Study of the multifunctional nuclear scaffold protein A kinase anchoring protein 95

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Table of Contents

Acknowledgements

Now that I am truly seeing the light at the end of this very long journey, I'll stop writing for a while about AKAPs, FUS, and nuclear biology, and write instead about the people that helped me and was there all along my PhD.

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Well, it is true.

List of abbreviations

AKAP95	A Kinase Anchoring Protein 95
ALS	Amyotrophic Lateral Sclerosis
APC/C	Anaphase Promoting Complex
BioID	Biotin Identification
cAMP	cyclic Adenosyl Monophosphate
CCFs	Chromatin Cytoplasmic Fragments
FUS/TLS	Fused in Sarcoma/Translocated in Liposarcoma
G2	phase 2
γ-H2AX	Gamma-Histone 2 A X
HA95	Homologous of AKAP95
HDAC	Histone Deacetylase
hnRNP	Heterogenous nuclear Ribonucleoprotein Particle
KD	Knocked-down
KT	Kinetochore
Mad1/2	Mitotic Arrest Deficient 1/2
MCC	Mitotic Checkpoint Complex
MCM	Minichromosome Maintenance
MT	Microtubule
NE	Nuclear Envelope
NEBD	Nuclear Envelope Breakdown
NLS	Nuclear Localization Signal
NM	Nuclear Matrix
NPC	Nuclear Pore Complex
PKA	Protein Kinase A
PP	Protein Phosphatase
RNAPII	RNA Polymerase II
SAC	Spindle Assembly Checkpoint
snRNAs	small nuclear RNAs
snRNPs	small nuclear Ribonucleoparticles
TPR	Translocated in Promoter Region
UTR	Untranslated Region

List of publications

Paper I

AKAP95 interacts with nucleoporin TPR in mitosis and is important for the spindle assembly checkpoint.

Graciela López-Soop, Torunn Rønningen, Agnieszka Rogala, Bernd Thiede, Philippe Collas, Thomas Küntziger. *Cell cycle,* 2017*, under review*

Paper II

Co-association of AKAP95 and FUS/TLS on promoters of active genes

Graciela López-Soop, Akshay Shah, Philippe Collas, Thomas Küntziger

1. Introduction

The cell, the functional unit of all living organisms, is an enclosed yet dynamic system where molecules reside and interact to perform the myriad of biochemical reactions needed for its correct functioning. How can cells possess such enormous levels of complexity and yet achieve high degrees of accuracy and precision? The answer falls on a solid cellular organizing system. Throughout evolution, molecules, structures and events emerged to collectively organize and rule the structure and functions of the eukaryotic cell. Examples include organelles and membranes, where functionally related proteins reside and perform their actions; a widespread polymer network, providing structure and enabling intracellular transport and whole cell motion. Multiprotein complexes and molecular machines, such as the nuclear pore complexes; non-membranous nuclear bodies and single-layered cytoplasmic vesicles, such as promyelocytic leukemia bodies and autophagosomes, respectively; and scaffold proteins. This thesis aims to shed light on the behaviour of one such cellular scaffold protein, A kinase anchoring protein 95 (AKAP95).

1.1. Scaffold proteins

Many scaffold proteins have been discovered in the 1980s and early 1990s[1]. Choi *et al.*[1] in 1994 set the stage for the recognition of the novel family of scaffold proteins. They described the function of Sterile5 (Ste5) in the mitogen-activated protein kinase (MAPK) signalling pathway for mating in *S. cerevisiae*. The role of Ste5 had remained ambiguous over the previous years. It was known that Ste11, a mitogen-activated kinase, phosphorylated Ste7, and in turn, Ste7 activated the MAPK Fus3 or Kss1, and in this manner the signal culminated in the phosphorylation of target effectors required for mating in *S. cerevisiae*[2]. Interestingly, different kinases were shown to interact, indicating the possibility of simultaneous binding of the four components to Ste5. This study established one of the first characteristics of a scaffold protein[3], namely, the ability to assemble more than one protein on the same molecular scaffold (**Fig. 1**).

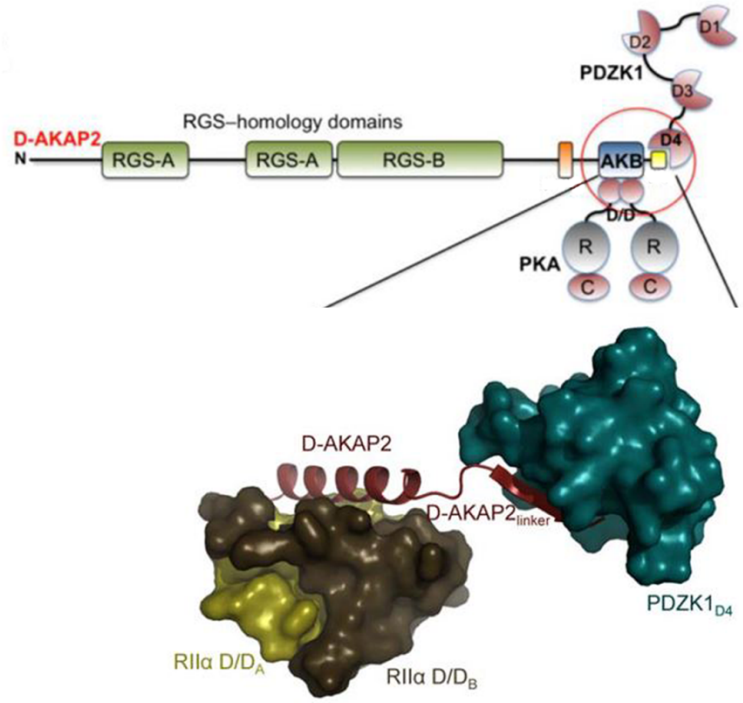

Figure 1. **Surface structure inferred from crystallographic data of a scaffold protein (AKAP2) in complex with two binding proteins (PKA and PDZK1)**. Adapted from[4].

The large family of A kinase anchoring proteins (AKAPs), exemplifies another important characteristic of scaffold proteins: the ability to localize the action of the associated (or binding) molecules to specific subcellular domains. AKAPs are a family of scaffold proteins discovered at the beginning of the 1980s[5,6]. AKAPs, were originally reported as structural proteins that bound and "anchored" the regulatory subunit of the cyclic-AMP-dependent protein kinase A (PKA) holoenzyme to different compartments of the cell[6]. PKA is a holoenzyme composed of two regulatory (R) and two catalytic (C) subunits involved in many cellular functions. The binding of two molecules of cyclic AMP (cAMP) to each R subunit of PKA catalyses the release of the C subunits. Next, released C subunits will phosphorylate nearby substrates. All AKAPs contain an R binding domain, which forms an amphipathic helix capable of binding PKA. Additionally, AKAPs harbour in their structure a targeting sequence specifying subcellular localization, where they spatially restrict PKA function and bind other interactor proteins (**Fig. 2**). For example, AKAPs can be targeted to membranes through myristoylation/palmytolation signals (AKAP18), to mitochondria (WAVE1), microtubules (MAP2), centrosome (pericentrin), perinuclear membrane

(mAKAP), nucleus (AKAP95), Golgi apparatus (AKAP220), cytoplasm (AKAP-Lbc), and to specialized cellular structures such as spermatozoid flagellar axonemes (AKAP110, AKAP82) and dendrites (AKAP150).

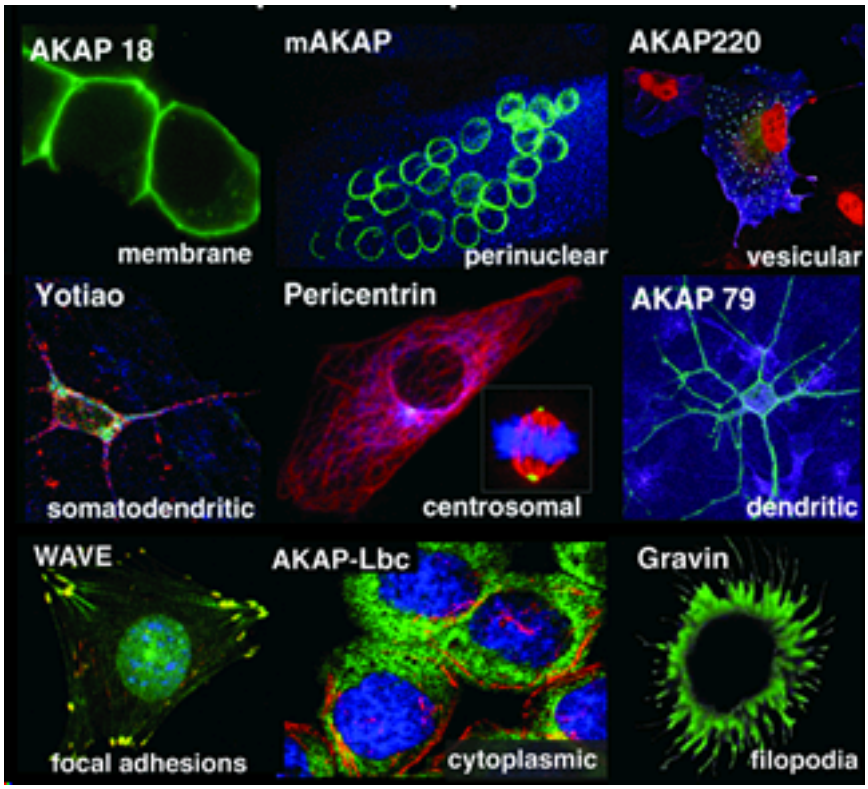

Figure 2. **Subcellular localization of different members of the AKAP family of scaffold proteins**. Taken from John D. Scott lab website (http://faculty.washington.edu/scottjdw/).

Another characteristic of scaffold proteins, in addition to their ability to coordinate and position signalling events within its appropriate cellular domain, is to confer protection from negative regulating effectors such as phosphatases. The scaffold protein IQGAP1 acting in the NFAT signalling pathway[7], is an example of this concept. In resting conditions, the transcription factor NFAT is localized in the cytoplasm heavily phosphorylated and in its inactive form in complex with IQGAP1. Upon a Ca^{2+} influx activation cue, NFAT is released from its inactivating complex with IQGAP1, and can translocate to the nucleus where it will exert its transcription factor function. Without the IQGAP1 scaffolding function, NFAT is prematurely dephosphorylated and imported to the nucleus[7]. In the same study, Sharma *et al.* show that a long-intergenic non-coding RNA (lncRNA), NRON, also played a role in providing structure and stabilization to the

NFAT/IQGAP1 complex, expanding the molecular targets scaffold proteins can interact with.

Zheng et al. went further and performed a very detailed and elegant study of the interactions and behaviour of a scaffold protein; Shc1, in the epidermal growth factor (EGF) signalling cascade[8]. They found Shc1 to make contact with 41 different protein mediators of the EGF response. Furthermore, they characterized the Shc1 phosphorylation pattern, showing how post-translational modifications are important in coordinating a signalling cascade[9]. The different pattern of phosphorylation on Shc1 will recruit different binding partners. Immediately after EGF stimulation, Erk and PI(3)K activating proteins including (1)Gab1/2, another scaffold protein; (2)Sos1/2, a Ras/Rho guanine-exchange factor; and (3) protein kinases and phosphatases (Pik3, Ptpn11, Lrrk1) bind to Shc1. Fifteen to twenty minutes after the response, another set of proteins involved in cytoskeletal reorganization and signalling termination was observed to interact with Shc1; including two different Ser/Thr phosphatases[8].
From this study, several conclusions can be drawn: (i) post-translational modifications maximize a scaffold protein´s activity and (ii) serve as accurate modulators of the response. Another important characteristic of scaffold proteins that this study exemplifies is the ability to coordinate positive inputs triggered by a signalling response and integrate the downregulating cues for proper termination of the signal. This latter function is of paramount importance for the ability to switch on and switch off, in a tightly controlled manner, cellular signalling. Scaffold proteins, consequently, constitute an important tool for the cell, and it is not surprising to find diseases where scaffold protein's action is compromised[10–14].

The large family of AKAPs also displays several examples of the consequences of disrupting the microsignaling environments they coordinate[13,14]. Yotiao (AKAP350) forms a complex with the voltage gated potassium channel (KCNQ1) and PKA in myocytes which is involved in cardiac repolarization[15]. Single nucleotide polymorphism studies have identified a single aminoacid substitution mutation (S1570L) in Yotiao which has been linked with long-QT syndrome[16].

This mutation reduces the ability of Yotiao to interact with KCNQ1. PKA, consequently, cannot fully phosphorylate the channel and this reduces its activity. Patients with long-QT syndrome, an inherited heart disease, display prolonged repolarization intervals in the heart, which can lead to cardiac arrhythmias and even sudden death. Pericentrin, an AKAP localized at the pericentriolar material of the centrosome, is involved in the microtubule organization network at interphase and at mitosis; Pericentrin mediates the formation and orientation of the mitotic spindle[17]. Loss-of-function mutations in the *PCTN* gene have been linked with the rare autosomal recessive disease Majewski/ microcephalic osteodysplastic primordial dwarfism type II (MOPDII)[12]. Patients suffering this genetic condition are characterized by extreme short stature (<100 cm), microcephaly and several bone and dental abnormalities[10]. Pericentrin interacts with numerous proteins and it is involved in multiple cellular pathways. Therefore, pericentrin dysfunction contributes to the disease onset through various mechanisms[18].

Cancer is another pathogenic condition where scaffold proteins are involved. In breast cancer, two members of the Cas protein family, p130Cas/BCAR1 and Nedd9, are overexpressed[19]. Particularly, the up-regulation of p130Cas inhibits the stimulation of SMAD2/3 by TGFβ signalling and diverts the response to non-canonical TGFβ pathways, such as p38 MAPK; a cell survival and transformation inducer, promoting growth and metastasis[20]. The mechanisms behind the up-regulation of p130Cas and Nedd9 in transformed cells are not yet clear. However, this outcome shows how scaffold proteins are important signalling transducers and how a fine tuned regulation of the cellular circuitry is necessary to maintain a stable balance between cell survival and cell arresting messaging[21].

1.2. A kinase anchoring protein AKAP95

1.2.1. General features

AKAP95 is the only member of the AKAP family of scaffold proteins localized at the nuclear interior. It is ubiquitously expressed and associates with the nuclear matrix[22]. In addition to binding the regulatory type II alpha (RIIα) subunit of

PKA[22,23], AKAP95 possesses two zinc-fingers that can bind nucleic acids[22,24] (**Fig. 3**).

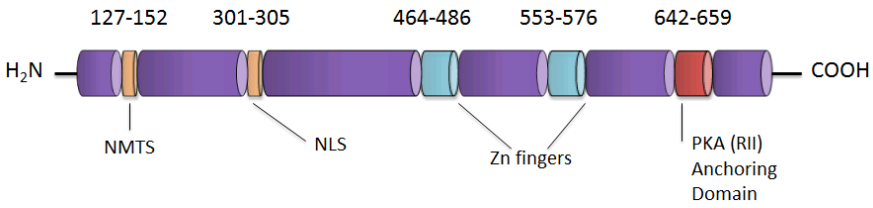

Figure 3. **AKAP95 primary structure and functional motifs**. NMTS, nuclear matrix targeting domain; NLS, nuclear localization signal.

AKAP95 has an homologous protein, HA95 (homologous to AKAP95)[25]. AKAP95 and HA95 display a 61% homology in its primary structure. HA95 contains a nuclear targeting signal and localizes to the nucleus. AKAP95 and HA95 genes are adjacent to each other (positioned at chromosome 19p13.1) arguing in favour of a gene duplication event. HA95, however, lacks the PKA anchoring domain, and therefore, does not bind RII and is not considered an AKAP protein.

1.2.2. Mitosis and cell cycle regulation

Our group found that AKAP95 was responsible for chromatin condensation at the onset of mitosis by recruiting a component of the condensin complex, hCAP-D2/Eg7, into condensing chromosomes and was important in the maintenance of condensed chromosomes through its association with PKA (RIIα)[26,27]. The Zn fingers of AKAP95 are responsible for targeting AKAP95 to mitotic chromatin and initiating the condensation process[24]. Both Zn finger domains and the RII/PKA binding domain are required to initiate and maintain chromatin condensed[24].

Another mitotic effector under the control of AKAP95 is histone deacetylase enzyme 3 (HDAC3)[28]. HDAC3 was found to form a complex with AKAP95, as well as with the homolog of AKAP95 (HA95)[25], during the G_2/M cell transition. The lack of HDAC3 abolishes the deacetylation of histone 3 residues. As a result, cells are unable to undergo mitosis normally. Li and coworkers showed that AKAP95 and HA95 are required for HDAC3 deacetylating activity and that this in turn affects the global level of histone 3 serine 10 phosphorylation (H3S10p). This

phosphorylation is catalysed by the protein kinase Aurora B and constitutes a prominent mark related to cells in mitosis[29]. Depletion of AKAP95 and HA95 resulted in reduced levels of H3S10p[28]. Overall, AKAP95 may have a role in targeting the HDAC3-Aurora B kinase complex to chromosomes for the initiation of chromosome condensation. However, the domain of AKAP95 interacting with HDAC3 has not been elucidated.

In line with AKAP95 role in mitosis, another type of events coordinated by AKAP95 are its association with distinct types of cyclins and the mini chromosomes (MCM2) proteins. Cyclins are important regulators of cell cycle progression[30] and work by Arsenijevic *et al.* uncovered AKAP95 interactions with cyclins D and E[31,32]. AKAP95-cyclin interaction only took place when CDKs (the catalytical protein partner of cyclins) were not binding cyclins. Interestingly, cyclin D, a G1/S cyclin, was found to co-immunoprecipitate along with AKAP95 and RIIα, suggesting the formation of a complex coordinated by AKAP95. MCM2 was reported to be another binding partner of AKAP95 and it was shown that AKAP95 and MCM2 are involved in the initiation of DNA replication at G1 as well as S phase[33]. The binding domain involved in this interaction is localized at the N terminal side of AKAP95. These results underscore a consistent link of AKAP95 with DNA replication and division processes, as AKAP95 is implicated in different stages of the cell division cycle, from DNA replication and elongation in S phase, to chromosome condensation in mitosis.

1.2.3. Transcription and differentiation regulation

A yeast two-hybrid screen unveiled the association of AKAP95 with RNA helicase p68 at the nuclear matrix in rat brain interphase nuclei[34]. The authors also mapped AKAP95 nuclear localization signal (NLS) and the nuclear matrix targeting sequence (NMTS) to residues 301-305 and 127-152, respectively. The NMTS is highly conserved among AKAP95 orthologues and it is essential for the binding to p68. This result suggests a potential role for AKAP95 in transcriptional processes. However, the functional relevance of this interaction was not addressed in this study.

Likewise, a binding partner purification screen uncovered AKAP95 as a novel dpy-30 interacting partner[35]. Dpy-30 is a protein shared by all members of the mixed-lineage leukemia (MLL) family of histone 3 methyltransferase protein complexes. Jiang *et al.* showed that AKAP95 co-immunoprecipitated with several subunits of the MLL2 complex. In fact, overexpression of AKAP95 and MLL2 stimulated by 30.000 fold the expression of a luciferase reporter gene. Similarly, a 50% increase of histone 3 lysine 4 di- and tri-methylation (H3K4me2/3) at the promoter region of the luciferase locus was observed on cells overexpressing AKAP95 compared to control cells. Interestingly, neither the matrix-targeting domain nor the RII-binding domains of AKAP95 are necessary for interaction with dpy-30 and for its role in transcription. The authors tested the role of AKAP95 in the all-*trans* retinoic acid (ATRA)-mediated developmental differentiation of human embryonic carcinoma cells (ECCs) and mouse embryonic stem cells (mESCs). AKAP95 depletion led to a marked decrease in the induction of several developmental genes including *IGFBP5, HAND1, MSX1* and *HoxC6* on both human ECCs and mESCs. H3K4me3 levels were diminished at the promoters of the corresponding target gene loci. Additionally, gene ontology analysis of the differentially expressed genes in ATRA-mediated differentiation revealed that neural differentiation was one of the gene categories most affected in both dpy-30 and AKAP95-depleted cells. These results show a direct link between AKAP95 and transcription regulation and suggest that different pools of AKAP95 might be acting in different transcriptional complexes and different gene expression programs[35].

In another study, AKAP95 was found as part of the Oct4 interacting network in mouse embryonic stem cells (ESCs) by affinity purification capture and mass spectrometry identification[36]. However, the role of AKAP95 in the Oct4 signalling network was not further investigated in this study[36]. The putative AKAP95:Oct4 interaction was not confirmed biochemically either; therefore, these results should be taken with care. Jiang and colleagues[35] did not detect any significant change on expression levels of several pluripotency genes including Oct4 on mESCs depleted for AKAP95.

1.2.4. RNA metabolism processes

Some studies are highlighting a role of AKAP95 in RNA metabolic processes. Two independent RNA-binding proteins identification screens identified AKAP95 as part of a human RNA-interactome[37,38], and another group found AKAP95 as a novel splicing protein in an affinity purification screen of spliceosomal complexes[39]. A study addressed a putative role of AKAP95 in the stability of the lactate dehydrogenase subunit A (LDH-A) mRNA[40]. The authors showed that AKAP95 was responsible for anchoring PKA at the 3' untranslated regions (UTR) site of the LDH-A mRNA[40], affecting the stability of the LDH-A mRNA. Depletion of either AKAP95 or PKA resulted in decreased LDH-A mRNA stability, leading to lower LDH-A mRNA levels and diminished protein production. It is interesting to note that formation of this complex would take place in the cytoplasm, as AKAP95, PKA and the LDH-A mRNA, were purified from ribosomal protein extracts in rat glioma cells[40]. Also, a link between AKAP95 and rRNA production has been established after observing a subpopulation of AKAP95 localized at nucleoli during interphase[41]. Marstad and colleagues found a close localization of AKAP95 with the nucleolar upstream binding factor, together with two other nucleoli markers (RPA43 and fibrillarin)[41]. Moreover, chromatin immunoprecipitation experiments revealed AKAP95 association with ribosomal encoding genes. Inhibiting RNA polymerase I or II (RNAPII) activity resulted in a specific recruitment of AKAP95 to the periphery of nucleoli, suggesting that nuclear distribution of AKAP95 is dependent on transcriptional activity. Furthermore, an inverse correlation between AKAP95 and ribosomal transcripts was established after siRNA mediated knock-down of AKAP95. The 47S and 18S ribosomal subunits were upregulated in AKAP95 depleted cells. Conversely, overexpression of AKAP95 resulted in decrease levels of 47S and 18S transcripts. Collectively, these reports seem to set the stage for a less characterized role for AKAP95 in RNA pathways.

1.2.5. Cell-type specific and other functions of AKAP95

In other cellular types, several associations have been found for AKAP95, including with the phosphodiesterase (PDE) 4 in Jurkat cells[42], fidgetin in NIH/3T3 cells during mouse embryonic development[43] and p105 in RAW cells[44]. Phosphodiesterases are cAMP hydrolysing enzymes. In Jurkat cells, PDE4 immunoprecipitated AKAP95 and an *in vitro* interaction between AKAP95 and PDE4 was also demonstrated. These preliminary results suggest the ability of AKAP95 to interact with a PDE, and possibly, with PKA within the same complex[42]. Following the role of AKAP95 in immune cells, AKAP95 was found to mediate the prostaglandin E2 induced-attenuation of the inflammatory response driven by TNF-α expression in activated macrophages. Inhibition or disruption of AKAP95-PKA complex resulted in loss of prostaglandin E-suppressive effects. Notably, the authors point to a less recognized role of AKAP95 in the cytoplasm through anchoring PKA type II with p105, a cytoplasmic NF-kB modulator in the LPS-induced inflammatory response. The authors proposed that the AKAP95-p105 interaction domain might be contained within the NMTS and NLS of AKAP95, thus retaining a fraction of AKAP95 in the cytoplasm. Immunoprecipitations using RAW cells (mouse macrophages) cytosolic extracts confirmed the AKAP95-p105-PKA complex formation outside the nucleus.

RSK1, a downstream kinase of the ERK1/MAPK signalling pathway, was reported to be another AKAP95-binding partner in HeLa cells[45]. In particular, upon epidermal growth factor (EGF) stimulation, RSK1 is phosphorylated at Ser221 by PDK1 and subsequently translocates to the nucleus. A mutant form of AKAP95 that cannot localize to the nucleus resulted in decreased nuclear accumulation of activated RSK1 after EGF stimulation. Gao and colleagues proposed that AKAP95 mediates retention of RSK1 at the nucleus, allowing the exertion of its nuclear activities.

Another protein partner described to bind AKAP95 is the ATPase fidgetin. The authors obtained co-association by immunoprecipitation experiments and mapped the interaction domain to the C-terminal end of AKAP95. AKAP95 was highly expressed during mid-gestation in mouse embryonic development,

overlapping the fidgetin expression profile. In fact, when the authors of the study generated fidgetin and AKAP95 knock-out mice, some presented cleft palate phenotypes, and succumb shortly after birth[43].

These results expand AKAP95's roles to developmental and differentiation programs. Altogether, there are several indications converging to a description of AKAP95 as a multifunctional cell-cycle scaffold protein providing support to numerous nuclear biology processes and various more functions still to be uncovered (**Fig.4**).

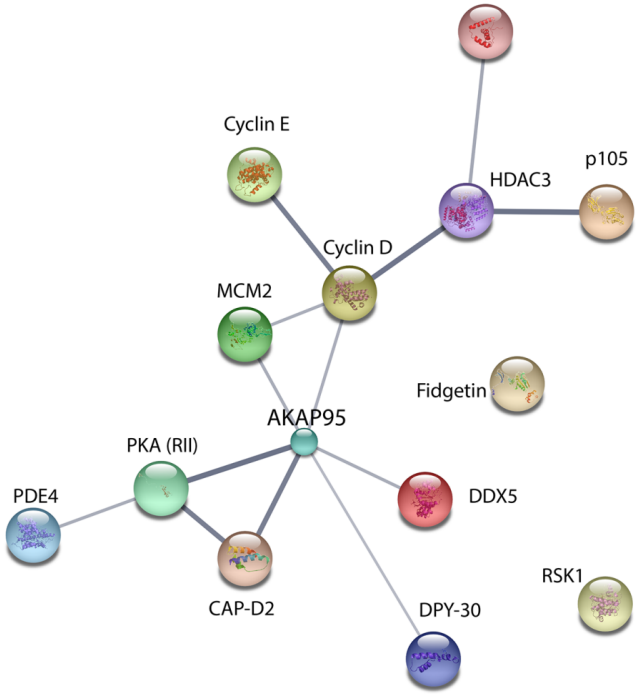

Figure 4. **AKAP95 interacting partners network from published reports**. Created using String platform (www.string-db.org).

1.3. TPR, a multifunctional protein of the nuclear pore complex

1.3.1. General overview

Translocated in *Promoter Region* (TPR) is a 267-KDa coiled-coil protein [46,47] localized at the nuclear basket of nuclear pore complexes (NPCs)[48] (**Fig. 5**). TPR homologs are widely distributed across the eukaryotic lineage. Examples of the most studied TPR homologs include Mlp1/Mlp2 (*S. cerevisiae*), Megator (*D.*

melanogaster) and NUA (*A. thaliana*). TPR is anchored to the NPC at the outer-most nuclear side by nucleoporin (Nup)153[49] and it can project towards the nuclear interior up to 350 nm. siRNA knockdown of Nup153 releases TPR from the nuclear periphery to the nuclear interior[49]. Likewise, TPR is one of the last nucleoporins to be disassembled and reassembled at the beginning and end of mitosis[49]. Notably, TPR has been found localized at discrete subdomains at the nuclear interior[50], often forming clusters at the periphery of nucleoli[48], as determined by EM studies.

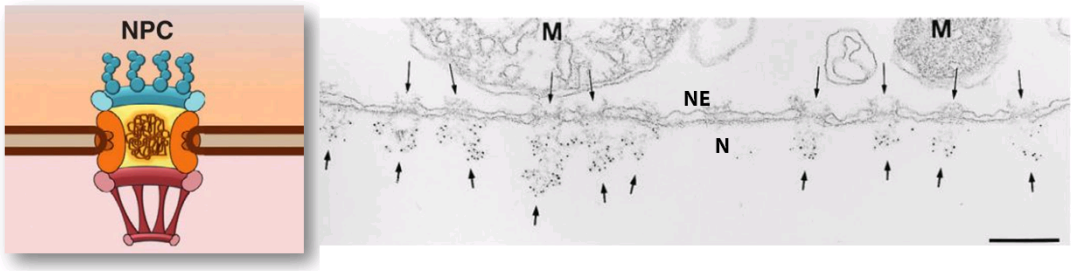

Figure 5. **TPR is localized at the nuclear basket of nuclear pore complexes (NPC).** Cartoon illustrating nuclear pore structure embedded in the nuclear envelope (left). Electromicrograph of *Xenopus* oocytes displaying a section of the nuclear envelope (Right). Nuclear interior black dots labelling TPR molecules. M, mitochondria; NE, nuclear envelope; N, nucleus. Images adapted from [51] and [48].

1.3.2. RNA and protein export

NPCs are the access and exit gates of the nucleus. A myriad of molecules and components are constantly shuttling in and out the nucleus. Therefore, it is not surprising that the first role of TPR to be uncovered was protein[50,52] and poly(A)mRNA[52] nucleocytoplasmic transport. Indeed, poly(A)mRNA was observed to significantly accumulate when TPR was blocked using specific antibodies. A study proposed TPR as a "gatekeeper" for mRNAs with retained introns (mRNAs that had not undergone proper intron splicing). TPR silencing resulted in fast export of mRNAs with retained introns that exploit the Nxf1/Nxt1 mRNA exit pathway[53,54]. This phenomenon was shown to be dependent on TPR's localization to the nuclear pore basket mediated by Nup153[54]. At the same time, proteins which harbour a nuclear export signal (NES), including p53, were substantially affected when TPR was knocked-down

and showed a marked nuclear accumulation[55,56]. Exportin-1 and CRM1 being the major protein-export receptors of proteins harbouring a NES[57]. TPR was implicated in this process interacting with CRM1 through NES-containing peptides[58].

1.3.3. Chromatin organization

TPR has additionally been shown to be involved in chromatin organization and metabolism. The Drosophila TPR homolog, Megator (Mtor), was reported to bind up to 25% of the genome. Mtor, together with its binding partner Nup153, covered 42% of the Drosophila genome. The regions covered by the two nucleoporins were described to be enriched in active transcriptional marks such as H4K16 acetylation[59] and with RNA polymerase II occupancy, comprising large chromatin domains ranging from 10kb to 500kb. While many of the nucleoporin-binding loci were located at the nuclear periphery as expected, a small pool of loci was localized at the nuclear interior. This finding supports the hypothesis for the less explored intranuclear role of TPR. In a study conducted in HeLa cells infected with poliovirus, TPR was shown to be responsible for maintaining heterochromatin exclusion zones around the NPC[60]. Chromatin at the nuclear periphery is generally found in a repressed state except for chromatin localized at the vicinity of NPC, which is found in a less-compact state. When TPR was silenced by RNAi, heterochromatin exclusion zones were lost and densely packed chromatin extended across the nuclear envelope[60].

In yeast, Mlp1 and Mlp2, have been implicated in telomere length control[61]. Mlp mutants show for each gene a 50bp increase in telomere length compared to controls. Furthermore, a study conducted by Zhao *et al.* implicated Mlp1/2 indirectly in DNA repair through desumoylation perturbation. Mlp1/2 anchors the desumoylating enzyme Ulp1 at the nuclear periphery. Delocalization of Ulp1 from the nuclear envelope resulted in "nibbled" colony-morphology growth. Similar results were obtained when Mlp proteins were silenced. Ulp1 desumoylating capacity is lost upon its delocalization from the nuclear envelope and sumoylated proteins accumulate in the *Mlp* double mutants[62].

1.3.4. Other nucleoporin functions and dysfunctions of TPR

TPR has also been linked with the extracellular-receptor kinase 2 (ERK2), an important transcription factor residing in the cytoplasm in its inactive form. Upon emission of a mitogenic extracellular signal, ERK2 is activated and is subsequently translocated to the nucleus. The authors showed that TPR and ERK2 interact via four ERK2 phosphorylation sites on TPR (Thr2102, 2123, 2200 and Ser2142). They also observed that after siRNA-depletion of TPR, ERK2 did not translocate to the nucleus following EGF stimulation[63]. Additionally, TPR pull-downs contained ERK2 phosphorylated substrates, suggesting that TPR anchors and restricts ERK2 phosphorylation activity to the nuclear periphery local environment. These findings extend the role of TPR in contributing to the localized actions of a transcription factor.

TPR has interestingly been implicated in pathologies caused by mutations in nuclear envelope proteins. Progeria is a rare genetic disorder characterized by premature aging (e.g progeria and other progeroid syndromes), among others pathological manifestations. Specific mutations in the *LMNA* gene are the precursors of this disorder. It has been observed that TPR nuclear envelope localization is lost in fibroblasts from progeria patients[64]. Furthermore, import of TPR mediated by the large protein cargo receptor Importin-α (also called karyopherin-α) is defective in cells containing Progerin[65] (the mutated lamin A protein). In these conditions, TPR cannot exert its many nuclear functions and the progerin phenotype is consequently exacerbated.

1.3.5. TPR in the spindle assembly checkpoint

TPR depletion results in several mitotic defects including chromosome lagging, micronuclei and multinucleated cell formations[66–69]. Additionally, it is well established that TPR interacts and anchors mitotic arrest deficient proteins (MAD) 1 and 2 at the nuclear envelope during interphase[66–68,70]. TPR-MAD1 interaction is dependent on the phosphorylation status of TPR[68]. Some groups argue that TPR/MAD1-2 interact during mitosis too[66,67,71]. However, TPR's

precise role during mitosis together with the spindle assembly checkpoint (SAC) components MAD1 and MAD2 still remains an open question with as many answers as complexity layers the SAC response possess. For instance, the assumption that TPR associates with MAD1 and MAD2 proteins during mitosis[66,67] and particularly, at kinetochores[66] (KTs), has lately been questioned[70,71]. Schweizer *et al.* did not observe large differences in MAD1 kinetochore localization between TPR-depleted cells and controls. Neither did they detect an interaction of TPR with MAD1 outside of interphase, nor presence of TPR at KTs. On the contrary, they did observe a marked decrease in the kinetochore MAD2 pool upon TPR silencing[71].

Along the same line, Rodriguez-Bravo *et al.* reported that neither MAD1, MAD2 or any other SAC component, was displaced from KTs upon TPR knockdown. Instead, they propose a model where TPR is important for a pre-mitotic checkpoint complex (MCC) assembly[70]. This would provide a new view for TPR-MAD1/2 scaffolding, where its functional interaction might take place during interphase and its effect exerted at mitosis. If this is true, however, it again raises the initial question of the role of TPR during mitosis. Other groups, however, have confirmed a TPR/MAD1/2 interaction at mitosis and the recruitment of this complex to KTs[67]. The initial question of TPR's role during mitosis might be unravelled by extending the list of identified interacting partners of TPR during mitosis, with the dynein complex (dynein, dynactin and DLC) proposed as a novel TPR binding partner[67], together with Aurora kinase A[69]. These last observations require further investigation for a better understanding of the roles of TPR s in mitosis.

Altogether, TPR, a nucleoporin residing at the nuclear side of NPCs, plays various nuclear homeostatic roles but also, and of great interest; a small nucleoplasmic pool of TPR has been identified and should be the subject of intense and compelling future research.

1.4. FUS/TLS: The DNA and RNA binding multifunctional protein

1.4.1. General overview

Fused in Sarcoma/Translocated in Liposarcoma (FUS/TLS) is a 75KDa multifunctional nuclear matrix protein[72] expressed in all human tissues. FUS belongs to the nucleic acids binding-FET family of proteins[73], comprising the members FUS, Ewing sarcoma breakpoint (EWS) and Tata-binding associated factor (TAF15). FET members are conserved across all multicellular organisms. FET proteins share a common domain structure consisting of a transcriptional activation domain (comprised of LC and RGG domains) localized at the N-terminal part and different RNA and DNA binding domains localized adjacently across their C-terminal part[74] (**Fig. 6**).

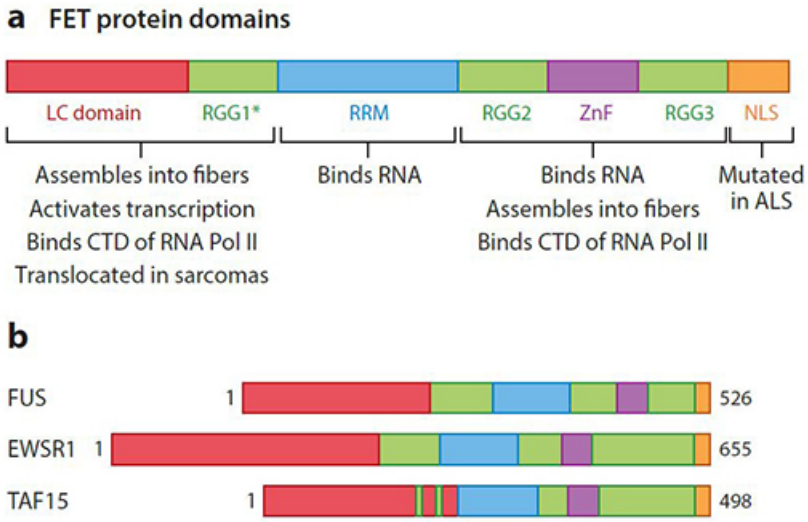

Figure 6. **Schematic representation of FET proteins and their structures**. Taken from[73].

Translocations of the transcriptional activation domain of FET members with the DNA-binding domains of different transcription factors have been reported in a large number of human sarcomas and leukemias[75]. In fact, FUS was originally identified as a fusion protein with the transcription factor CHOP in myxoid liposarcoma[74]. Furthermore, certain FUS point mutations have been observed in patients with neurodegenerative disorders[76], especially in familial amyotrophic lateral sclerosis (ALS). The majority of these mutations tend to be clustered at

the NLS of FUS, impeding its nuclear import. FUS expression in cerebral tissue is among the highest (www.gtexportal.org) and several reports support the bivalent hypothesis of a nuclear FUS loss-of-function combined with a toxic gain-of-function in the cytoplasm to explain the pathological mechanisms triggered by FUS mislocalization mutations in ALS patients.

1.4.2. FUS as a transcriptional regulator

FUS is extensively associated in the literature with transcription regulation. FUS has been shown to interact with RNA polymerase II (RNAPII) in numerous reports[77–81], as well as with general transcription factors (TF) including transcription factor II D (TFIID)[77], TAFII 100[77] and Tata binding protein (TBP)[82]. Some members of the nuclear receptor family have also been shown to be associated with FUS; retinoid X receptor, estrogen receptor, thyroid hormone and glucocorticoid receptor[83] . Additionally, interactions with gene-specific TFs have been reported for FUS, for instance with the p65 subunit of NFkB[84], RUNX family transcription factors[85] and SPI-1/PU.1[86] (a myeloid lineage transcription factor that plays a role in both transcription and splicing).

Chromatin immunoprecipitation studies coupled to promoter microarrays, revealed that FUS approximately associates with 1,000 human promoters[87], and up to 10,000 transcription start sites (TSS) using sequencing[80]. FUS binds to single-stranded DNA[87] through its N-terminal domain[88] and preferentially associates at the TSS[80] of active regions of chromatin[88]. FUS has been implicated in RNAPII pausing[80]. Depletion of FUS resulted in an increased occupancy of RNAPII at the TSS of many genes. Serine 2 phosphorylation of the C-terminal domain (CTD) tail of RNAPII was increased at the vicinity of the TSS when FUS is depleted, suggesting that FUS specifically prevented phosphorylation of CTD at serine 2. Additionally, the authors showed by an *in vitro* pull-down assay that the ability of FUS to interact with the CTD of RNAPII was independent of nucleic acids binding.

FUS regulates gene expression both positively[84,85,87,88] and negatively[80,82,85,87]. Tan and colleagues observed both induction and reduction of some FUS-bound genes mRNAs upon FUS depletion[87]. In another study, FUS overexpression resulted in repression of RNA polymerase III-transcribed genes[82]. In mouse cortical neurons, close to 200 genes show altered expression[89], all of which are involved in signalling cascades and metabolism. However, other studies report no major differences in gene expression in $FUS^{-/-}$ mouse brains[90]. A limited number of differentially expressed genes has been observed when FUS is knocked down[80,91]. Interestingly, inhibition of RNAPII activity results in a dramatic relocalization of FUS from its normal homogenous nucleoplasmic distribution to a nucleoli-associated pattern[92]. This suggests that FUS might be actively recruited from the nucleoli to chromatin, and that this activity is dependent on RNAPII function.

FUS research in gene expression shows variable results. This might be due to the fact that FUS main role may not lie in gene transcription *per se* but more in bridging gene expression and mRNA processing and maturation, as accumulating research is starting to view these two processes as coupled together[93].

1.4.3. FUS in mRNA splicing

The spliceosome is a multi-megadalton macromolecular machine in the eukaryote cell. It is constituted of 5 small nuclear RNAs (snRNAS) (U1, U2, U4, U5 and U6) and hundreds of splicing proteins which together form small nuclear ribonucleoprotein particles (snRNPs)[94] that assemble on pre-mRNAs .
Gene expression and RNA splicing (the excision of introns from pre-mRNAs) are intimately related in eukaryotes and both processes occur simultaneously[95]. There are several examples of proteins that perform dual functions in both DNA transcription and mRNA splicing, including the transcription cofactor TAT-SF1, SKIP, proteins involved in coupling transcription to mRNA export such as Aly or UAP56 and many others[96]. Mounting evidence suggests that FUS belongs to the same category[73,76,81,89,97].

An increasing number of reports are showing new FUS binding proteins with roles in mRNA editing[72,81,86,98,99]. A FUS interactome screen identified around 30 FUS interactors all involved in RNA processes[98]. For instance, heterogeneous nuclear ribonucleoproteins (hnRNPs)[98,100], U1 small nucleolar RNA (U1 snRNA)[81,98], general splicing factors[98] (SFSR1, SFSR3) and poly-adenylation factors such as CPSF-160, among others[98].

Functional studies have been carried out that reveal the role FUS plays in RNA splicing. For instance, FUS has been shown to promote the transcription of the 9S splice variant of the EIA adenovirus gene[86], favouring the 5´most distal splicing site. Reed´s lab reported that FUS bridges the essential U1 snRNA splicing factor with RNP2[81]. Furthermore, splicing of CMV-Ftz[81] and β-globin pre-mRNAs[72] in FUS-depleted cell extracts was inactive after 30 and 90 minutes incubation, respectively. In line with these findings, knocking down FUS protein levels resulted in 250 significantly altered splicing events in HeLa cells[98], and up to 3000 altered exons in mouse primary cortical neurons[89].

FUS has been shown to be associated with poly(A) RNA[74,101]. In a genome-wide RNA immunoprecipitation study, Hoell and colleagues found 7,000 FUS RNA targets in HEK cells[102]. Lagier-Tourenne and colleagues obtained similar numbers (~8,000 and ~6,000 genes) in mouse brains and human brain cortex, respectively[97]. Using photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) data, Hoell *et al.* revealed that approximately 80% of FUS signal was localized at intronic regions, preferentially binding near splice acceptor sites[102]. Ishigaki and colleagues observed FUS concentrated at 3'UTR and intronic regions in the mouse brain, supporting previous observations[102], as well as preferring genes with alternative start/end sites[89]. Furthermore, FUS-dependent mRNA regulation primarily affects genes encoding important proteins for neuronal function in cells derived from nervous tissues[89–91,97] and RNA metabolism proteins in HeLa cells[98]. FUS binding to RNA appears to follow a *trans*-regulation. Attempts to identify FUS binding motifs in RNA failed or gave variable targeting sequences[89,102,103]. Instead, secondary structure recognition seems to be determining FUS binding[89,102]. FUS has

recently been associated in survival of motorneuron (SMN) regulation[98]. SMN proteins are essential for the biogenesis of snRNPs. SMN proteins reside mainly at the cytoplasm. However, a minor fraction is localized as intranuclear aggregates, named Gems. Nuclear extracts from HeLa and neuronal cells showed selective co-immunoprecipitation of FUS and SMN proteins. FUS and SMN interaction was notably enhanced in neurons compared to HeLa cells, in spite of having similar protein levels in both cell types. Furthermore, FUS and SMN interaction appears to be RNA-dependent. Truncations of the NLS of FUS result in markedly decreased levels of Gem bodies in the nucleus and decreased interaction with components of the U1-snRNP. The authors suggest that FUS is sequestering SMN in the cytoplasm through its respective RGG/Tudor interaction motifs, thereby disrupting its nuclear functions. Altogether, there is a large body of data pointing FUS as an important factor in pre-mRNA maturation.

1.4.4. Additional roles of FUS

FUS most notable roles are in DNA transcription and RNA splicing in the nucleus. However, some studies have collected data that show FUS being involved in mRNA transport in the cytoplasm[104,105]. In particular in mouse brain, FUS has been observed to localize throughout neuronal dendrites[104–106]. FUS recruitment and mRNA molecules at post-synaptic spines is notably enhanced after metabotropic glutamate receptor (mGluR) activation[104,105]. Furthermore, FUS somatic translocation appears to be actin and microtubule dependent, as cytoskeletal depolymerizing drugs markedly reduced FUS signal at dendrites. The authors reinforced this statement by showing no major change of RNA density in neuronal dendrites after mGluR stimulation in the absence of FUS[105]. Specifically, the authors identified a FUS target involved in actin filaments growth; Nd1-L. Nd1-L recruitment to dendrite spines was greatly increased after mGluR stimulation. In the absence of FUS, Nd1-L dendrite localization was drastically inhibited[105]. Additionally, a screen for FUS mRNA targets in the cytoplasm of motoneurons identified hundreds of candidates[103]. These results point to a FUS-dependent cytoplasmic mRNA transport of a subset of genes, a process that is essential for local protein translation and synaptic plasticity in nerve cells[107].

An emerging new function for FUS is its role in DNA repair, genome stability[108] and the DNA damage response (DDR)[109]. FUS was shown to play a role in both homologous recombination (HR) and non-homologous end joining responses to double stranded DNA breaks affecting both proliferating cells and primary mouse cortical neurons[109]. It was further shown that induction of DDR signalling was impaired in FUS KD conditions, as exemplified by decreased numbers of foci of phosphorylated H_2AX (γH_2AX), 53BP1 and p-ChK2 (mediators of the DDR). Furthermore, FUS seems to be one of the earliest members in the DDR to be recruited to double-stranded DNA breaks. Lack of FUS results in the absence of HR effectors, such as pATM and NBS1, at sites of double-stranded DNA breaks. The authors of the same study showed the interaction of FUS with HDAC1. Remarkably, inducing DNA damage resulted in a notably stronger interaction, suggesting that both proteins might be involved in the DDR pathway. When FUS was knocked-down, HDAC1 levels in DNA damage sites were reduced, as was its occupancy in double stranded DNA break repair foci. The authors did not reveal the specific role FUS plays together with HDAC1 in DDR. Nevertheless, these data support an additional role for FUS in mediating DNA repair responses.

1.4.5. FUS proteinopathies: Amyotrofic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS)/Lou Gehrig´s disease, is a fatal neurodegenerative disorder affecting primarily motor neurons. Progressive weakening and atrophy of voluntary skeletal muscles characterize ALS. The majority of ALS cases arise sporadically, however, 15% are of familial inheritance[110]. 5% of familiar ALS cases[111,112] are caused by mutations in the coding sequence of FUS[113,114]. ALS-associated mutations in FUS tend to be clustered at the C terminal site in its NLS [76]. FUS nucleocytoplasmic shuttling is disrupted and ALS patients show FUS cytoplasmic aggregates[115]. Considering the large number of physiological functions where FUS plays a role (**Fig.7**), it is not surprising that loss of its compartmentalization leads to detrimental consequences for the cell. In fact, a large amount of studies supports a loss-of-function/gain-of-function hypothesis for FUS mysregulation[76,88,98,109]. For instance, the double-stranded DNA repair pathway is impaired in cells where FUS harbours some ALS mutations[109]. FUS ALS-mutant forms R521G and R495X

shift from euchromatin bound to nuclear soluble [88]. On the other hand, mutations that disrupt the NLS of FUS significantly increased its interaction with SMN in the cytoplasm and conversely, decreased the nuclear Gem bodies[98], important components of the biogenesis of spliceosomal factors. Similarly, FUS mutants were not able to rescue many of the altered splicing events resulting from FUS depletion. The RNA-binding properties of ALS dysfunctional FUS proteins are also found altered[102]. In this light, mutant FUS bound predominantly to the 3'UTR site of genes in contrast to intronic regions, and targeted a much lower fraction of mRNAS compared to wild-type FUS.

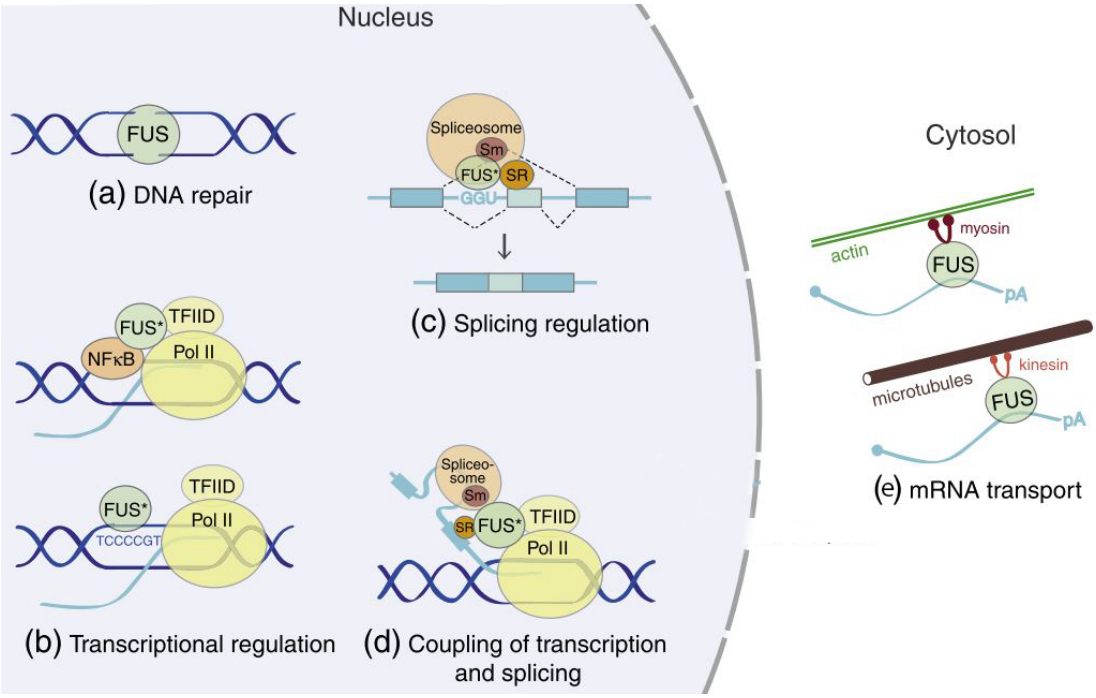

Figure 7. **Overview of FUS nuclear and cytoplasmic functions**. Adapted from [76].

1.5. The cell cycle: general overview

The cell is the basic vital unit of all living organisms[116,117]. The ultimate purpose of cells is to survive, both as single units and as components of larger cellular organizational arrangements called tissues. Similar to any other organism, cells possess a "life cycle" where they periodically go through the different stages of their so-called cell cycle; named gap1 (G1), synthesis (S), gap2 (G2) and mitosis (M). The cell cycle is commonly divided into two main phases; **interphase**-the

time period in between cellular divisions- and mitosis –the actual cell division. In interphase, chromatin is predominantly loosed and decondensed, and in mitosis, chromatin is condensed and chromosomes are distinguishable under the microscope[118].

1.5.1. Interphase: Chromatin is loose and highly active

Interphase is the longest stage of the cell cycle. Almost all vital functions of the cell occur during interphase. Interphase comprises G1, S and G2 phases (in most, but not all, cells). The transition to each of these phases is regulated by a family of protein kinases; the cyclin-dependent protein kinases (Cdk) together with their regulatory binding partners, cyclins. Additionally, cells can enter a "resting" condition named G0, where they exit the cell cycle and thus stop dividing. This cell cycle stage is normally triggered by extracellular conditions and many terminally differentiated mammalian adult cells such as neurons and skeletal muscle cells are found in G0. At G1, the cell goes through a series of checkpoints that will determine its commitment to replication of nuclear DNA. During S phase, the cell synthesizes a whole new copy of its genome, and G2 phase is devoted to the last cell growth and cytoplasmic organelles duplication before entering mitosis. Furthermore, the cell monitors the structure of the newly replicated DNA, making sure there are no major replicative errors, in which case, the cell might pause and spend time in G2 to allow DNA repair to take place. Once DNA integrity requirements are met, chromatin will start condensing and the cell will proceed to mitosis[119].

1.5.2. Mitosis: Segregation of genetic information and cellular division

The first reports on cellular division date back to the 17th century. At the same time, Hooke and Van Leeuwenhoek using the light microscope first observed the cell. However, it was not until the work of Walther Flemming, at the end of the 19th century, that the process by which a single cell gives rise to a multicellular organism was unveiled[120,121] (**Fig. 8**).

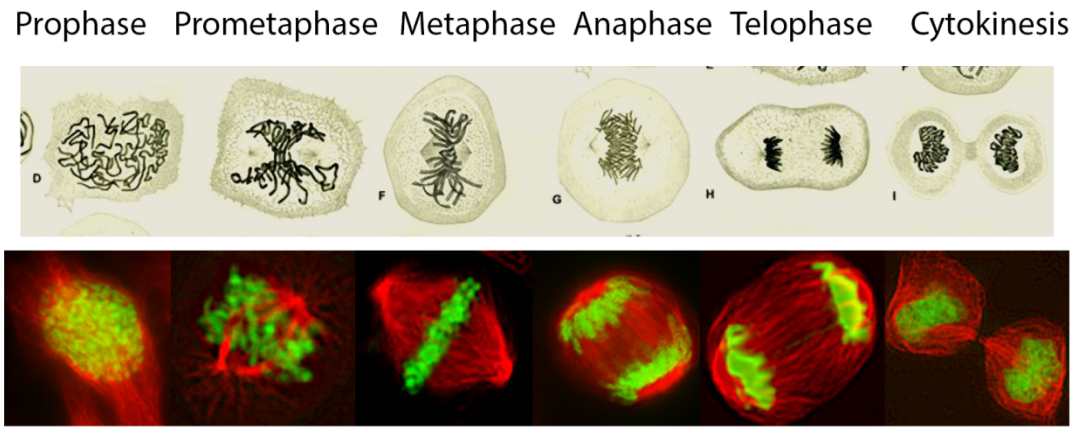

Figure 8. **Phases of eukaryotic cell mitosis**. Walther Flemming illustrations (1882) (up) and confocal microscopy images (down) of cell division. Modified from [121] and [122].

As Flemming accurately depicted in his early cellular division representations, mitosis can be subdivided into five well-distinguishable phases: prophase, prometaphase, metaphase, anaphase and telophase[123]. All of these phases are characterized by the condensation of chromosomes and the formation of the mitotic spindle. At prophase, interphase chromosomes start to condense into physically visible entities. At the same time, the two duplicated pairs of centrioles move to opposite poles of the cell and will start generating the mitotic spindle. Prophase ends when the nuclear envelope disassembles and prometaphase ensues. Condensed chromosomes can now be reached by the mitotic spindle microtubules emanating from the opposing spindle poles. Chromosomes are attached to spindle microtubules through a highly specialized proteinaceous structure named kinetochore (KT), localized at chromosome centromeres. The balance between tension forces arising from each side of the spindle, places chromosomes at the equatorial plane of the cell. Metaphase starts once all chromosomes are placed and aligned at the equatorial plate to form the metaphase plate and all kinetochore-microtubule attachments have taken place. Anaphase begins with the rapid degradation of cohesin rings holding together both sister chromatids. Microtubules of the mitotic spindle start to depolymerize and hence pull chromosomes towards the spindle poles. At telophase, chromosomes have reached their destination poles and chromatin starts to decondense. The nuclear envelope reforms and the cell undergoes cytokinesis, a

process where the cytoplasm containing the two resulting daughter nuclei progressively contracts in the middle, giving rise to the two independent daughter cells.

Time spent in mitosis can vary extensively between cell types and between developmental stages. For instance, yeast cells have very dynamic cell cycles, dividing every 90 minutes under normal growth conditions, whereas many laboratory cell lines have 24h cell cycles and divide within 1 hour. On the contrary, many adult cells cease to divide or only divide occasionally when needed (tissue maintenance, for instance). Blastomeres found at the early embryonic stages, however, possess the fastest rates of cell division[118] (albeit these cells show a reduced and thus, faster, cell cycle, where S/M phases alternate).

The Spindle Assembly Checkpoint (SAC) as the mitotic proofreader

At the background of faithful chromosome segregation, lies the spindle assembly checkpoint (SAC) response, an intricate machinery with the main purpose of preventing chromosome distribution errors[124,125]. The SAC impedes premature chromatid separation through assembly of the mitotic checkpoint complex (MCC) at unattached kinetochores. The MCC will remain active until all KT-MT attachments have taken place. The KMN network links centromeric chromatin with spindle MTs and is formed by the association of several protein complexes (KNL1-C, MIS12-C, NDC80-C). In the absence of MT binding, Aurora B will phosphorylate several subunits of the KMN network. Next, the SAC kinase Mps1 together with polo-like kinase 1 (Plk1)[126] will be recruited and further phosphorylate the MELT domains of KNL1. This, in turn, will serve as new docking points for the assembly of the MCC components, including Bub3, Bub1, Bub1R, Mad1, Mad2 and Cdc20. The collective action of the MCC is to inhibit Cdc20 from binding and activating the Anaphase Promoting Complex (APC/C), an E3 ubiquitin ligase. Free APC/C will target Cyclin B and Securin for proteosomal degradation. Securin degradation will release Separase, which in turn cleaves cohesin rings, allowing anaphase onset (**Fig. 9**).

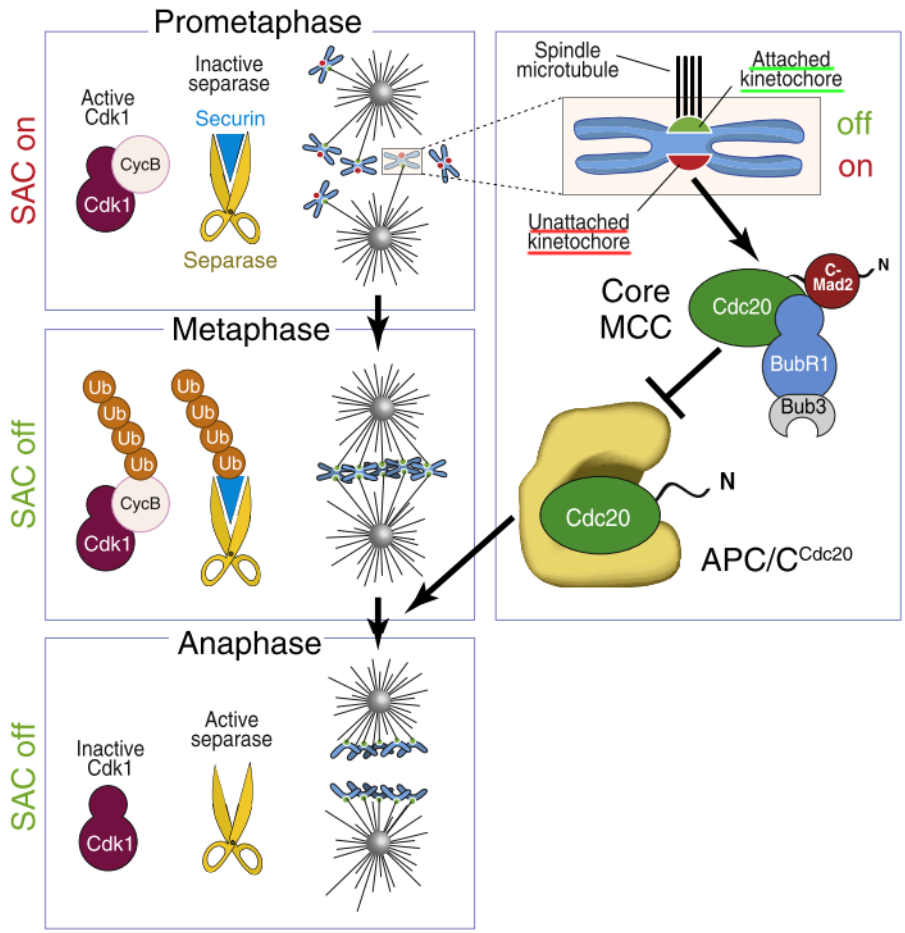

Figure 9. Simplified overview of the mechanistical aspects of the spindle assembly checkpoint (SAC) response. Adapted from [124]

The mitotic checkpoint (or SAC), therefore, safeguards cells from aneuploidy[127] (a condition given when cells inherit an abnormal set of chromosomes). Aneuploidies that arise during embryonic development are lethal[128] (except sex chromosomes aneuploidies and trisomy of the chromosome 21, which is the cause of Down syndrome. Individuals with Down syndrome have normal lifespans, albeit display severe cognitive impairments). The fatal character of constitutional aneuploidies (arising from meiotic errors of germ cells) exemplifies the vital role of inheriting the correct set of genetic material, especially during development stages. Possibly, the cause of its high lethality is an imbalance of the inherited genetic dosage[129].

Aneuploidies of sex chromosomes, however, might be less lethal due to the lower genetic content at these chromosomes (<1% of nuclear DNA) and to the genetic dosage compensation ability of extra X chromosomes (X inactivation). Nevertheless, gain or loss of whole chromosomes at gamete formation is often linked to non-disjunctive divisions and to chiasmatic problems during meiosis I[128]. Homolog chromosomes pair with each other during meiosis I, and homologous recombination takes place. A decreased recombination rate is often associated with human trisomies.

An intriguing question is that while all SAC proteins are found in mouse oocytes and seem to be functional, yet aneuploidies rates are higher in meiosis of germ cells (specially maternal) than in mitosis of somatic cells[130]. This could be explained, partly, by fundamental differences between meiosis and mitosis. There are two chromosomal divisions in meiosis compared to one in mitosis. Various studies have monitored SAC ability to stall mitosis under improper KT-MT attachments and found that SAC is less sensitive in oocyte meiosis[131–134]. For example, the SAC response does not need all homologous chromosomes to be paired at meiosis I of mouse oocytes to be satisfied[132,133]. On the other hand, aneuploidies that arise in somatic cells are a common genetic alteration in many cancers[135]. Effects associated with defective mitosis include DNA damage, p53 activation, oxidative stress, proteotoxicity (stress elicited by protein misfolding), proliferation defects and in some cases tumorigenesis[136]. The effects caused by somatic aneuploidy rather than favouring a cellular unviability phenotype tend to promote tumour formation. For instance, different types of leukaemia have been shown to have extra copies of chromosome 8, which harbours the protooncogene MYC[137]. Therefore, amplification of a protooncogene or loss of a tumour suppressor gene might be contributors to malignant transformation. Altogether, containing the correct karyotype is vital for the proper functioning of the cell and the ultimate survival of the organism. Therefore, cells devised a mechanism for keeping euploidy at almost constant levels and minimizing aneuploidy.

1.5.3. Transcription and epigenetic marks

Gene transcription is a vital cellular process that takes place throughout the cell cycle[138]. Gene transcription (or gene expression) is the way cells convert passive hereditary information contained in chromosomes, into an active and functional cellular metabolism orchestrated by proteins. In eukaryotic cells, the DNA encoded within genes in chromosomes is first transcribed into a long RNA molecule that after subsequent steps of splicing and maturation will exit the nucleus and will dictate the sequence of the protein polypeptide chain it codes for. Ribosomes, in the cytoplasm, are the molecules that assemble the polypeptide chain. RNAPII is the main enzyme responsible for the transcription of protein-coding DNA sequences into messenger RNA (mRNA) molecules. RNAPII does not act alone but requires the presence of multiple transcription-associated proteins and factors, that specify, stabilize and aid in the initiation of DNA transcription[139]. Collectively, this group of proteins is termed the pre-initiation complex (PIC) and is comprised of numerous factors and protein complexes[140]. The PIC usually assembles throughout a couple of hundreds of nucleotides upstream of the transcription start site (TSS). This region is termed the gene promoter, which on average spans 1-2kb upstream of the TSS. Promoters contain numerous structural and chemically modified patterns on nucleosomes that help proteins of the PIC and other factors to assemble onto them and initiate transcription. Other factors involved in gene transcription include enhancers and cell type-specific transcription factors (TFs). Enhancers bind to distal sequences from the genes they regulate and help to recruit and stabilize the PIC. Cell type-specific TFs perform a similar function; however, TFs bind closer to the promoter region of the gene they regulate and they also show cell-type specific expression.

Chromatin epigenetic marks are combinations of chemical groups (methyl and acetyl groups as the most studied ones) added to specific aminoacid residues found at different positions of histone protein tails. Correlation studies have observed that particular combinations of these three factors (chemical group, residue and position) are enriched in distinct types of chromatin. For example,

actively transcribed genes are enriched at their promoter regions in trimethylated histone 3 on lysine 4 (H3K4me3), and at their gene bodies in trimethylated H3K36 and H3K79. Constitutively silent genes are enriched in H3K9me3 and facultative heterochromatin is enriched in H3K27me3. Distal regions where enhancers bind to are marked by H3K4me1 and H3K27ac[141]**.** Altogether, histone post-translational modification marks serve as another regulatory layer of gene expression and chromatin architecture and are involved in transcription, DNA repair and condensation processess[142]. Thereby, epigenetic marks are considered to be essential to provide functional meaning to the genetic code.

1.6. Features of nuclear architecture

1.6.1. Nuclear Envelope

The contents of the nucleus and the processes that occur within (except for red blood cells that lack a nucleus) are kept physically separated from the cytoplasm by two lipid bilayer membranes: the inner (INM) and outer nuclear membranes (ONM); together with its integral and associated proteins constitute the nuclear envelope (NE)[143] (**Fig. 10**). The NE not only acts as the nucleocytoplasmic physical barrier but is also involved in a large number of essential nuclear functions. The NE is the framework that provides structure to the nucleus and performs activities including regulation of nucleocytoplasmic transport, gene expression, chromatin organization, genome integrity and nuclear division. These functions are the result of the interplay of NE proteins, such as Lamins and nucleoporins, with chromatin[144]. Furthermore, chromatin at the nuclear periphery is generally heterochromatic, except, remarkably in areas in proximity to NPC which consists of largely decondensed chromatin[145].

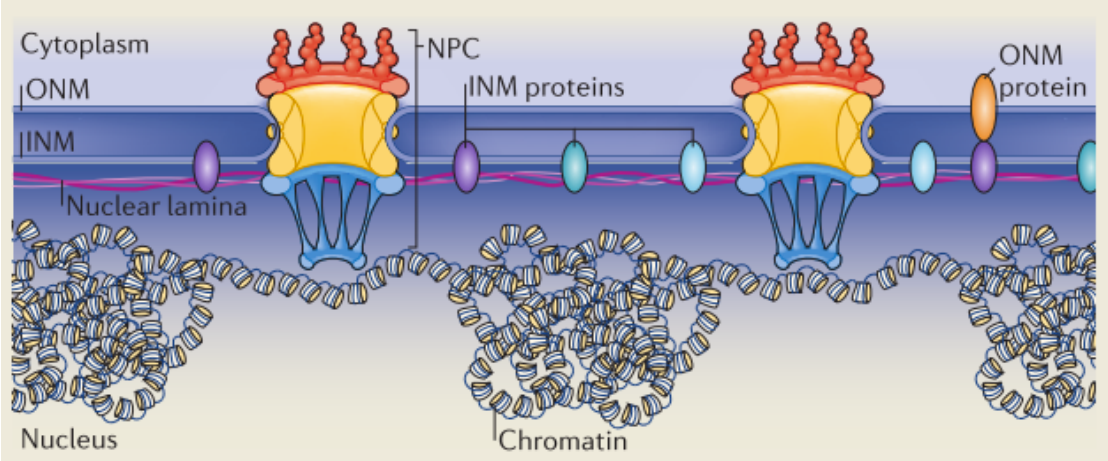

Figure 10. **Nuclear envelope representation with its principal components**. Taken from[146].

1.6.2. The nuclear lamina

The nuclear lamina lies right beyond the INM and forms a protein meshwork covering the entire nucleoplasmic-facing surface of the INM[147]. The product of two genes; *LAMIN A* and *LAMIN B*, constitutes the building blocks of the peripheral nuclear Lamina meshwork. *LAMIN A* gene products (LAMIN A and C) are anchored to the INM through their farnesylated C-terminal domains[148].

In the past, Lamins were mostly thought of being passive structural membrane proteins that functioned in nuclear morphology maintenance[149]. However, the discovery of mutations in the *LAMIN A* gene leading to distinct pathophysiological disorders[150], strongly contributed to changing the classical view of Lamins to a more sophisticated one, where Lamins are also important for gene expression and differentiation[151]. In fact, Lamins have been shown to associate through extensive domains with chromatin[152], to be associated with heterochromatin-rich regions[153], to interact with transcription factors and signalling molecules[154] and to be involved in the spatial organization of chromosomes[152]. All these attributes converge to make Lamins signalling and chromatin organizing nuclear platforms.

1.6.3. Nuclear Pore Complexes

Nuclear pore complexes (NPCs) are multiprotein channels embedded throughout the NE that mainly regulate the transit of nuclear and cytoplasmic macromolecules. NPCs are formed by multiple copies of 30 different nucleoporins (Nups). Overall, NPCs consist of a cytoplasmic-facing ring, an inner central channel, a nuclear ring and filaments that protrude from each ring[155] (**Fig. 11**). The nuclear ring filaments are further connected to a second ring in the nucleoplasm named 'nuclear basket'. The number of NPC per cell ranges from 2 to 8 per μm^2 as accounted in C2C12 cells[145] (although in other cell types or cell stages it might vary). Small molecules or proteins (<30-40KDa) can freely transit through the pore. Proteins or particles larger than 40KDa need an active shuttling in and out the nucleus through the association with different nuclear transport receptors with the NPC[146,156]. Some of these, such as NXF1 and CRM1, target mRNA and rRNA molecules to the NPC, others such as the THO-TREX complex, couple mRNA transcription to RNA export[157] and even function in RNA processing before export[158].

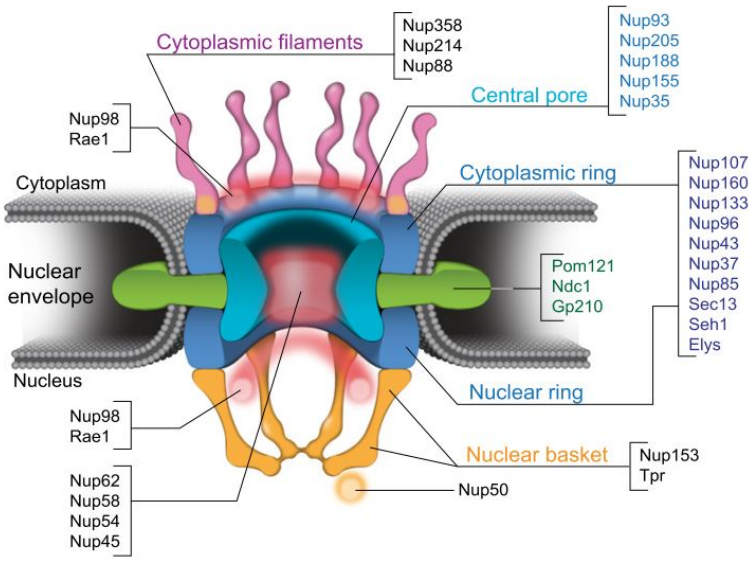

Figure 11. Schematic of the nuclear pore complex structure and nucleoporins composition and localization. Taken from[159].

NPCs have been shown to be involved in chromatin organization, gene expression and cell differentiation[146]. For instance, TPR, a component of the nuclear basket, establishes heterochromatin-free zones around the NPC[60].

Temperature and nutrient-inducible yeast genes, are tethered to the NPC by means of specific DNA sequences[159,160]. Mlp1 (*S. cerevisiae* orthologue of TPR) has been shown to maintain chromatin loops between the promoter and termination sites of *HXK1*, a gene involved in glucose metabolism, as means of gene memory for rapid re-expression[161]. Various Nups have been shown to bind to distinct chromatin regions in *D. melanogaster,* covering silent and actively transcribed loci[59,162–164]. Additionally, other Nups have been shown to bind cell cycle and developmental gene loci in *Drosophila*[163]. Increasing evidence supports the novel idea that the set of Nups that compose the NPC is cell type and cell-stage dependent[165–168]. In fact, NUP50 and NUP210 have been shown to be important for myogenic differentiation of C2C12 cells and its depletion abolished myotube formation[169–171]. Additionally, NUP210 was further found to play a role in neural differentiation programs[169] as well as NUP133[167]. Furthermore, NUP133 was shown to have a differential expression during mouse embryonic development; especially at neuroepithelium and paraxial tissues. Nucleoporin 153 was reported to be important in maintaining pluripotency in mESC through targeted inhibition of lineage-specific genes[168].

These observations are changing the status of Nups from passive building blocks of NPCs to dynamic and important cell cycle and cell differentiation modulators. Overall, it is not surprising that NPCs are involved in a large number of nuclear processes given the versatility of its structure, its localization and properties.

1.6.4. Nuclear Matrix

The nuclear matrix constitutes the biochemical fraction of the nucleus resistant to detergents, nuclease and high-salt extraction. It is thought to constitute an insoluble filamentous network inside the nucleus, structurally resembling that of the cytoskeleton in the cell cytoplasm[172].

Visualization using electron microscopy on high-salt extracted cells was the only method that yielded images of a dense fibrous-like structure[172]. Studies have rather focused on unveiling the protein composition of the nuclear matrix, and several proteins, including AKAP95[26,173], Megator (*D.melanogaster* TPR orthologue)[174], FUS[72,173], Lamins[173] and hnRNPs[173], have been shown to be part

of the nuclear matrix. However, the NM is starting to be seen as a dynamic structure with an invariable 'core' of proteins and another variable set of NM-associated proteins that will differ depending on cell stage and cell type[174,175].

On the other hand, there is no robust evidence to define a clear role for the NM. Some reports propose the NM to act as a platform that coordinates and provides support to essential nuclear functions such as DNA replication[176], DNA transcription, DNA repair[177], RNA splicing[178] and chromatin remodelling[179]. Some studies aimed to collect information on putative matrix attachment regions (MARs) on DNA. MARs can be classified as constitutive, providing a structural role of DNA anchoring and nuclear architecture maintenance, and facultative, where MARs are involved in cell-type specific transcription or origin of replication. A report using two different extraction protocols (one harsher, using high-salt extraction and another milder using LIS) on HeLa cells found that high-salt extracted MARs are associated with intragenic, gene poor and silenced genes and that less-stringency extraction conditions, yielded MARs overlapping expressed genes[180]. However, no consensus sequence motif was found and rather, authors investigating the association of DNA to the NM, proposed it might be a 3D-structural regulation.

An interesting role for the NM in initiation of replication was elegantly exemplified by a study of Radichev and colleague where they treated late G1 cells with DNase I to remove all loose, DNA loop and non-NM-associated DNA contacts and subsequently incubated DNAse I-treated cells with S-phase nuclear extract to induce replication. Cells were able to initiate replication, indicating that origins of replication are associated with the DNase-resistant insoluble fraction of the nucleus[181].

Collectively, NM investigation indicates that this structure might be involved in multiple routine nuclear metabolism tasks as means of giving structure and support between nucleic acids and its protein effectors.

2. Aims of the study

AKAPs are fundamental signaling hubs distributed throughout the cell needed for the precise and specific coordination of the local targets they regulate. AKAP95 is a nuclear scaffold protein that plays various roles within the nucleus. It assists chromatin condensation as well as DNA transcription and replication. AKAP95 associates to a larger extent with the nuclear matrix and to a lesser extent with chromatin. Little is known about AKAP95 association with DNA or its chromatin distribution. This thesis aims to:

i) Investigate novel binding partners of AKAP95 and their function

ii) Elucidate AKAP95 genome-wide promoter occupancy sites and the genomic landscape of AKAP95

3. Summary of publications

Paper I: AKAP95 interacts with nucleoporin TPR in mitosis and is important for the spindle assembly checkpoint

Graciela López-Soop, Torunn Rønningen, Agnieszka Rogala, Bernd Thiede, Philippe Collas, Thomas Küntziger.

Cell Cycle, 2017. Manuscript under revision at the time of this writing

In **paper I** we report that AKAP95 depletion leads to defects during mitosis as shown by lagging chromosomes, faster prometaphase-anaphase transitions and appearance of micronuclei. Using a proximity-based labelling approach, we identify TPR as a novel AKAP95 interacting partner. Interestingly, TPR depletion has been reported to cause similar defects during mitosis[66,67,70,71,182] . AKAP95 shows a distinctive enrichment during the phases of mitosis comparable to that of TPR[67,182]. Particularly, AKAP95 and TPR show an enriched localization at the vicinity of the metaphase plate. After AKAP95 depletion, the specific TPR localization at mitosis is diminished, whereas AKAP95 mitotic localization is not affected when TPR is depleted. However, TPR total protein levels do not vary upon AKAP95 silencing.

Similar to TPR knock-down phenotypes, MAD1[66,67,71,182] protein levels are deregulated in AKAP95-depleted cells. MAD1 localization at KTs is >50% reduced. Altogether, our findings point towards a regulatory role of AKAP95 in the spindle assembly checkpoint response.

Paper II: Co-association of AKAP95 and FUS/TLS on promoters of active genes

Graciela López-Soop, Akshay Shah, Philippe Collas, Thomas Küntziger

Manuscript

In **paper II** we show the novel interaction between AKAP95 and Fused in Sarcoma/Translocated in Sarcoma (FUS/TLS) in HeLa cells. FUS is a multifunctional nuclear protein with prominent roles in DNA transcription and RNA splicing. We observed through co-immunoprecipitation experiments and imaging analysis that AKAP95 interacts with a fraction of the total FUS pool. We also inspected AKAP95 chromatin landscape as means of understanding AKAP95/FUS interaction. Using chromatin immunoprecipitation coupled to microarray analysis, we report that AKAP95 binds to approximately 1000 human promoters, of which, 400 are shared with FUS. We also characterized AKAP95 occupancy sites. We found that AKAP95 preferentially binds 1000-500bp upstream of transcription start sites. AKAP95 is enriched in active promoters; bearing transcription-promoting marks such as H3K4me3, H3K36me3. Furthermore, AKAP95-occupied genes show higher levels of transcription compared to the average genome. Interestingly, FUS promoter profile is different on AKAP95 co-occupying genes; where it displays a notable enrichment 2000 and 500 bp upstream of the TSS. Our preliminary data, argues in favor of a role of AKAP95 together with FUS in DNA transcription that will have to be further confirmed.

4. Discussion

4.1. AKAP95 micronuclei: nuclear blebbing or lagging chromosomes after mitosis?

Nuclear blebbing has been reported as a process that triggers the appearance of chromatin cytoplasmic fragments (CCFs)[183]. Unlike lagging chromosomes arising from defective cellular divisions[184], nuclear blebbing has been associated with senescent cells[183]. CCFs and micronuclei generated by defective mitosis are indistinguishable when visualized by DAPI stain. However, CCFs show a particular biochemical and structural profile. Ivanov *et al.* reported that CCFs triggered by cellular senescence are strongly enriched in the heterochromatic mark H3K27me3 and in the DNA damage histone mark γ-H2AX. Additionally, euchromatin histone modifications such as H3K9ac and nuclear lamin A/C were absent from senescent CCFs[183]. This is the opposite of what we observed in the AKAP95-induced micronuclei, which contained histone modification marks and nuclear envelope components with similar distributions to normal nuclei. The γ-H2AX staining pattern was variable in AKAP95-induced micronuclei (unpublished results), supporting previous observations where γ-H2AX is detected in micronuclei from S phase onwards[185]. The distinctive biochemical composition of CCFs and micronuclei exemplifies the opposed origins of both structures. For instance, Ivanov *et al.* noted that lamin A/C and lamin B protein levels were markedly downregulated in senescent cells, leading them to propose a partial loss of nuclear envelope integrity whereby targeted chromatin fragments would bud off. Indeed, depletion of Lamin B genes leads to a higher proportion of nuclei showing blebs[186]. Cancer cell lines show more permeable nuclear membranes and nuclear envelope herniations which aggravate upon altered Lamin organization[187]. Of note, depletion of nucleoporins did not result in any significant nuclear permeability or structural changes[187], highlighting the specific role of Lamins in conserving nuclear envelope integrity[153]. Another difference between micronuclei and CCFs is their fate. In their study, Ivanov showed that CCFs were targeted with autophagy markers including p62 and the protein ubiquitination marker FK2, whereas we and others[185] did not detect any

specific enrichment of several autophagy markers in micronuclei. Instead, micronuclei persisted throughout interphase and entered the subsequent mitosis with unsuccessful outcome.

Micronuclei consist of whole chromosomes or acentric fragments, as well as double minutes (MN), which are small chromatin fragments lacking telomeres and centromeres generated by DNA poisons[188]. In fact, micronuclei genetic activity varies largely across cell types and the type of chromatin fragment excluded from the nucleus[188]. Some studies have shown micronuclei with normal DNA replication rates[189,190] yet others reported inefficient and asynchronous DNA replication[185,189,191], which have been associated with reduced levels of both Lamin B expression[189] and micronuclear import[185] of important DNA replication mediators such as MCM subunits. DNA transcription in micronuclei shows similar trends than replication[188], with the most limiting factor being the correct structural and functional assembly of micronuclei. For example, acentric MN derived from chromatin bridges at anaphase show diminished levels of NPC components with concomitant nuclear import defects which lead to transcriptional inactivation [192]. Similar to DNA replication, absence of Lamin B in micronuclei resulted in gene transcription incompetent MN[193]. However, not all Lamin B-positive MN were transcriptionally active[193]. These data shows the importance of a functional nuclear envelope to undertake the main nuclear tasks and the essential role of Lamin B.

The mechanisms by which CCFs bud off from nuclear membranes[183,187] is not quite yet understood. A recent study by the Berger lab[194] implied that oncogenic-induced lamina-positive senescence CCFs are processed by autophagy in the cytoplasm. More importantly, Berger and colleagues showed that the LC3-LaminB1 interaction plays a significant role in the budding of CCFs. When LC3/LaminB1 interaction was prevented using a peptide block, the number of CCFs was significantly reduced compared to control oncogenic-induced senescent cells. The authors of this study claim that Lamin B is degraded by autophagy upon oncogenic stimuli and that LC3 both triggers and participates in

the generation of CCFs, shedding some light into the enigmatic nuclear blebbing mechanism.

It is worth mentioning that apart from the two studies cited above[183,194], CCFs in senescent cells have not been reported. This could be due to the specificity of this process, as CCFs were only detected upon replicative- or oncogenic-induction senescence[183]. Autophagy response triggers, on the contrary, did not elicit CCFs[194]. This also exemplifies that CCF research is still at an early stage.
Nevertheless, micronuclei and CCFs, even though sharing partial biochemical structure, are functionally divergent; whereas the former arise by defective anaphases in mitosis which later on try to follow the cell cycle, the latter arise as by-products of oncogenic-induced cellular senescence, which are targeted towards autophagic degradation pathways. In any case, both micronuclei and CCFs seem to result in an apparent loss of genetic material, which automatically leads to forms of aneuploidy and potentially to cancer development.

4.2. From DNA condensation to proper chromosome segregation regulated by AKAP95

In **paper I** we observed a significant number of micronuclei in HeLa cells after AKAP95 depletion (Fig.1A), that we later on discovered originated from lagging chromosomes arising from defective anaphases (Fig.1B). We also observed KT delocalization of MAD1 (Fig.4A) and faster mitotic progression (Fig.4C). Furthermore, TPR, a conserved nucleoporin implicated in SAC regulation[66–68,71,182], was identified as a novel AKAP95-binding protein. These evidences lead us to suggest that AKAP95 is involved in correct chromosome segregation at anaphase through the regulation of the spindle assembly checkpoint (SAC) response.

AKAP95 has been shown to play different roles throughout most cell cycle stages[195]. Previous work in our laboratory uncovered the interaction of AKAP95 with the condensin I subunit CAP-D2/Eg7 at mitosis, as well as the recruitment of RIIα to mitotic chromosomes[26]. Collas *et al.* observed that *Xenopus* nuclei

immunoblocked for AKAP95 did not condense chromatin when incubated in mitotic cell extracts. In fact, immunoblocking AKAP95 prevented CAP-D2/Eg7 chromatin recruitment at the onset of mitosis. Similarly, maintenance of condensed chromatin during metaphase was also lost by disrupting AKAP95-PKA interaction. AKAP95 was proposed to recruit CAP-D2, and later PKA to mitotic chromatin, sustaining chromatin condensation at mitosis[26,27].

Mammals possess two condensin complexes (I and II) which are both heteropentamers consisting of two common SMC subunits (CAP-E and CAP-C) and three non-SMC subunits: CAP-D2, CAP-G and CAP-H for condensin I and CAP-D3, CAP-G2 and CAP-H2 for condensin II[196]. Selective inactivation of condensin I does not impede chromatin condensation upon mitosis onset[197]. On the contrary, inactivation of condensin II results in a delay in chromosome condensation before NEBD. Despite that, cells manage eventually to condense chromatin and undergo mitosis, suggesting that both complexes might be compensating for each other's lack of function. Accordingly, siRNA-selective depletion of the SMC2/CAP-E subunit common to condensins I and II results in abnormal and incomplete chromosome condensation at metaphase[198]. Therefore a model was proposed whereby chromatin condensation initiation in late G2 depends on condensin II, whereas the primary role of condensin I would be after NEBD from prometaphase onwards. These data correlates with the subcellular distribution of Condensin complexes[197,199]: condensin I is cytoplasmic whereas condensin II is nuclear.

In addition to CAP-D2/Eg7, AKAP95 BioID analysis led to the detection of two other non-SMC Condensin I subunits (CAP-G and CAP-H), supporting our previous findings[27]. However, the role of AKAP95 in targeting condensin I to chromosomes it is still somewhat unclear. The C-terminal extremity of CAP-D2 was shown to be sufficient to mediate direct interaction with mitotic chromatin[200]. Therefore, AKAP95's role during chromatin condensation might be distinct from specifically targeting the condensin complex to mitotic chromosomes. Rather, AKAP95 might be important as regulating and/or recruiting additional protein factors required for the proper functioning of

condensin. Furthermore, both condensin complexes have been associated with roles in establishment of proper centromeric heterochromatin [199,201,202] and in kinetochore function, both in an Aurora B-dependent manner[199]. Depletion of non-core condensin subunits does not impede chromatin condensation or entry into mitosis. However, centromeric chromatin stiffness seems to be affected, which leads to chromosome segregation defects including chromatin bridges and irregular chromatin arms [201–203]. Notably, condensin subunits-depleted cells did not display an accelerated transit through mitosis. On the contrary, cells spent more time in mitosis. This suggests that the robustness of the SAC response is not affected by depletion of condensin subunits[204].

Interestingly, the *C. elegans* homolog of human CAP-G2, NCAPG2, has recently been associated with proper microtubule-kinetochore attachment through recruitment of Polo-like kinase 1 (PLK1)[205]. Depletion of NCAPG2 affected the recruitment of BubR1 and PLK1 to KTs, with diminished levels of phosphorylated BubR1, a substrate of PLK1. Altogether, this suggests that condensin subunits might possess a role in SAC signalling that extends beyond their classical condensation chromatin function.

Studies on the mitotic function of PKA reported that it was responsible for histone 1.4 serine 35 phosphorylation (H1.4Ser35p) at mitosis[206]: inhibition of PKA resulted in increased mitotic chromatin compaction, as visualized by accumulation of larger chromatin fragments after micrococcal nuclease digestion, and defective chromosome alignment at the metaphase plate[207]. However, no report described a decondensation phenotype following PKA inhibition, and it is believed that PKA contributes to the correct chromatin compaction degree during mitosis[206]. The AKAP95 knockdown decondensation phenotype from previously published observations[24,26,27,208] could be partly explained by the reported role of AKAP95 and HA95 in regulating histone 3 deacetylation prior to mitosis onset during G2 phase[28]. Double AKAP95/HA95 siRNA-mediated knock-down resulted in G2/M arrest. The association of AKAP95/HA95 with HDAC3 promotes a hypoacetylated chromatin landscape. This chromatin state is required for phosphorylating histone 3 on serine 10

which primes chromatin to undergo mitosis[209] and is required for complete chromosomal condensation[28]. Depletion of AKAP95 and HA95 resulted in accumulation of histone 3 acetylation that hindered the correct sequence of events for cells to divide.

Additionally, a role in chromatin condensation orchestrated by AKAP95 might be taking place at the beginning of mitosis, where we observe AKAP95 enriched in the vicinity of the metaphase chromosomes. As soon as anaphase starts, AKAP95 relocalizes from a peripheral chromatin-bound fraction to the spindle mid-zone plate, whereas the colocalization with chromatin of both condensin complexes persists throughout mitosis[199]. Altogether, the SAC function that we report here for AKAP95 seems to be independent of the previously reported role of AKAP95 in mitotic chromosome condensation prior to mitosis. Rather, we propose it as an additional function of AKAP95 in the nucleus, which is yet to be fully understood.

4.3. A scaffold protein as a novel SAC player

We show in **paper I** that AKAP95 is important for normal mitotic progression, and we propose the AKAP95/TPR axis as a possible mechanism. Our observations raise the possibility that AKAP95 is important for TPR's proper function in mitosis. An interesting avenue to explore is the hypothesis that TPR regulation by AKAP95 is mediated by regulatory protein post-translational modifications (PTMs). Indeed, Rajanala *et al.*[68] showed that a specific Serine phosphorylation (S2094) catalysed by PKA, appears on TPR at mitosis onset and decreases in G1. Moreover, a second phosphorylation on S2059 catalysed by CDK1, seems to be constitutive and necessary for Mad1 anchoring at the nuclear periphery during interphase. Even though the phosphorylation on S2059 showed a stronger distinctive mitotic localization and was required for Mad1 peripheral localization, both phosphorylation sites were associated with similar ratios of micronuclei formation when the phosphorylated serine residues were substituted, suggesting that specific TPR modifications are needed for its proper role in mitosis. AKAP95 might be a good candidate for coordinating

phosphorylation of TPR S2094 as it binds the RIIα subunit of PKA at mitosis[22,26] . In the context of this work, it would be of great interest to analyse TPR S2094 phosphorylation levels in AKA95-depleted cells.

TPR has been proposed to regulate MAD1/MAD2 proteostasis (protein homeostasis) via sumoylation[71]. Schweizer *et al.* reported that TPR depletion results in delocalization from the nuclear periphery of the SUMO-isopeptidase sentrin-specific protease 1 (SENP1), and inhibition of the proteasome in TPR-depleted cells partially recovered MAD1/MAD2 proteins levels. SUMOylation is regarded as an important regulatory mechanism for proper chromosome segregation during mitosis[210]. Several centromere and kinetochore proteins need to be SUMOylated to perform their correct function during kinetochore spindle assembly, including Aurora B[211] and BubR1[212]. Interestingly, in our BioID analysis we detected the SUMO activating subunit Sae1, the SUMO conjugating enzyme Ubc9 (SUMO E2) and the E3 SUMO-protein ligase RanBP2. Furthermore, *Mlp1/2* and *Ulp1/2* which are respectively the TPR and SENP1/2 homologs in budding yeast, have been functionally linked[62]. *S cerevisiae* Mlp proteins are responsible for tethering Ulp enzymes at the nuclear envelope and affecting their desumoylating activity. Given the apparent evolutionary conservation of this process, it is tempting to speculate about the possibility of AKAP95 being involved in a SUMOylation pathway together with TPR at mitosis. However, the detailed mechanism by which TPR might affect SUMOylation during SAC response is yet to be uncovered.

Protein kinases are also key players in the upstream regulation of SAC response, particularly Aurora kinase B and Mps1. In addition, some studies have identified PP1[213–215] and PP2A[216,217] to play a role in SAC silencing. Both PP1γ[218] and some PP2A[219] isoforms have already been localized at kinetochores in mammalian cells. The catalytic subunit of PP1 is an enzyme with pleiotropic functions and its specific activity therefore vastly relies on the regulatory protein it is associated with and with its subcellular localization. PP1γ is recruited to the outer kinetochore by KNL1[214], a member of the KMN network, and it participates in the phosphorylation of SAC kinases substrates, thus gradually inactivating the

previously activated SAC response and also stabilizing the bi-oriented KT-MT attachments.

Specifically, PP1 recruitment to KTs is maximal at metaphase, while barely observed during prometaphase, even in the presence of nocodazole[214]. This is in accordance with SAC signalling dynamics; at prometaphase, the SAC response is active, and it is not until most chromosomes have properly attached at metaphase, that the SAC response is gradually deactivated. On the contrary, PP2A seems to possess an earlier role in SAC dynamics. It reaches its peak of enrichment at unattached prometaphase kinetochores and gradually decreases until its complete absence at anaphase onset[216,219]. Indeed, PP2A is important for KT-MT attachments as dephosphorylation of Aurora B substrates is necessary to counteract the high phosphorylation activity that takes place at KTs and prevents contact with MTs[216]. PP2A is recruited to KTs via a phosphorylated motif in the pseudokinase BubR1[217]. We detected two regulatory subunits A of PP2A (PP2R1A and PPP2R5D) and two other more regulatory subunits belonging to PP1 and PP4 in our BioID screen. In fact, PPP2R5D is one of the regulatory subunits of PP2A detected at mitotic centromeres from prometaphase to metaphase[216].

Given the clear need of a tight feedback regulation on SAC signalling where activation (phosphorylations) and deactivation (dephosphorylations) events must take place with the right timing, magnitude and localization, a scaffold protein such as AKAP95 could be a major player in the intricate and highly dynamic SAC response. In addition, TPRS2094 mitotic phosphorylation reaches its basal levels at G1[68]. It is possible that the dephosphorylation of TPR is coordinated by AKAP95 recruiting a protein phosphatase.

4.4. TPR and AKAP95 role in SAC at mitosis: before or throughout?

There is little doubt that TPR does possess a role in SAC response. However, the precise mechanisms and site of action is still an unsettled question. The first studies to report the TPR/MAD1/MAD2 interaction claimed it took place during

interphase but also during mitosis[66]. A study by Schweizer[71] *et al*. suggested a role for TPR in SAC robustness at interphase by regulating MAD1/MAD2 proteostasis, even before cells commit to divide at G2. The authors propose that decreased protein levels of MAD1/MAD2 (and concomitant mitotic defects) in the absence of TPR arise from enhanced MAD1/MAD2 protein degradation. In particular, they propose a premitotic role for TPR in regulating MAD1/MAD2 stability at the nuclear periphery and preventing its premature degradation.

In elegant experiments, Rodriguez-Bravo[70] *et al.* give support to the pre-mitotic hypothesis. They show NPCs are important during interphase for assembling the premitotic MCC complex that establishes the minimum MCC levels necessary to impede premature APC/C activation at the early stages of mitosis[220]. TPR mediated-MAD1/MAD2 recruitment to the nuclear periphery[66,221] assists the conversion of open MAD2 to closed MAD2[222] when MAD2 shuttles from the nucleus to the cytoplasm. This closed active form of MAD2 will be able to interact with CDC20 in the cytoplasm and establish a premitotic MCC together with other SAC components.

The premitotic MCC formation hypothesis is coherent since it should transmit a rapid signal to inhibit CDC20 from binding the phosphorylated (and pre-active) APC/C complex[223]. In fact, the APC/C complex enters the nucleus at prophase[223] (before NEBD takes place) just as the kinetochore structure assembles at centromeres and starts recruiting its multiple components and SAC members[224] . Therefore, it is of paramount importance that a sufficient non-KT pool of the MCC is already assembled to outbalance the activation cues directed to APC/C and prevent the premature degradation of Securin and Cyclin B. Rodriguez-Bravo *et al.* exemplified this by generating a MAD1 mutant incapable of binding to TPR at the NPC, which showed five-times less MAD2-CDC20 binding and almost five-times more lagging chromosomes at anaphase compared to control situations[70].

Given that (i) TPR has not been identified at KTs[70,71], (ii) we did not detect AKAP95 at KTs either, (iii) TPR has been suggested to play a role in maintenance of MAD1/MAD2 proteostasis before mitosis[70,71] involving SENP1 and SENP2[71]

and (iv) our BioID screen identified several SUMO-related enzymes, the pre-mitotic hypothesis that AKAP95 and TPR act in the SAC response before mitosis onset might be plausible.

Favouring the other side of the argument, various reports[66–68,182] have shown the specific enrichment of TPR at the vicinity of mitotic chromosomes during metaphase formation and its relocalization to the spindle midzone upon anaphase onset. We also observed similar dynamics for AKAP95. Knowing that metaphase is the last stage before all chromosomes have engaged with proper KT-MT attachments, one could speculate that AKAP95 and TPR might be involved in the silencing of SAC response, equally important for proper chromosome segregation[225], and thus perform its action during metaphase.

Overall, there are indications favouring both temporal aspects of AKAP95 and TPR role during the SAC response. Further research will need to be conducted to delineate both the mechanistic and temporal views of AKAP95 action in proper chromosome segregation together with TPR.

4.5. AKAP95 and FUS: transcription and RNA processing linkers?

In **paper II**, we report a novel interaction between AKAP95 and FUS. We also report the genome-wide promoter enrichment profile of AKAP95 and show that AKAP95 is preferentially associated with post-translational histone modifications typical of euchromatin, some of which, such as H3K4me3, mark promoters of transcribed genes. Furthermore, of the approximately 1000 promoters bound by AKAP95, FUS is found to co-occupy approximately 45 % of those

FUS is a DNA- and RNA-binding protein[80]. In fact, it was first identified for its potent protooncogene action when ectopically fused with the transcription factor CHOP, leading to liposarcomas[74]. This work notably highlights the transcription promoting action of FUS. The fact that we found endogenous AKAP95 and FUS to co-immunoprecipitate underscores an unknown role

between these two proteins. FUS has been reported to be a positive regulator of transcription[84,85,87,88] and to bind diverse transcription factors[84–86]. ChIP-seq analysis of FUS distribution along the genome indicates that FUS is specifically enriched at the TSS of genes[80]. Our ChIP-human promoter chip analysis of AKAP95 strikingly shows a similar genomic profile to that reported earlier for FUS[80,88]. AKAP95 was found in expressed gene promoter areas and its promoter binding profile shows an enrichment ~200 base pairs upstream of TSSs. It is therefore tempting to speculate on a transcription initiation role of AKAP95 together with FUS. Transcription initiation begins with the assembly of the pre-initiation complex (PIC), which encompasses association of multiple transcription activator factors along the 5' end of genes upstream of the TSS[140]. These general transcription factors and activators recruit RNAPII, and together with the action of other effector proteins controlling phosphorylation of the c-terminal domain (CTD) of RNAPII, will initiate transcription[226]. FUS interacts with RNAPII and controls Ser2 phosphorylation of the CTD of RNAPII[80]. The two main protein kinases that phosphorylate the CTD of RNAPII are CDK-9 (a.k.a. p-TEFb) and CDK-12[226]. Our AKAP95 BioID screen did not detect these kinases. Interestingly, although not validated, subunit two of RNAPII was found in our AKAP95 BioID screen (**paper I**) together with the negative elongation factor B (NELF-B). This data suggests a possible role of AKAP95 in transcription initiation and elongation, together with FUS, RNAPII and NELF-B. Additionally, AKAP95 has been shown to accumulate at nucleoli when RNAPII activity is inhibited[41]. A similar phenotype has been described for FUS[101]. Thus, both AKAP95 and FUS seem to be RNAPII activity-dependent proteins. Nonetheless, the extent of their functional interdependence remains to be investigated.

On the other hand, mRNA splicing is an activity that can no longer be separated from DNA transcription in eukaryotic cells[93,227,228]. Indeed, FUS is a well described example of this paradigm [81,228]. Recently, Hu *et al.* showed for the first time a role of AKAP95 in RNA splicing[229]. Indeed, they show that AKAP95 binds to the exon-intron junctions of many transcribed pre-mRNAs in a manner that involves the Zn fingers of AKAP95, while the N-terminal domain seems to be involved in RNA splicing factors protein scaffolding[229]. This study strongly

supports our findings in **paper II** and validates the hypothesis of AKAP95 as an RNA-binding protein described in the Introduction section. The recent work is also consistent with our data showing that AKAP95 mainly associates with actively transcribed genes.

It would be interesting to determine whether the interaction between AKAP95 and FUS is DNA- and/or RNA-dependent, or whether the interaction is direct, as Hu and colleagues reported for AKAP95 and hnRNPs[229]. Moreover, it would be interesting to examine whether the RNA-splicing function of AKAP95 is dependent on AKAP95 anchoring FUS to its targets. Additionally, performing an overlap of FUS and AKAP95 mRNA binding sites and genome-occupancy sites would be very useful to study the identity of mRNAs AKAP95 and FUS co-regulate. This would be particularly interesting in a neurogenic differentiation context, as both AKAP95[230] and in particular FUS[114] protein dysregulations lead to neuropathologies. Furthermore, it would be informative to determine AKAP95 contribution to other RNA metabolism processes. In fact, our AKAP95 BioID screen detects AKAP95 putative partners involved in 5' pre-mRNA capping, such as nuclear cap-binding protein, RNA-export complexes such as the THO complex, and cleavage and polyadenylation factors (unpublished results). These results, although not yet confirmed, suggest a role for AKAP95 extending beyond RNA splicing scaffolding, in assisting many steps of mRNA maturation.

4.6. Overall conclusions: new abilities of scaffold proteins

Increasing evidence indicates that scaffold proteins are not only targeted to specific subcellular structures to anchor other proteins, but can also be recruited to specific sites to carry out specialized tasks. An interesting example is illustrated by SLX4, a nuclear scaffold protein involved in DNA repair[231]. In the double-strand break repair (DBS) response, SLX4 is targeted to DNA lesions by means of a sumoylation recognition motif present in its structure[232]. This idea also expands the scope of scaffold proteins, which recognize their partners via post-translational modifications. Another example of different scaffold proteins activities is the nuclear and cytoplasmic MAGE protein family, which act as

ubiquitin-ligating enhancers[233]. KAP1, a transcriptional corepressor, has been shown to have intramolecular sumoylation-conjugating activity[234]. These discoveries imply a new level of sophistication in the regulation of scaffold proteins, where they not only provide structure and linkage to different components but also may act as enzyme cofactors.

Altogether, the present investigation has revealed two novel functions of AKAP95: i) regulation of the SAC response during mitosis (**paper I**) and interaction with the DNA transcription and RNA splicing factor FUS (**paper II**). These findings add to the growing list of AKAP95 functions, which seems to play a role in all stages of the cell cycle (**Fig. 12**). This contributes to the growing idea that many proteins perform multiple distinct functions[235], e.g. depending on sub-cellular localization or the nature and composition of molecular complexes they may associate with. This view is entirely consistent in the context of the so-called C-paradox[236], where an organism may not necessarily need a larger genome to carry out a larger number of cellular functions, but rather, may optimize the use of its proteome by enabling a scaffolding of a wide array of combinatorial protein complexes from a fixed number of proteins.

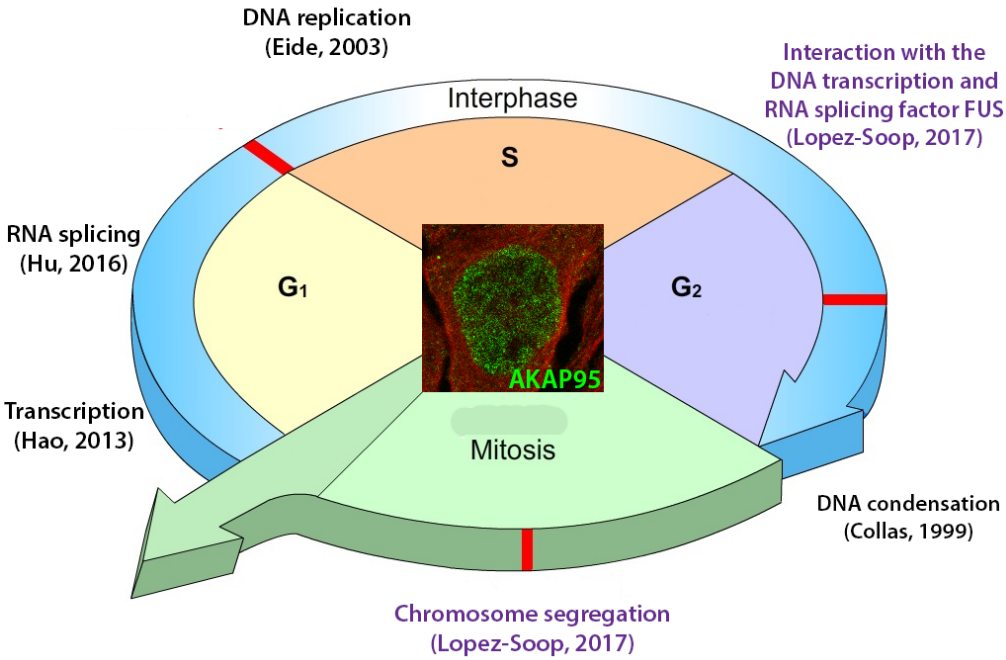

Figure 12. Diagram of AKAP95 nuclear biological functions[26,33,35,229].

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