ENSG00000187764	HsaEX0057112	-19.91
C4orf21	ENSG00000138658	HsaEX0010993	-19.85
NPHP4	ENSG00000131697	HsaALTA0005796-2/2	-19.76
SMCHD1	ENSG00000101596	HsaALTD0000367-2/2	-19.71
AGER	ENSG00000204305	HsaEX0003069	-19.69
CSMD1	ENSG00000183117	HsaEX0017492	-19.47
LRRC23	ENSG00000010626	HsaEX0036544	-19.38
EGF	ENSG00000138798	HsaALTD0002136-2/2	-19.06
ATP2A1	ENSG00000196296	HsaEX0006867	-18.78
WDR27	ENSG00000184465	HsaEX0071048	-18.67
ANKRD33B	ENSG00000164236	HsaEX0004470	-18.52
C18orf45	ENSG00000134490	HsaEX0009897	-18.35
PLA2G6	ENSG00000184381	HsaALTA0006364-2/2	-18.27
DNAJB6	ENSG00000105993	HsaALTA0002559-2/3	-18.16
NAPEPLD	ENSG00000161048	HsaINT0109951	-18.12
ZNF30	ENSG00000168661	HsaEX0073383	-18.06
41518	ENSG00000214765	HsaEX0057274	-18.01
ZNF12	ENSG00000164631	HsaEX0073008	-17.69
PMS2CL	ENSG00000187953	HsaEX0048689	-17.51
C21orf33	ENSG00000160221	HsaALTA0001226-2/3	-17.5
GBAP	ENSG00000160766	HsaALTD0002728-2/2	-17.36
RP11-245J9.2		HsaEX0054554	-17.33
WRNIP1	ENSG00000124535	HsaALTA0009555-2/2	-17.27
ZNF354B	ENSG00000178338	HsaEX0073509	-17.16
ZNF2	ENSG00000163067	HsaEX0073164	-17.04
RP4-694B14.5	ENSG00000213742	HsaEX0055286	-16.92
ARHGEF11	ENSG00000132694	HsaEX0005606	-16.9
MTRF1	ENSG00000120662	HsaEX0040712	-16.84
PMEPA1	ENSG00000124225	HsaALTD0004889-2/2	-16.78
AC008734.2		HsaEX0000972	-16.72
LRDD	ENSG00000177595	HsaALTA0004825-2/2	-16.66
TMEM102	ENSG00000181284	HsaINT0168223	-16.09
TTC23	ENSG00000103852	HsaALTD0006874-2/2	-16.01

Mitosis			
GENE	**Ensemble id**	**EVENT-VAST ID**	**delta PSI**
FAM13A	ENSG00000138640	HsaEX0024006	-15.98
CARD9	ENSG00000187796	HsaINT0027762	-15.6
VWCE	ENSG00000167992	HsaEX0070793	-15.42
RHBDD1	ENSG00000144468	HsaEX0053432	-15.39
SCARB1	ENSG00000073060	HsaINT0145437	-15.36
TC2N	ENSG00000165929	HsaALTD0006417-2/2	-15.34
PAQR6	ENSG00000160781	HsaALTD0004574-2/2	-15.25
RBM12	ENSG00000244462	HsaALTA0007044-2/2	-15.24
FLOT1	ENSG00000137312	HsaALTD0002606-2/2	-15.22
ZNF250	ENSG00000196150	HsaALTA0009771-2/2	-15.16
KCNC4	ENSG00000116396	HsaEX0032957	-15.13
VPS13B	ENSG00000132549	HsaEX0070478	-15.13
ZCWPW1	ENSG00000078487	HsaEX0072407	15.02
SNED1	ENSG00000162804	HsaEX0060675	15.05
NPHP4	ENSG00000131697	HsaINT0115245	15.14
SLC22A4	ENSG00000197208	HsaEX0058901	15.15
CEACAM19	ENSG00000186567	HsaEX0014471	15.18
PNPLA6	ENSG00000032444	HsaEX0048735	15.27
AAK1	ENSG00000115977	HsaINT0000188	15.29
VIM	ENSG00000026025	HsaALTA0009404-2/2	15.37
RHPN1	ENSG00000158106	HsaEX0053550	15.41
C4orf21	ENSG00000138658	HsaEX0010992	15.43
C1orf113	ENSG00000214193	HsaEX0010117	15.47
SLC7A6	ENSG00000103064	HsaALTD0006048-2/2	15.47
DEPDC5	ENSG00000100150	HsaALTA0002449-2/3	15.48
SLC10A3	ENSG00000126903	HsaALTD0005939-2/2	15.58
ATF7IP2	ENSG00000166669	HsaEX0006523	15.65
LOC155060		HsaINT0093515	15.71
CCNO	ENSG00000152669	HsaALTD0001249-2/2	15.82
C1orf156	ENSG00000171806	HsaALTD0000898-2/2	15.83
LACE1	ENSG00000135537	HsaEX0035176	15.84
SMG6	ENSG00000070366	HsaEX0060452	15.89
ASB1	ENSG00000065802	HsaALTA0000705-2/2	15.89
ESAM	ENSG00000149564	HsaEX0023149	16.03
PTPRU	ENSG00000060656	HsaALTD0005176-2/2	16.04
KIAA0430	ENSG00000166783	HsaALTA0004521-2/2	16.09
NEK8	ENSG00000160602	HsaINT0112631	16.19
DDAH1	ENSG00000153904	HsaEX0018811	16.34
NUDT17	ENSG00000186364	HsaINT0116639	16.39
ZCWPW1	ENSG00000078487	HsaEX0072409	16.49
N4BP2	ENSG00000078177	HsaEX0041416	16.5
CCDC163P	ENSG00000236624	HsaEX0013139	16.64
DAPK2	ENSG00000035664	HsaEX0018512	16.66
ZMAT3	ENSG00000172667	HsaALTA0009706-2/2	16.8
NBR2	ENSG00000198496	HsaEX0041901	16.81
AARSD1	ENSG00000108825	HsaEX0000166	16.93
SEC31B	ENSG00000075826	HsaINT0146952	17.06
TPCN2	ENSG00000162341	HsaEX0066649	17.08
C5orf45	ENSG00000161010	HsaEX0011184	17.1
C17orf48	ENSG00000170222	HsaALTD0000853-2/2	17.11
BBS9	ENSG00000122507	HsaEX0007783	17.22
EPHA5	ENSG00000145242	HsaEX0022761	17.43
SLC12A3	ENSG00000070915	HsaEX0058589	17.47

Mitosis			
GENE	**Ensemble id**	**EVENT-VAST ID**	**delta PSI**
TPCN2	ENSG00000162341	HsaEX0066654	17.47
RCAN3	ENSG00000117602	HsaALTD0005342-2/2	17.5
SCRN3	ENSG00000144306	HsaALTD0005706-4/4	17.59
ENTPD5	ENSG00000187097	HsaEX0022544	17.71
RANBP17	ENSG00000204764	HsaINT0137118	17.83
ZNF383	ENSG00000188283	HsaEX0073540	17.94
TPCN2	ENSG00000162341	HsaEX0066653	17.96
LTBP4	ENSG00000090006	HsaEX0036960	18.14
TPCN2	ENSG00000162341	HsaEX0066639	18.22
ACOX3	ENSG00000087008	HsaEX0001993	18.25
NCF2	ENSG00000116701	HsaEX0041994	18.47
ADAMTS10	ENSG00000142303	HsaINT0003903	18.93
FOXP1	ENSG00000114861	HsaEX0026271	18.94
RHEBL1	ENSG00000167550	HsaEX0053494	19.12
FOXP2	ENSG00000128573	HsaEX6010003	19.39
CCDC78	ENSG00000162004	HsaALTD0001217-2/3	19.46
TPCN2	ENSG00000162341	HsaEX0066652	19.54
NFYA	ENSG00000001167	HsaALTA0005715-2/2	19.7
ACOX3	ENSG00000087008	HsaEX0001994	20.08
TTLL3	ENSG00000214021	HsaALTD0006893-2/3	20.26
NPHP4	ENSG00000131697	HsaEX0043551	20.32
DPY19L4	ENSG00000156162	HsaEX0020923	20.5
CSTF1	ENSG00000101138	HsaINT0042542	20.9
ELMO2	ENSG00000062598	HsaEX0022151	20.99
ZNF320	ENSG00000182986	HsaALTA0009789-2/2	21.13
PPM1K	ENSG00000163644	HsaEX0049500	21.14
NSMCE4A	ENSG00000107672	HsaALTD0004401-2/2	21.15
ZCWPW1	ENSG00000078487	HsaEX0072403	21.67
STOML1	ENSG00000067221	HsaEX0062579	22.03
SUMO1	ENSG00000116030	HsaALTA0008356-2/2	22.49
ZCWPW1	ENSG00000078487	HsaEX0072400	23.41
ITFG2	ENSG00000111203	HsaEX0032300	23.46
DLG4	ENSG00000132535	HsaALTD0001950-2/2	23.48
ZNF780B	ENSG00000128000	HsaALTA0009921-2/2	23.52
XRRA1	ENSG00000166435	HsaEX0071902	23.57
DLG4	ENSG00000132535	HsaALTA0002523-2/3	23.61
FAM119A	ENSG00000144401	HsaALTA0003098-2/2	23.93
OSBPL7	ENSG00000006025	HsaEX0044937	24.05
MTHFD2L	ENSG00000163738	HsaEX0040498	24.11
RNF207	ENSG00000158286	HsaEX0054018	24.19
SYTL4	ENSG00000102362	HsaEX0063329	24.36
C15orf52	ENSG00000188549	HsaINT0020995	25.92
KIF22	ENSG00000079616	HsaALTA0004589-2/2	25.93
GALK2	ENSG00000156958	HsaALTA0003539-3/3	26.52
LOC57653		HsaINT0094747	27.05
PRR5	ENSG00000186654	HsaALTA0006726-2/2	27.86
HM13	ENSG00000101294	HsaALTD0003056-2/2	32.52
BAT1	ENSG00000198563	HsaALTA0000890-3/3	35.7
AC005726.6	ENSG00000167524	HsaEX0000879	36.42

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Résumé

Titre de la thèse : Un nouveau rôle pour Aurora-A dans l'épissage des pré-ARNm

L'ADN constitue une source d'informations génétiques dans la plupart des organismes vivants. L'ADN hautement stable est transcrit en ARN hautement réactif et instable. Le pré-ARNm transcrit subit trois processus principaux avant d'être reconnu par la machinerie de traduction à décoder en protéines. Ces processus incluent le coiffage en 5 ', l'épissage d'ARN et la polyadénylation en 3'. Tous ces processus sont principalement co-transcriptionnels et contribuent à l'efficacité et à la fidélité de l'expression des gènes.

Les séquences d'ADN non-sens intervenantes sont présentes entre les séquences d'ADN codantes et sont ensuite transcrites en tant que telles à la molécule d'ARN. Ces séquences intermédiaires sont retirées de la molécule d'ARN nouvellement formée et ce processus est appelé épissage d'ARN. L'épissage des pré-ARNm engendre la diversité des protéines et joue donc un rôle important dans la détermination de la complexité d'un organisme. L'épissage d'ARN est également connu pour être important pour le développement d'un organisme, la diversité tissulaire, le cycle circadien, la santé et la maladie.

Le pré-ARNm contient des éléments qui sont des séquences courtes et conservées telles que le site d'épissage en 5 '(SS), le système 3′SS et le site de branche (BS) qui définit les introns qui doivent être reconnus et éliminés par l'spliceosome. Ce spliceosome est une machine complexe et dynamique. Les spliceosomes sont constitués de cinq protéines snRNP différentes et de leurs protéines non-snRNP associées. En plus du noyau spliceosomal snRNP, le spliceosome contient également de nombreuses sous-unités multiprotéiques pré-organisées telles que le complexe Prp19 (Prp19), le complexe Intron-Binding Complex (IBC), le complexe pré- ARNR REtention et Splicing (RES) et le complexe Exon. Complexe de jonction (EJC). Contrairement aux autres types de machines, le spliceosome n'est pas un complexe pré-assemblé. Au lieu de cela, il s'assemble par étapes sur un pré-ARNm. Tout au long de l'assemblage des spliceosomes, de nombreux réarrangements structurels et compositionnels permettent la formation de différents complexes spliceosomaux.

L'épissage alternatif des pré-ARNm est un mode de régulation des gènes et conduit à la synthèse de différents ARNm à partir d'un transcrit primaire unique et est donc responsable de la diversité des protéines chez les eucaryotes. Les isoformes de protéines provenant d'un épissage alternatif peuvent avoir différentes activités, interactions et localisation différentielle et peuvent donc remplir différentes fonctions. Il est également important pour tous les processus biologiques et détermine la spécificité cellulaire et tissulaire et le fonctionnement

normal des cellules. En fonction des conditions physiologiques, les modèles d'épissage alternatif des gènes changent constamment, permettant à un organisme de réagir aux changements de l'environnement.

L'épissage alternatif peut être réglé à l'aide des «éléments à action cis supplémentaires» et des facteurs d'épissage à action trans. Outre les éléments agissant en cis (5'SS, 3'SS et site ramifié), le pré-ARN contient également des éléments régulateurs en cis (également appelés éléments agissant en cis supplémentaires) qui sont divisés en quatre groupes en fonction de leur position dans le pré-ARNm (exon/intron) et leur fonction (amplificateurs/silencieux). Ces éléments cis-régulateurs sont ciblés par les protéines de liaison à l'ARN (RBP) appelées régulateurs d'épissage agissant en trans. Ces facteurs d'épissage agissant en trans comprennent les ribonucléoprotéines nucléaires hétérogènes (hnRNPs) et les protéines riches en sérine-arginine (protéines SR). Ces RBP faciliter ou empêcher le recrutement de composants spliceosome versdes sites d'épissage particuliers et majorly régulent l'épissage alternatif. Les défauts de la régulation de l'épissure ont été impliqués dans de nombreuses maladies.

Les hnRNPs appartiennent à une grande famille de protéines de liaison à l'ARN (RBP) impliquées dans le métabolisme de multiples acides nucléiques, y compris l'épissage alternatif, la stabilisation de l'ARNm et la régulation de la transcription et de la traduction. Les protéines SR portent le nom de leur domaine RS typique, riche en sérine (Ser, S) et en arginine (Arg , R). Ils contiennent des RRM N-terminaux, suivis d'un domaine RS en aval .

Les modifications post-traductionnelles (PTM) des protéines SR constituent un signal important qui module divers aspects des protéines SR, à savoir la localisation, l'activité, les interactions et la stabilité. On sait que la phosphorylation sur les domaines SR est responsable de leur recrutement sur le site de transcription à partir les domaines nucléaires SC35. SRPK1 est spécifique de la phosphorylation du domaine SR présent dans toutes les protéines SR. Outre les kinases spécifiques d'épissage, telles que les CLK et les SRPK, qui sont connues pour phosphoryler les protéines d'épissage et réguler leur localisation et leur fonction, il existe également de nombreuses kinases de signalisation et de cycle cellulaires qui fonctionnent également dans l'épissage. De telles kinases incluent NEK2, MAPK, GSK3β, Akt, PKA et Aurora-A. Ma thèse était axée sur l'exploration de la fonction de la kinase Aurora-A dans l'épissage pré-ARNm.

Aurora-A est une sérine/thréonine kinase connue pour réguler les événements mitotiques dans les cellules. Sa surexpression dans les tumeurs à promouvoir la survie des cellules s par modulation de la prolifération cellulaire et l' apoptose surmonter. Auparavant, il

a été démontré qu'Aurora-A est important pour la stabilité d'une protéine riche en sérine/arginine (SR), ASF/SF2, modifiant ainsi l'épissage alternatif des gènes liés à l'apoptose. Dans le but d'identifier les nouveaux partenaires et les nouvelles fonctions d'Aurora-A, Olivia Gavard et Jean-Philippe Gagné (anciens membres de notre équipe) ont identifié de nombreuses protéines d'épissage dans l'interactome d'Aurora-A. Étant donné que l'interactome d'Aurora-A suggérait son rôle dans l'épissage pré-ARNm, j'ai cherché à comprendre les mécanismes moléculaires qui sous-tendent sa fonction précise dans l'épissage.

L'interactome Aurora-A contenait 444 protéines potentiellement interactives. L'analyse d'enrichissement des partenaires supposés en interaction Aurora-A a fortement suggéré le rôle d'Aurora-A dans l'épissage des pré-ARNm. On sait que plus de 200 protéines sont associées au spliceosome à différents stades de l'épissage . De manière remarquable, l'interactome Aurora-A contenait 51 protéines d'épissage sur 263 protéines d'épissage connues. J'ai découvert que sur 51 protéines d'épissage interagissant avec Aurora-A, 11 protéines provenaient du noyau et 37 protéines des composants pas de noyau du spliceosome. L'analyse d'enrichissement relatif de l'interactome Aurora-A a indiqué que les protéines dans les complexes PRP19 sont les plus abondantes parmi le composant noyau-spliceosomal. L'analyse d'enrichissement relatif des protéines d'épissage dans l'interactome a également révélé que les protéines hnRNP et SR étaient des interacteurs importants de Aurora-A. Il est donc possible qu'Aurora-A régule l'épissage du pré-ARNm via les protéines hnRNP et SR.

À travers s in vitro de dosage pull-down, j'ai montré une interaction directe de l' Aurora-A avec l' ensemble des 12 protéines SR et protéines hnRNP (hnRNP-C2, RALY, PCBP2 et PTBP1). Étant donné que la plupart de ces protéines sont connues pour contenir un domaine commun appelé RRM, il est possible qu'Aurora-A puisse spécifiquement interagir avec ces protéines via le domaine RRM. L'analyse d'enrichissement de domaine des données d'interactivité Aurora-A a également montré l'enrichissement pour le domaine RRM. Cependant, ces résultats doivent être complétés par les études d'interaction *in vitro* avec des mutants de délétion des protéines hnRNP et SR. Étant donné que la phosphorylation joue un rôle crucial lors de l'épissage et que Aurora-A est une protéine kinase, j'ai vérifié si les protéines interagissant directement avec Aurora-A pouvaient être phosphorylées par Aurora-A à l'aide du dosage kinase *in vitro*. J'ai trouvé que les protéines d'épissage hnRNP-C2, RALY, PCBP2, PTBP1 et eIF4A3 sont phosphorylées par Aurora-A *in vitro*.

Comme Aurora-A interagissait avec de nombreuses protéines d'épissage et en phosphorylait également quelques-unes *in vitro*, j'ai examiné si les niveaux de protéines SRSF étaient affectés par la déplétion d'Aurora-A induite par l'ARNi. J'ai observé que l'épuisement d'Aurora-A entraînait en effet une diminution dramatique des taux de nombreuses protéines du SRSF, notamment l'ASF/SF2 (SRSF1) et le SC35 (SRSF2). Ce résultat indique qu'Aurora-A est important pour maintenir le niveau de «protéines SR». Cependant, il faut déterminer si ce type de régulation par Aurora-A sur les protéines SR se situe au niveau de l'ARN ou des protéines.

Les protéines d'épissage du pré-ARNm sont enrichies dans un domaine spécifique situé dans la région interchromatine du nucléoplasme, appelé «domaines nucléaires SC35», qui se présente sous la forme de 20 à 50 structures de forme irrégulière de tailles variables. Comme de nombreuses protéines d'épissage ont été identifiées dans l'interactome d'Aurora-A, j'ai décidé de déterminer si Aurora-A se localisait dans les domaines nucléaires SC35. Par le test d'immunofluorescence, j'ai montré la co-localisation de Aurora-A avec le marqueur SC35. Cela indique clairement que Aurora-A peut localiser des domaines nucléaires SC35. J'avais révélé la localisation de Aurora-A au niveau des domaines nucléaires SC35 et de sa périphérie, où l'épissage post et co-transcriptionnel sont connus pour avoir lieu respectivement. Grâce dosage d'épissage *in vitro,* j'ai montré que Aurora-A peut améliorer l'efficacité de l' épissage de β - globine pré-ARNm. J'ai examiné si l'inhibition de Aurora-A aurait une incidence sur l'épissage pré-ARNm dans les cellules en culture. L' inhibition de Aurora-A est connue pour perturber le cycle cellulaire. L'épissage pré-ARNm est également connu pour être modifié d'une manière dépendante du cycle cellulaire. D'où l'effet d' inhibition de Aurora-A dans l'épissage de pré-ARNm a été analysé dans des cellules synchronisées. J'identifie que 414 événements d'épissage ont été modifiés par l'inhibition d'Aurore-A, surtout sans effet significatif sur l'expression de leurs gènes. De manière surprenante, les protéines kinases SR importantes, CLK1 et CLK4, sont également identifiées comme étant épissées de manière alternative d'une manière dépendante de Aurora-A.

Dans l'ensemble, mes travaux ont révélé le rôle important et novateur d'Aurora-A dans l'épissage pré-ARN et ont suggéré des moyens moléculaires permettant à Aurora-A de moduler l'épissage pré-ARN.

Titre : Un nouveau rôle pour Aurora-A dans l'épissage des pré-ARNm

Mots clés : Aurora-A, Protéines SR, hnRNP, phosphorylation, d'épissage de pré-ARNm, CLK.

Résumé : Aurora-A est une sérine/thréonine kinase connue pour réguler les événements mitotiques dans les cellules. Sa surexpression dans les tumeurs favorise la survie cellulaire en modulant la prolifération cellulaire et en inhibant l'apoptose. Auparavant, il avait été montré qu'Aurora-A était important pour la stabilité d'une protéine riche en sérine / arginine (SR), ASF/SF2, modifiant ainsi l'épissage alternatif des gènes liés à l'apoptose. En cohérence avec ce rôle dans l'épissage, nous avons découvert que l'interactome d'Aurora-A contient un grand nombre de composants du spliceosome - des protéines spliceosomales centrales et non essentielles.
Mon travail de thèse a consisté à caractériser la fonction de Aurora-A dans l'épissage pré-ARNm et à étudier sa fonction au niveau moléculaire. J'ai montré qu'Aurora-A interagissait directement avec des facteurs d'épissage, tels que les protéines SR et les protéines hnRNP, et phosphorylait également quelques-unes d'entre elles in vitro. J'ai démontré qu'Aurora-A est important pour la stabilité de ASF/SF2 et de nombreuses autres protéines SR.
En utilisant la microscopie à immunofluorescence, j'ai démontré en outre qu'Aurora-A localise vers les taches nucléaires, les sites où les protéines d'épissage sont stockées, ainsi qu'à la périphérie des taches nucléaires où une majorité d'épissage de pré-ARNm est connue. L'ajout d'Aurora-A recombinant améliore notamment l'efficacité d'épissage du pré-ARNm de la bêta-globine in vitro. En analysant la méthode RNA-seq à l'aide de l'outil VAST, j'ai identifié 414 événements d'épissage alternatifs qui sont modifiés lors de l'inhibition de Aurora-A. De manière surprenante, les protéines kinases SR importantes, CLK1 et CLK4, sont également identifiées comme étant épissées de manière alternative d'une manière dépendante de Aurora-A.
Dans l'ensemble, mes travaux révèlent le rôle important et novateur d'Aurora-A dans l'épissage des ARNm et suggèrent des voies moléculaires permettant à Aurora-A de moduler les facteurs d'épissage et d'épissage..

Title A novel role of Aurora-A in pre-mRNA splicing

Keywords : Aurora-A, SR proteins, hnRNP, phosphorylation, pre-mRNA splicing, CLK.

Abstract : Aurora-A is a serine/threonine kinase known to regulate mitotic events in cells. Its over-expression in tumours promote cell survival by modulating cell proliferation and overcoming apoptosis. Previously, it has been shown that Aurora-A is important for the stability of a serine/arginine (SR)-rich protein, ASF/SF2, thereby altering the alternative splicing of apoptosis-related genes. In consistence with this role in splicing, we have discovered that the interactome of Aurora-A contains a large number of spliceosome components - both core and non-core spliceosomal proteins.
My PhD work aimed to characterize the function of Aurora-A in pre-mRNA splicing and to investigate its function at the molecular level. I show that Aurora-A interacts directly with splicing factors such as SR proteins and hnRNP proteins and also phosphorylates few of them *in vitro*. I demonstrate that Aurora-A is important for the stability of ASF/SF2 and many other SR proteins.
Using immunofluorescence microscopy, I further demonstrate that Aurora-A localizes to nuclear speckles, the sites where splicing proteins are stored, as well as to the periphery of nuclear speckles where a majority of pre-mRNA splicing is known to take place. Notably, addition of recombinant Aurora-A enhances the splicing efficiency of beta-globin pre-mRNA *in vitro*. Through RNA-seq analysis using the VAST tool, I identified 414 alternative splicing events that are altered upon inhibition of Aurora-A. Surprisingly the important SR protein kinases, CLK1 and CLK4 are also identified to be alternatively spliced in an Aurora-A dependent manner.

Altogether my work reveals a broad and novel role of Aurora-A in pre-mRNA splicing and suggests molecular means through which Aurora-A could modulate the spliceosome and splicing factors.