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Regulated interactions of the SUMO ligase RANBP2 with the mitotic spindle and kinetochores during mitotic progression

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GLOSSARY

APC: anaphase-promoting complex
Bir1: baculoviral IAP repeat 1
BRAF: B-Rapidly Accelerated Fibrosarcoma
BubR1: budding uninhibited by benzimidazole-related 1
CCAN: constitutive centromere-associated network
CENP: centromere protein
CDK1: cyclin dependent kinase-1
CRM1: chromosome region maintenance 1
CPC: chromosomal passenger complex
Dox: Doxycycline
FG: phenyl/glycine motif
FOXO: Forkhead box O
GTP: guanosine triphosphate
HAUS: homologous to augmin subunits
IF: Immunofluorescence
IkB: inhibitor of kappa B
INCENP: inner centromere protein
K-fiber: kinetochore fiber
KMN: KNL1, Mis12 complex and Ndc80 complex
KNL1: kinetochore null protein 1
KRAS: Kirsten rat sarcoma
KT: kinetochore
LMB: Leptomycin B
MCAK: mitotic centromere-associated kinesin
MEF: mouse embryo fibroblast
MIP: Maximum Intensity Projection
Mis12: mis-segregation 12
MT: microtubules
MTOC: microtubules-organizing centers
NDC80: nuclear division cycle 80
NE: nuclear envelope
NES: nuclear export signal
NLS: nuclear localization sequence

NOC: Nocodazole
NPC: nuclear pore complex
NUP: nucleoporin
OP18: oncoprotein 18
PIAS: protein inhibitor of activated STAT
PLA: proximity ligation assay
Par4: prostate apoptosis response 4
RAN: Ras-related nuclear
RANBP1: RAN-binding protein 1
RANBP2/NUP358: RAN-binding protein 2/Nucleoporin 358
RANGAP1: RAN GTPase activating protein 1
RANGEF: guanine nucleotide exchange factor for RAN
Rb: Retinoblastoma
RBD: RAN-binding domain
RCC1: Regulator of chromosome condensation 1
RNAi: RNA interference
RRSU: RANBP2, RANGAP1-SUMO1 and Ubc9 complex
SAC: spindle assembly checkpoint
SAE1/UBA2: SUMO-activating enzyme subunit 2/Ubiquitin-like 1-activating enzyme 2
SAF: spindle assembly factor
SENP: sentrin specific protease
SIM: SUMO-interaction motif
Ska: spindle and kinetochore-associated
Spc24: Spindle Pole Body Component 24
Spc25: Spindle Pole Body Component 25
SUMO: small ubiquitin-like modifier
Topo II alpha: Topoisomerase II alpha
TPX2: Targeting protein for Xklp2
gamma-TuRC: tubulin ring complex
Ubc9: Ubiquitin carrier protein 9, E2-conjugating enzyme
WB: Western blotting

SUMMARY

RANBP2 is a large nucleoporin (NUP) residing at nuclear pore complexes (NPCs) in interphase and plays a role in nucleo-cytoplasmic transport of macromolecules across the NPC. In mitosis, when nuclear envelope (NE) breaks down and NPCs disassemble, RANBP2 localizes on mitotic structures.
RANBP2 has SUMO (small ubiquitin-related modifier) E3 ligase and SUMO-stabilizing activities and regulates protein SUMO conjugation, a relevant post-translational modification in dynamic processes such as the DNA damage response, stress response, signalling pathways and mitosis. A characterized SUMOylated RANBP2 target is RANGAP1, the GTP-hydrolysis activating factor for the GTPase RAN. RANBP2 and RANGAP1, together with Ubc9 (a SUMO E2 enzyme), form a complex, called RRSU (RANBP2/RANGAP1-SUMO/UBC9), that has enhanced SUMO ligase activity and localizes to kinetochores (KTs) in metaphase with a mechanism that is incompletely understood.
The goal of my PhD project was to identify the molecular mechanisms regulating the RRSU complex localization in space and time during mitosis, particularly to KTs, given the importance of these structures as the connecting structures between chromosomes and the mitotic spindle and their crucial role in chromosome segregation.
Both RANBP2 and RANGAP1 are known to interact with nuclear transport receptors, Importin beta and CRM1, during nuclear transport in interphase. In my project I have developed in situ proximity ligation assays (PLA) to visualize their interactions with these transport factors, follow their dynamics during cell division and assess whether nuclear transport receptors have themselves a functional role in the RRSU complex localization in mitotic cells.
PLA results show that the RRSU complex engages in dynamic interactions with Importin beta and CRM1 during mitotic progression: it preferentially interacts with Importin beta in early mitotic stages along the spindle MTs. In metaphase, after MTs

attach all KTs, this interaction decreases. The RRSU complex also interacts with CRM1: this interaction becomes up-regulated in metaphase and becomes visible at MT attached-KTs. Thus, the RRSU complex appears to "switch partners" from prometaphase (prevalent engagement with Importin beta along the spindle) to metaphase (increased PLA signals with CRM1 at KTs), suggesting that protein SUMO conjugation takes place with a spatially and temporally regulated programme in mitosis.
To validate the "switch partner" model I generated inducible cell lines, both for Importin beta and CRM1, to assess whether unbalancing one or the other would influence the RRSU complex localization in mitosis. Results from experiments with the inducible cell lines show that the mitotic localization of the RRSU complex depends on the antagonistic actions of Importin beta and CRM1: indeed, unbalancing each one of them impairs the RRSU complex localization and concomitantly generates segregation defects, suggesting that KT functions are defective. Overall, the results of my project highlight the importance of localized SUMOylation of proteins at the mitotic apparatus and KTs for balanced chromosome segregation, and indicate a role of nuclear transport receptors as upstream regulators in the process. It is of note that several cancer types overexpress these transport factors, which may contribute to the high level of genetic instability observed in these cancers.

INTRODUCTION

Eukaryotic cells divide to form two daughter cells through the complex and elegant process of mitosis. In the process, the newly duplicated genome of the cell is faithfully segregated to generate two genetically identical daughter cells. To do this, cells build a bipolar spindle composed of microtubules (MTs) endowed with dynamic activity. Every chromosome binds the growing end of a MT via its kinetochore (KT), a multiprotein structure assembled on centromeric DNA. Through this interaction, chromosomes are then segregated at the opposite poles of the dividing cell. This process is critical to the transmission of the genetic identity from a cell to its daughters. If errors occur daughter cells can become aneuploid, i.e. harbour a gain or loss of chromosomes, which can predispose them to become transformed. KTs act as functional units that attach the MTs in preparation of chromosome segregation. To achieve this, KTs orderly recruit factors that play critical roles establishing, stabilizing and monitoring the attachment to the spindle MTs. In my PhD project I have studied one such factors, i.e. the nucleoporin and SUMO ligase RANBP2/NUP358, the mechanisms underlying its recruitment to KTs during mitosis and the consequences of its mislocalization on mitotic progression.

1. An overview of mitosis

Mitotic entry is governed by the activity of the master mitotic kinase, cyclin dependent kinase-1 (CDK1), which functions in complex with cyclin B (*Pines and Hunter, 1991*).

In eukaryotic cells mitosis is conventionally subdivided into five stages: prophase, prometaphase, metaphase, anaphase and telophase (**Figure 1**). After telophase, cytokinesis allows the physical division of the two daughter cells.

At prophase, chromosomes condense within the nucleus and mitotic spindle assembly begins. The duplicated centrosomes, which act as the major (but not unique) MT-organizing centres

(MTOC), move apart in opposite directions and begin to nucleate MTs that project randomly in all directions in the cytoplasm: thus aster-like structures of growing MTs start forming (*Karsenti and Vernos, 2001*). During prometaphase the nuclear envelope (NE) breaks down. The spindle MTs stochastically encounter chromosomes and attach them via their KTs. During this process MTs, coming from centrosomes, are highly dynamic and randomly project throughout the cell until they encounter chromosomes in a process defined "search-and-capture".
Other mechanisms, independent on centrosomes, are involved in the formation of the spindle MTs. These mechanisms collectively form the acentrosomal MT pathway (reviewed by *Meunier and Vernos, 2016*), briefly described in the next chapter.
Metaphase is achieved when all KTs are attached by MTs, and all chromosomes, due to the symmetrically balanced forces applied onto them by the MTs emanating from each pole, are aligned at the cell equator (metaphase plate) (*Tanaka, 2013*). This moment is very short but, to avoid errors that can lead to aneuploidy, it is strictly controlled by the spindle assembly checkpoint (SAC), a signalling network devoted to monitor the attachment of all chromosomes to MTs prior to triggering the onset of anaphase (*Rieder and Khodjakov, 2003*). If no errors are detected, the checkpoint signals anaphase onset. At this point, the cohesion complexes that hold together the sister chromatids are degraded, so that sister chromatids can segregate. Finally, in telophase, chromosomes decondense, forming the two daughter nuclei, and the nuclear membrane re-forms around each of them. The final degradation of cyclin B leads to loss of activity of the master mitotic kinase, CDK1, initiating cytokinesis and mitotic exit.

2. The mitotic spindle formation: the acentrosomal MT assembly machinery

Early lines of evidence for the existence of a centrosome-independent MT assembly mechanism in dividing cells were

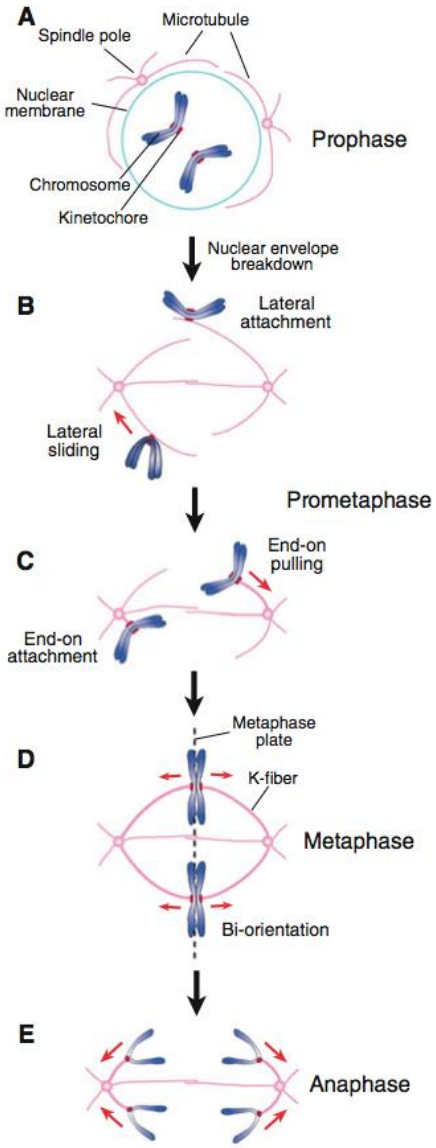

Fig. 1: **The phases of mitosis**. The progression of mitosis through the canonical morphological stages is shown. From Tanaka, 2013.

obtained in the 1970-80s (*McGill and Brinkley, 1975; Telzer et al., 1975; Witt et al., 1980; De Brabander et al., 1981; Karsenti et al., 1984*). We now know that these acentrosomal MTs are essential, and can be sufficient under certain cell types, for the assembly of a functional bipolar spindle. Two main mechanisms drive acentrosomal MT assembly in the dividing cells: the first one is dependent on chromosomes, while the other one is dependent on nucleation of pre-existing MT themselves (reviewed by *Meunier and Vernos, 2016*). These different mechanisms are linked to one another in a sequence of events that ultimately leads to the formation of kinetochore MTs, often referred to as KT fibers (K-fibers) within the bipolar spindle.

2.1 The chromosome-dependent mechanism of MT nucleation

Central to this mechanism is the signalling network mediated by the GTPase RAN (*Ciciarello et al., 2007; Clarke and Zhang 2008; Kalab and Heald, 2008*). RANGTP, i.e. the GTPase active form, promotes the local release of free spindle assembly factors (SAFs) in a biologically proficient form for MT nucleation. Many SAFs, which contain short nuclear localization sequences (NLSs) are otherwise inhibited by their binding with importins (Importin alpha/beta complex). RANGTP dissociates the import complexes and promotes SAF activity and hence spindle assembly. The RANGEF (guanine nucleotide exchange factor) RCC1 (regulator of chromosome condensation 1) is associated with chromatin throughout in mitosis, and induces the formation of RANGTP around chromosomes. SAFs are therefore released in their active state around chromosomes; RANGTP is highly concentrated and can induce MT nucleation therein, but not in the cytoplasm at a distance from them (*Kalab et al., 1999; Zhang et al., 1999; Caudron et al 2005; Tulu et al. 2006; Torosantucci et al 2008*). The polymerized MTs are then stabilized in the vicinity of KTs through a phosphorylation-dependent mechanism involving Aurora B in the chromosomal passenger complex (CPC) (reviewed by *Weaver and Walczak 2015*). The CPC resides at

KTs in metaphase. Here Aurora B, the catalytic component of the complex, phosphorylates and inactivates the MT-destabilizing factors MCAK (mitotic centromere-associated kinesin) and OP18 (oncoprotein 18). This creates a local environment around the KTs acting as a "hot spot" for MT stabilization (*Tulu et al., 2006*). MTs are therefore preferentially stabilized in the KT area.

2.2 The MT-dependent pathway of MT nucleation

An additional mechanism for acentrosomal MT assembly in mitosis was identified. This pathway is dependent on the octameric augmin complex termed HAUS (homologous to augmin subunits) (*Goshima et al., 2008; Lawo et al., 2009; Hsia et al., 2014*). This complex is recruited to both i) MT arrays that are being nucleated and stabilized through the RANGTP and CPC pathways, and II) "canonical" centrosome-nucleated MTs. The recruitment of gamma-TuRC (tubulin ring complex) to nucleated MTs induces extra-nucleation and branching of a new MT *(Petry et al., 2011; Uehara et al., 2009)*. This amplification mechanism drives the rapid increase of the MT mass within the spindle. Moreover, a study reported the co-immunoprecipitation of augmin with TPX2 (targeting protein for Xklp2) (*Petry et al., 2013*), one of the "SAFs" activated by RANGTP after release from Importin alpha/beta complexes *(Carazo-Salas et al. 1999)*. This suggests a potential direct link between RANGTP-dependent and augmin-dependent MT assembly pathways. The newly “branched” MTs are then captured through their plus-ends, and stabilized at KTs through their interaction with KT-associated proteins, including: i) the KMN complex [KNL1 (kinetochore null protein 1)/MIS12 (mis-segregation 12)/NDC80 (nuclear division cycle 80)], and ii) the Ska (spindle and kinetochore-associated) complex. Conversely minus-ends are pushed away towards the spindle poles, such that MTs are organized in bundles and form a K-fiber (*Rieder, 2005; Khodjakov et al., 2003, Maiato et al., 2004*).

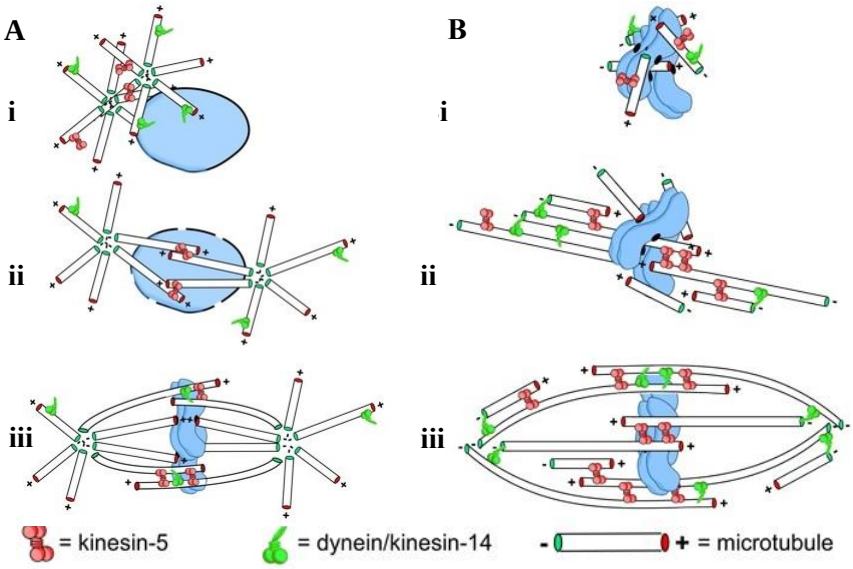

Fig. 2: **Spindle assembly pathways.** Pathways to spindle assembly are typically defined by the source of the microtubules used to construct the spindle. Some mitotic systems rely more heavily on one pathway or the other but spindle assembly likely involves some combination of both centrosome- and chromosome-nucleated microtubules. **A.** Centrosomal pathway. In early prophase, astral microtubules emanate from a pair of centrosomes clustered at a single locus on one side of the intact nucleus which bears condensed chromosomes (**i**). The centrosomes are then forced apart by motor-dependent microtubule-microtubule sliding (e.g. kinesin-5) (**ii**). After nuclear envelope breakdown, centrosomally derived microtubules can search for and capture targets such as kinetochores and other microtubules ultimately adopting a spindle-like shape (**iii**). **B.** Acentrosomal spindle assembly is characterized by a burst of microtubule nucleation around chromosomes that requires localized signals from the RANGTP and CPC pathways (**i**). Newly nucleated microtubules are arranged by sliding filament mechanisms eventually forming two prominent loci of focused minus ends, precursors to the spindle poles (**ii**). Eventually, microtubule motors continue to shape the microtubule arrays until it achieves the bipolar, fusiform shape of a typical spindle (**iii**). Modified from Gatlin and Bloom, 2010.

To summarize available evidence (**Figure 2**), RANGTP triggers the initial activation of MT nucleation and stabilization around mitotic chromosomes. The chromosomal and centrosomal MTs are then stabilized in the proximity of the KTs in an Aurora-B/CPC-dependent manner. Concomitantly, chromosomal MTs act as a template for augmin-dependent MT nucleation, providing an efficient mechanism for MT amplification around chromosomes (*Meunier and Vernos, 2016*). Upon MT plus-end capture by KTs, minus-ends are pushed away towards the spindle poles aided by motor proteins (*Gatlin and Bloom, 2010*). The model highlights therefore the crucial role of KTs both for MT nucleation and MT stabilization. The RAN GTPase emerges as a key regulator in both processes.

3. Post-translational modifications during mitosis: the role of SUMOylation

In addition to the well-established of phosphorylation of key structures (i.e. the nuclear envelope, centrosomes, microtubules and chromosomal proteins) in mitotic progression, another post-translational modification, SUMO conjugation, is proving of growing importance for many mitotic factors.

SUMO proteins are small ubiquitin-like modifiers that become covalently conjugated to cellular proteins carrying the consensus motif ψ-K-X-E (ψ, any hydrophobic amino acid, e.g. A, I, L, M, P, F, V or W; X, any amino acid residue) (*Zhao et al., 2009*).

SUMO conjugation affects neither the catalytic activity (unlike phosphorylation), nor the stability (unlike ubiquitination) of target proteins, but it modifies their surface of interaction, and hence their association/dissociation form partner proteins and their subcellular localization.

The quick and reversible attachment of SUMO peptides to specific proteins is essential for multiple cellular events, including transcription (*Hay, 2006*), DNA repair (*Moschos and Mo, 2006; Morris, 2010; Dou et al., 2011*), DNA recombination

(*Potts, 2009*) and, of interest to this work, mitotic chromosome segregation (*Wan et al., 2012*).
The SUMO pathway structurally resembles the ubiquitin pathway (**Figure 3**) and consists of:
- the dimeric SUMO E1 SAE1/UBA2 (SUMO-activating enzyme subunit 2, also known as Ubiquitin-like 1-activating enzyme 2),
- the single SUMO conjugating enzyme Ubc9, acting as an E2 ligase in the conjugation pathway,
- several E3 ligases that catalyze the multimerization of SUMO peptides on target proteins. These include PIAS (protein inhibitor of activated of STAT) family members, RANBP2 (RAN binding protein 2), and a few other E3 ligases.

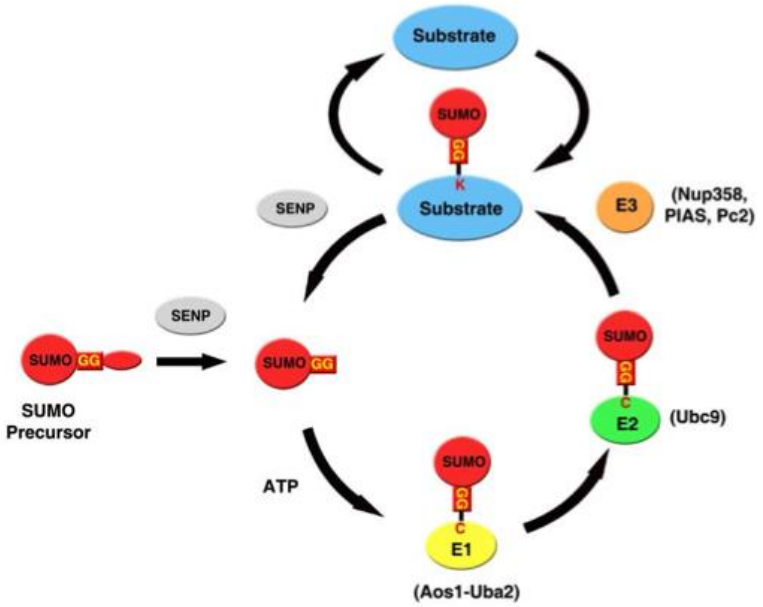

Fig. 3: **The SUMO pathway**. See legend in the following page

Fig. 3: **The SUMO pathway.** The SUMO precursor is processed by SENP proteases to expose a C-terminal double-glycine motif (GG). The mature SUMO is then activated by the E1 activating enzyme to form a thioester bond between the GG residue of SUMO and the cysteine (C) residue of E1. SUMO is then transferred to the catalytic C residue of the E2 conjugating enzyme. Finally, SUMO is transferred from E2 to the substrate by forming an isopeptide bond between the G residue of SUMO and a lysine (K) residue in the substrate. The last step is facilitated by an E3 ligase that promotes SUMO conjugation to specific protein substrates. At the end of the cycle, SUMO is deconjugated from its substrate by a SENP isopeptidase. From Wan et al., 2012

SUMOylation of proteins enables their covalent interaction with partners via SUMO interaction motifs (SIMs) in these proteins (*Johnson, 2004*). SUMOylation is reversible by SUMO proteases called SENPs (Sentrin specific proteases), which remove SUMO peptides from target proteins. Several studies have revealed the importance of SUMO modification in KT function.

3.1. Sumoylation of Topoisomerase II alpha

A well-characterized SUMOylation substrate is Topoisomerase II alpha (TopoIIalpha) (*Bachant et al., 2002; Azuma et al., 2003*). During mitosis TopoIIalpha re-localizes from chromosome arms to the centromeres of sister chromatids (*Christensen et al., 2002; Tavormina et al., 2002*), where it decatenates DNA in sister centromeres to enable chromosome segregation (*Lee and Bachant, 2009*). SUMOylation plays a critical role in regulation of TopoIIalpha-mediated decatenation of centromeric DNA (*Ryu et al., 2010; Porter and Farr, 2004*): PIASgamma is required for SUMO2/3 modification on TopoIIalpha in *Xenopus* extracts (*Azuma et al., 2005*). This SUMOylation inhibits the function of TopoIIalpha and temporally prevents the premature resolution of centromeric DNA until the onset of anaphase (*Ryu et al., 2010*). RANBP2 has been found to be the SUMO E3 ligase for TopoIIalpha in mice. Indeed, in mouse mutant embryonic

fibroblast (MEF) cells with reduced expression of RANBP2, TopoIIalpha is defective for SUMOylation and fails to localize at inner centromeres in mitosis (*Dawlaty et al., 2008*).

3.2 SUMOylation of the CPC complex

The chromosomal passenger complex (CPC) is composed of the Aurora B kinase and three non-enzymatic subunits: INCENP, Survivin and Borealin (*Ruchaud et al., 2007*). This complex has a dynamic localization during mitosis, residing to both chromosome arms and inner centromere at the entry of mitosis, and then concentrates to the inner centromere at metaphase. Upon sister chromatid separation at the onset of anaphase, the CPC is re-localized from the inner centromere to the spindle midzone (*Ruchaud et al., 2007*).

CPC functions are i) to correct erroneous kinetochore-microtubule attachments during prometaphase, and ii) ensure correct cytokinesis (*Ruchaud et al., 2007; Carmena and Earnshaw, 2007*). Aurora B is modified by SUMO2/3 at lysine 202 (K202) near its kinase domain (*Ban et al., 2011; Fernandez-Miranda et al., 2010*). This SUMOylation is reported to be mediated by PIAS3 in in vitro conjugation assays (*Ban et al., 2011*). This modification of Aurora B is required for proper chromosome congression (*Fernandez-Miranda et al., 2010*), supporting the idea that SUMOylation is a novel mechanism regulating processes that depend on Aurora B activity, although mechanistic details remain to be elucidated (*Ban et al., 2011; Fernandez-Miranda et al., 2010*).

Analysis of SUMOylation of the CPC in mammalian cells has also revealed that the non-enzymatic subunit Borealin is modified by SUMO2/3, with a higher level of SUMOylation in metaphase compared to anaphase (*Klein et al., 2009*). RANBP2 is the SUMO E3 ligase for Borealin both *in vitro* and *in vivo*, while the SUMO isopeptidase SENP3, which also resides at KTs, is responsible for its deSUMOylation. However, SUMO modification of Borealin does neither affect the CPC assembly

nor its localization at either centromeres or the spindle midzone (*Klein et al., 2009*). It is thought that borealin-conjugated SUMO peptides serve as a "reservoir" for the specific conjugation of other KT-associated protein substrates. Interestingly, the yeast Survivin homolog Bir1 has also been identified as a SUMO target, but the role of its SUMOylation is currently unknown (*Monpetit et al., 2006*).

3.3 SUMOylation of inner KT proteins

The CENP-H/I/K complex (consisting of CENP-H, CENP-I and CENP-K) belongs to the constitutive centromere-associated network (CCAN) associated with the so-called inner kinetochore. The CCAN is assembled onto, and associated with CENP-A- (H2A homologous histone) containing chromatin throughout the cell cycle (*Perpelescu and Fukugawa, 2011*). CENP-H and CENP-I are modified by conjugation with polymeric SUMO2/3 chains (*Mukhopadhyay et al., 2010*). Because the CENP-H/I/K complex is recruited to the constitutive inner kinetochore in S-phase, it is hypothesized the SUMOylated form of this complex promotes inner kinetochore assembly (*Mukhopadhyay and Dsso, 2010; Mukhopadhyay et al., 2010*).

***3**.4. SUMOylation at outer kinetochore and fibrous corona*

Proteins that constitute the fibrous corona and the outer kinetochore, which directly interacts with the spindle MTs, are also subjected to SUMO conjugation (**Figure 4**).

A SUMO2/3 interacting motif has been identified at the C-terminal KT-binding domain of CENP-E. This motif is necessary for CENP-E binding to polymeric SUMO2/3 chains, which is essential for its targeting to kinetochores (*Zhang et al., 2008*). Both known CENP-E-interacting proteins, i.e. Nuf2 (*Liu et al., 2007*) and BubR1 (*Yao et al., 2000; Chan et al., 1998*) are also specifically modified by SUMO2/3 *in vivo* (*Zhang et al., 2008*).

The Ndc80/Hec1 complex (comprising Ndc80/Hec1, Nuf2, Spc24 and Spc25), a key component of the KMN network at the

outer kinetochore, plays a major role in stabilising KT-MT attachments prior to chromosome segregation (*Tooley and Stukenberg, 2011; Ciferri et al., 2007*). Nuf2 interacts with CENP-E and is required for its targeting to kinetochores in mammalian cells (*Liu et al., 2007*). Nuf2 is specifically modified with SUMO2/3 (*Zhang et al., 2008*), Ndc80 has also been identified as a SUMO substrate in budding yeast, though the functional significance of this modification is unknown (*Monpetit et al., 2006*).

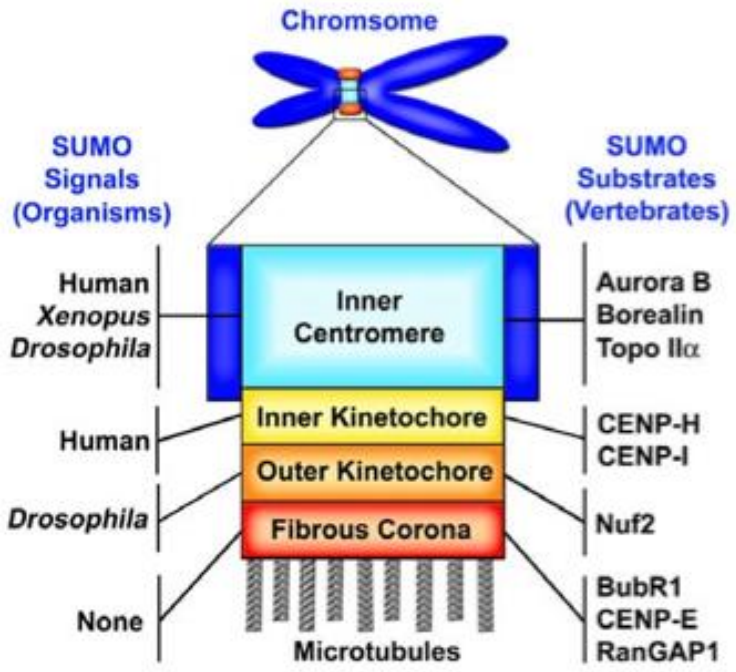

Fig. 4: **SUMO substrates at kinetochores.** Known SUMO targets are shown in association with their exact localization at the inner centromere, the inner and outer KTs and at the fibrous corona. From Wan et al., 2012

BubR1, a key component of the SAC, localizes on unattached KTs in early prophase and dissociates from MT-attached KTs

following chromosome congression in metaphase (*Cheeseman and Desai, 2008*). BubR1 is SUMOylated at Lysine 250 (K250), strongly stimulated by prolonged mitotic arrest caused by MT-inhibitory drugs (nocodazole or taxol). BubR1 SUMOylation regulates neither its activation nor its KT localization, but, rather, plays a critical role in BubR1 dissociation from KTs and checkpoint inactivation for resumption of anaphase onset and accurate chromosome segregation (*Yang et al., 2011*).
These data, together with the finding that SUMO-specific isopeptidases reside at centromeres and at KTs (*Zhang et al., 2008; Cubeñas-Potts e al., 2015*) and have roles in KTs functions (*Mukhopadhyay et al., 2010; Cubeñas-Potts e al., 2013*), suggest that cycles of SUMOylation and de-SUMOylation modulate proteins in KT-mediated process.
In conclusion, SUMOylation of different proteins appears to be required at several steps of kinetochore assembly and function (**Table 1**), and hence, ultimately, for accurate chromosome segregation.

Table 1. Centromere- and KT-protein that undergo SUMOylation and SUMO-deconjugation cycles

Protein	Localization	Reference
Borealin	centromere	Klein et al., 2009
Topoisomerase II α	centromere	Azuma et al., 2003
CENP-H	inner kinetochore	Mukhopadhyay et al., 2010
CENP-I	inner kinetochore	Mukhopadhyay et al., 2010
Nuf2	outer kinetochore	Zhang et al., 2008
BubR1	fibrous corona	Zhang et al., 2008
CENP-E	fibrous corona	Zhang et al., 2008
RANGAP1	fibrous corona	Matunis et al., 1996

4. RANBP2: a nucleoporin with SUMO-ligase activity

RANBP2 (RAN-binding protein 2), also called NUP358 (nucleoporin of 358 kDa), is the largest nucleoporin (NUP) and resides at the nuclear pore complex (NPC) cytoplasmic face in

interphase. Therein. it plays a role in nucleo-cytoplasmic transport of macromolecules throughout NPCs.
When NPC disassemble at mitosis, RANBP2 associates with the spindle MTs and a fraction is recruited to the outer KTs specifically at metaphase (*Salina et al., 2003*) (**Figure 5**).
RANBP2 comprises different functional domains (**Figure 6**):
- four RAN GTPase-binding domains (RBDs), hence its name
- phenyl/glycine (FG)-rich regions common to other NUPs and important to enable passage of nuclear import complexes throughout the NPC into the nucleus.
- a zinc-finger domain and a cyclophilin-homologous domain (*Wu et al., 1995; Yokoyama et al., 1995*).
- a most important domain that is not shared with other NUPs is a SUMO E3-ligase domain that renders RANBP2 able to SUMOylate other proteins (*Pichler et al., 2002*).
- overlapping the E3 domain, RANBP2 has a SUMO-interacting motif (SIM), through which it binds SUMOylated proteins and stabilizes them in the SUMO-conjugated form (*Werner et al., 2012*). RANBP2 provides a major source of SUMO-conjugating and SUMO-stabilizing activities in vertebrate cells.

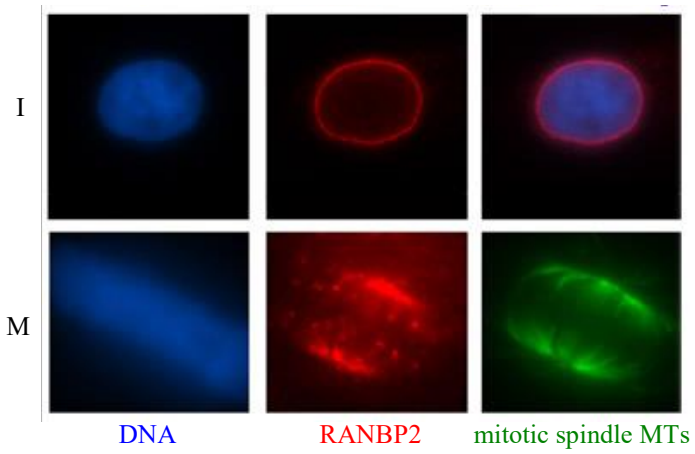

Fig. 5. See legend in the following page

Fig. 5. The localization of RANBP2 in human Hela cells. Top row: RanBP2 distribution in an interphase (I) HeLa cell; note the punctuate red staining around the nucleus (blue), which identifies the regular distribution of nuclear pore complexes (NPC)s. Bottom row: a metaphase (M) cell with aligned chromosomes (left panel). RANBP2 (red) co-localizes with mitotic MTs (green) with an accumulation at the kinetochore level, appearing as red spots proximal to the MT growing ends (Di Cesare and Lavia, 2014).

Figure 6. A Schematic of RANBP2 domains. Boxes 1-4 identify four RAN-binding domains, Cy indicates a cyclophilin-like domain, vertical dashes mark the position of FG-repeats that interact with transport receptors (modified from Werner et al., 2012).

5. The RANBP2/RANGAP1-SUMO/Ubc9 (RRSU) complex

A major target of RANBP2 SUMOylation activity is RANGAP1, the GTP-hydrolysis activator factor for RAN, as mentioned above. RANBP2 binds and stabilizes the SUMOylated form of RANGAP1 (SUMO-RANGAP1) through its SIM domain. This is required to localize SUMO-RANGAP1 at NPCs, while unconjugated RANGAP1 is soluble in the cytoplasm (*Matunis et al., 1996; Mahajan et al., 1997; Matunis et al., 1998*). SUMO-RANGAP1 then activates RANGTP hydrolysis on the NPC cytoplasmic side during transport cycles, thereby allowing the dissociation of the export complex and the release of the exported protein in the cytoplasm (*Ritterhoff et al., 2016*).

On the other hand, RANGAP1 association with RANBP2 reinforces the SUMO E3 ligase activity of the latter. RANBP2 and RANGAP1 are components of a multimeric SUMO ligase unit which, together with the E2 SUMO conjugating enzyme Ubc9, form a complex called RRSU (RANBP2/RANGAP1-SUMO/Ubc9) complex (*Werner et al., 2012*) (**Figure 7**). SUMO-

RANGAP1 remains associated with RANBP2 throughout the cell cycle (*Swaminathan et al., 2004*).

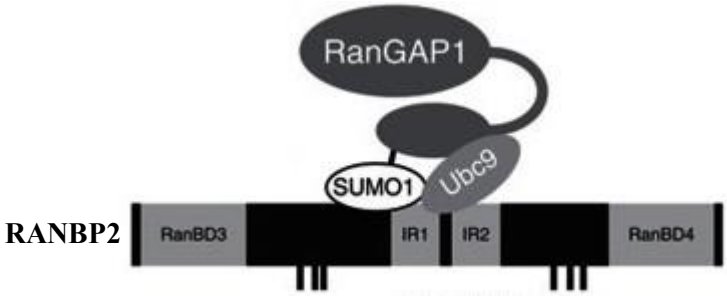

Fig. 7: Schematic of the RRSU complex (from Ritterhoff et al., 2016)

At mitosis onset, both RANBP2 and RANGAP1 localize to MTs of the mitotic spindle and a fraction reaches KTs in metaphase (*Joseph et al., 2002*) (**Figure 8**). Importantly, RanGAP1 localization at KTs requires SUMOylation by RANBP2 (*Joseph et al., 2004*).

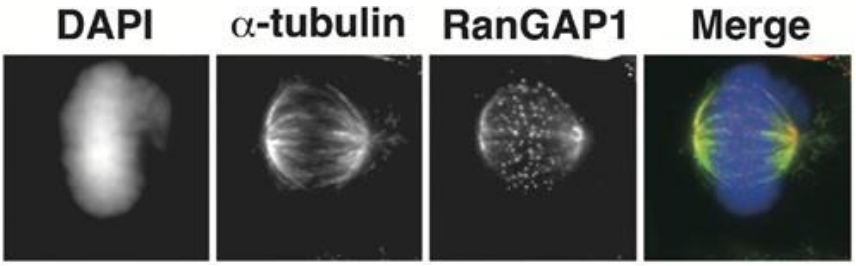

Fig. 8: RANGAP1 localization at the mitotic spindle and at kinetochores in a human metaphase cell (from Joseph et al., 2004).

5.1 The RRSU complex functions at KTs

The RRSU complex localizes at metaphase KTs and can play two important functions therein.

First, as previously described, RANGTP induces MT nucleation from KTs (*Tulu et al., 2006*) and thus contributes to mitotic spindle assembly (*Cavazza and Vernos, 2015; Meunier and Vernos 2016*). RANGAP1 recruitment to metaphase KTs decreases the local concentration of RANGTP: a first important

function is played in modulating GTP turn-over on RAN, which is critical to regulate the MT polymerizing activity of KTs (*Torosantucci et al., 2008*).
Second, SUMOylation is important to mitotic KT organization and chromosome segregation (*Wan et al., 2012*). RANBP2 acts in SUMO modification of KT-associated proteins, and/or stabilization in the SUMOylated form, as described above (*Wan et al., 2012*).

6. RANBP2 and RANGAP1 interact with nuclear transport receptors

Both RANBP2 and RANGAP1 interact with nuclear transport receptors during nuclear transport cycles.
In particular, RANBP2 interacts, via its FG-rich domains, with Importin beta, the main vector of protein import in interphase nuclei. RANBP2 is the most cytoplasmic of all NUPs. The interaction with importin beta occurs when nuclear import complexes initially bind NPCs to traverse them and eventually reach the nucleus.
At mitosis onset, Importin beta associates with the spindle MTs via dynein (*Ciciarello et al., 2004*). As mentioned, importin beta binds several NLS-containing SAFs that are kept inactive in the interaction (*Ciciarello et al., 2007; Clarke and Zhang, 2008; Kalab and Heald, 2008*). Importin beta binding prevents the premature activation of several factors in spindle assembly pathways (reviewed by *Forbes et al., 2015*). Indeed, altering the Importin beta expression, and hence its abundance, by either down-modulating (*Hashizume et al., 2013*) or increasing its expression (*Nachury et al., 2001; Ciciarello et al., 2004; Kalab et al., 2006; Roscioli et al., 2012*) induces deregulated activity of mitotic factors, yielding an array of mitotic abnormalities.
RANBP2 and RANGAP1 also interact with exportin-1/CRM1 (chromosome region maintenance 1), the export vector of proteins out of the nucleus.

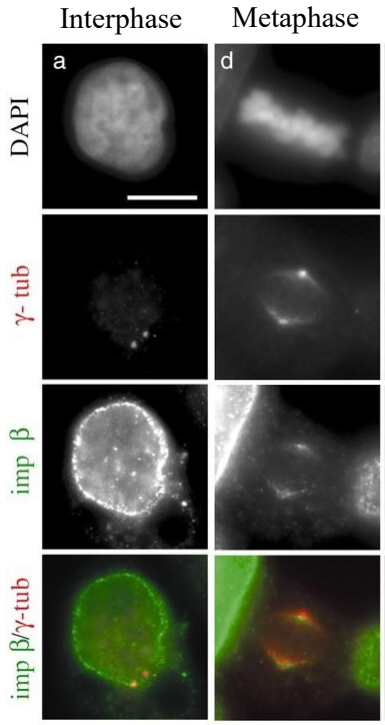

Fig. 9: Importin beta localization (from Ciciarello et al., 2004).

In the export cycle, CRM1 interacts with cargo proteins carrying nuclear export signal (NES). RANGTP stabilizes CRM1/NES cargo complexes, which become export-competent. On the NPC cytoplasmic side, RANGAP1 hydrolyses GTP on RAN, inducing the dissociation of export complexes and the release of the NES protein in the cytoplasm (*Ciciarello and Lavia, 2005*).
RANGAP1 itself contains several NES motifs (*Matunis et al., 1995*) with which CRM1 interacts. RANGAP1 localization at NPCs and at KTs, upon SUMOylation and interaction with RANBP2, requires CRM1 (*Cha et al., 2015*). Thus, an interplay exists between CRM1 and RANGAP1: on the one hand, RANGAP1 is a CRM1 export cargo via its NES motifs; on the other hand, it regulates export complex disassembly by causing

RANGTP hydrolysis in interphase (*Ritterhoff et al., 2016*). This suggests the existence of a self-limiting loop between export complex assembly and disassembly, in which RANGAP1 can be viewed as a pivotal factor. CRM1 can also directly interact with RANBP2 via the zinc finger-containing domain of RANBP2 (*Singh et al., 1999*). In mitosis, CRM1 fractions localize at centrosomes (*Forgues et al., 2003; Budhu et al., 2005*), at MTs and at KTs (*Arnaoutov et al., 2005; Zuccolo et al., 2007*) and recruit NES-containing proteins.

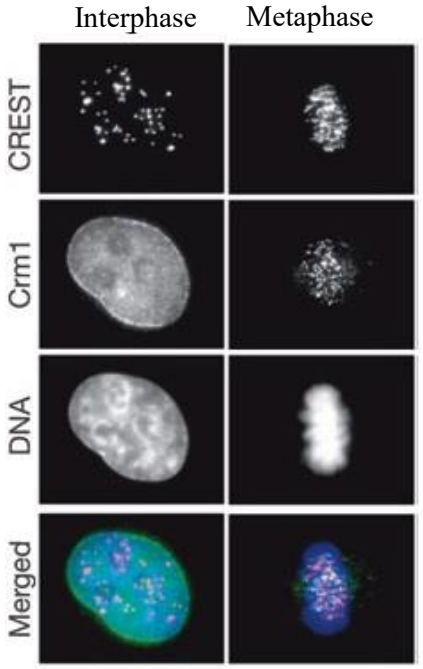

Fig. 10: CRM1 localization (from Arnaoutov et al., 2005).

CRM1 and RANGTP are both required to localize RANBP2 and SUMO-RANGAP1 at MT-attached KTs (*Arnaoutov et al., 2005*), suggesting that a loop takes place at KTs: RANGTP and CRM1 recruit RANGAP1, in complex with RANBP2, at MT-attached

KTs; at that point, RANGAP1 would hydrolyse GTP on RAN, changing its functional state at biorented KTs (*Dasso, 2006*).

7. RANBP2, Importin beta and CRM1 are de-regulated in cancer cells

As previously described, RANBP2, Importin beta and CRM1 play fundamental roles in cell physiology by regulating protein transport between the nucleus and the cytoplasm. They also play key roles by associating with mitotic structures, particularly the spindle MTs and KTs. Their cellular localization has an impact on the activity of factors implicated in the build-up of the mitotic apparatus and chromosome segregation. Deregulated activity of nucleo-cytoplamic transport vectors is associated with pathological situations (listed for example in *Di Cesare and Lavia, 2014; Giubettini et al., 2013; Ruggero et al., 2012*). These pathological situations, in addition to transport defects, may also be caused by mitotic dysfunction of these proteins, leading to abnormalities in chromosome segregation and causing genetic instability, a cancer hallmark.

7.1 RANBP2 and cancer

As summarized above, RANBP2 and RANGAP1 modulate the interaction of KTs with K-fibers and regulate KT functions (*Arnaoutov et al., 2005; Clarke, 2005*), at least in part via localized GTP hydrolysis on RAN (*Clarke and Zhang, 2008*).
Vecchione et al. have recently pinpointed a pro-oncogenic activity of RANBP2 specifically elicited in a subset of colon cancers, carrying mutant BRAF V600E (BRAF-like cancers). Transcriptomics studies showed that RANBP2 is overexpressed in BRAF-like colon cancers and this renders these cancers more sensitive to the microtubule poison vinorelbine (*Vecchione et al., 2016*). RANBP2 is essential for survival of the colon cancer cells: indeed, RANBP2 silencing induces mitotic defects and prolonged mitotic arrest, eventually triggering cell death in mitosis. Moreover, these cells are defective in MT outgrowth from KTs;

RANBP2 depletion reduces this defect (*Vecchione et al., 2016*). The defects in MTs formation in RANBP2-overexpressing cells unveiled a potential vulnerability of such tumors to MT disrupting agents. Vecchione et al. found that these cells are 10- to 10,000-fold more sensitive to vinorelbine than colon cancer cells in which RANBP2 is not overexpressed (*Vecchione et al., 2016*). These results, together with the requirement for RANBP2 for colon cancer cell survival, and for MT nucleation from KTs during mitosis, indicate that the RANBP2 status can be used as a prognostic indicator of the sensitivity of these cancers to treatment with MT disrupting agents.

7.2 Nuclear transport receptors and cancer

7.2.1. Importin beta

As reported, Importin beta acts as a negative regulator of mitotic spindle formation by preventing the premature activation of spindle regulatory factors (*Ciciarello et al., 2004; Nachury et al., 2006; Tedeschi et al., 2007; Roscioli et al. 2012*). This can rationalise the finding that many cell types that overexpress importin beta become genetically unstable and hence transformed (*Rensen et al., 2008; Giubettini et al., 2012),*

In particular, Importin beta is overexpressed in cervical cancer cell lines and is important for proliferation and survival of those cells (*van der Watt et al., 2009*). Indeed, Importin beta silencing impairs cancer cell proliferation and induces cancer cell death (*van der Watt et al., 2009*). Moreover, Importin beta inhibition in these cancer cell lines results in elevated levels of p53, p21, p27 and p18, suggesting that Importin beta silencing triggers apoptosis (*van der Watt et al., 2009*). Importin beta expression is also upregulated in ovarian cancer (*Smith et al, 2010*), head and neck and lung cancers (*Martens-de Kemp et al., 2013),* gastric carcinoma (*Zhu et al., 2015*), breast carcinoma (*Van der Watt et al., 2013*) and some leukaemias (*Van der Watt et al., 2013*). These data suggest that aberrant expression of Importin beta can

lead to uncontrolled cell growth. These findings support the idea that inhibition/targeting of importin beta may have potential value in cancer therapy (*Mahipal and Malafa, 2016; Stelma et al. 2016; Van der Watt et al., 2013*).

7.2.2. CRM1

As recalled, CRM1 operates nuclear export of NES-containing tumor suppressors and cell cycle regulators, including retinoblastoma (Rb), adenomatous polyposis coli (APC), p53, p21, p27, FOXO, IkB, topoisomerase II and Par4 (*Senapedis et al., 2014*). Nuclear export of these proteins can lead to impaired apoptosis and aberrant cellular growth (*Kau et al., 2004*).

Like Importin beta, CRM1 is also overexpressed in cervical cancer cell lines and its absence affects cell death via apoptosis (*van der Watt et al., 2009; Ruggiero et al. 2013*). Moreover, CRM1 overexpression is associated with poor prognosis, higher grade and advanced disease in several tumor types (*Noske et al., 2008; Turner et al., 2012*). This suggests that high levels of CRM1 may be essential for cancer cells to maintain their high rate of proliferation and metabolic activity.

It has been proposed that CRM1inhibitors have promising value in cancer therapy. Van der Watt et al. showed that treatment with LMB (Leptomycin B), a CRM1 inhibitor, was highly cytotoxic on cervical cancer cells, while normal cervical epithelial cells were much less sensitive. Recently Kim et al. also reported that CRM1 is a valuable target for specific drugs in a subset of lung cancer cells, with KRAS mutated (*Kim et al., 2016*).

These studies highlight the importance of regulated expression of both CRM1 and importin beta, and indicate that altered levels of either transport receptor severely affects cell growth and division.

Transport factors interact with the RANBP2/RANGAP1 complex, RANBP2 is required for cancer cell survival. These observations raise the possibility that at least part of the pro-oncogenic effects of both importin beta and CRM1 is exerted by deregulating the proper localization of RANBP2 in mitotic cells.

AIM OF THE WORK

How is RANBP2 localization controlled in mitosis?

Previous work (*Roscioli et al., 2012*) showed that importin beta co-immunoprecipitates with RANBP2 and SUMO-RANGAP1 in mitotic cell extracts (**Figure 11**).

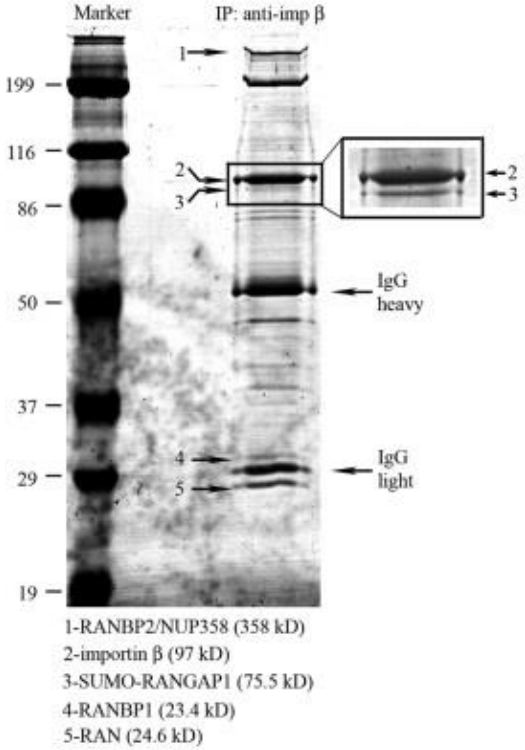

Fig. 11: **Importin-β partners in coIP assays from HeLa cells.** Coomassie blue–stained proteins in the importin-β coIP from HeLa mitotic cells. Bands were excised and processed for mass spectrometry. The inset shows an enlarged section to resolve importin-β and SUMO–RANGAP1, which migrate very close (Roscioli et al., 2012).

Furthermore, importin beta overexpression inhibits RANGAP1 recruitment to KTs (**Figure 12**).

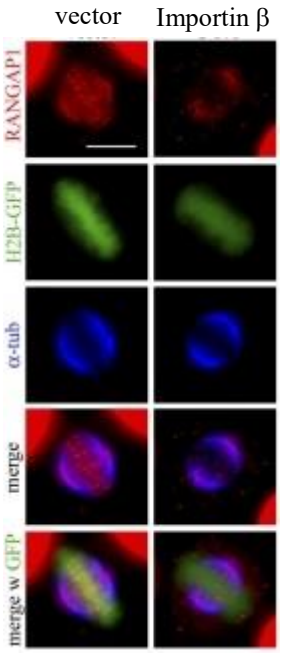

Fig. 12: **Importin-β excess hinders RANGAP1 recruitment at KTs in metaphase cells.** The panels show RANGAP1 at metaphase KTs in cells transfected with vector but not with importin-β (Roscioli et al., 2012)

On the other hand, RANGAP1 localization to KTs requires CRM1 function (**Figure 13**).

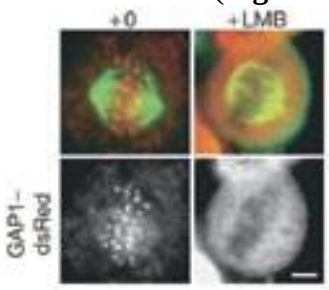

Fig. 13: RANGAP1 localization at kinetochores requires CRM1. Asynchronous untreated (+0) or LMB-treated cells expressing RNAGAP1^{dsRed} (in red) were fixed and stained with anti-α-tubulin antibodies (green) (Arnaoutov et al., 2005)

Thus, nuclear transport receptors regulate the mitotic localization of RANGAP1. These findings suggest that nuclear transport

receptors could also control the mitotic localization of RANBP2, and hence the sites of SUMO modification during mitosis.
Currently, no direct evidence is available to clarify whether or how importin beta, or CRM1, operate in spatial and temporal control of the RRSU complex during mitotic progression. It is also unclear whether during mitosis each nuclear transport receptor operates in a self-contained manner (as in interphase transport, in which the existence of the nuclear envelope spatially constrains each transport vector in its compartmentalised pathway), or whether the export and import systems cross-talk at the mitotic apparatus. Clarifying which one of these scenarios - which we could define "separation" versus "cross-talk" - applies is of relevance to fully understand the consequence of the aberrant expression observed in many cancer types.
To gain insight into these questions, in my PhD project I have developed for the first time proximity ligation assays (PLA) to visualize the interactions between transport factors - importin beta or CRM1 - and the RRSU complex during mitosis. This has enabled me to visualize and quantify RANBP2 interactions at the specific sites at which they take place and follow up their dynamics during mitotic progression.
In addition, I have taken advantage of inducible cell lines engineered in our laboratory, in which the abundance of each transport factor, i.e. either importin beta or CRM1, can be manipulated in a controlled manner to perturb the system. I have used these cell lines to ask how the RRSU complex would perform in mitotic cells under altered expression of nuclear transport factors. This has enabled me to demonstrate that importin beta and CRM1 play opposite functions in a finely-tuned control of the RRSU complex at MTs and at KTs, respectively. Ultimately, this finely tuned mechanism determines the sites of RANGTP hydrolysis and protein sumoylation in human mitotic cells.

RESULTS

1. Proximity Ligation Assay (PLA) is a valuable technique to visualize interactions between RAN network components

Proximity Ligation Assay (PLA) can detect protein interactions *in situ* in intact cells. By using this technique, it is possible to visualize protein interactions, their localization on cellular structures and their dynamics during the cell cycle.

PLA combines principles of immunofluorescence and DNA amplification to detect protein interactions *in situ*. Initially the proteins of interest are recognised by specific primary antibodies; the latter are then allowed to interact with secondary antibodies conjugated with two oligonucleotide tails (called PLA probes PLUS and MINUS). Connector oligonucleotides, which are complementary to the secondary antibody-conjugated DNA tails, are then added: if the two proteins of interest are in close proximity (within 10-30 nm), the connector oligonucleotides can pair with each one of them in a ligation step, forming a circle of DNA. Finally, a rolling circle DNA amplification occurs, whose product is visualized by a fluorescent probe complementary to the amplification product generated by the oligonucleotides tails (*Soderberg et al., 2006, 2008*) (**Figure 14**). The technique offers an important advantage compared to other techniques used to detect protein interactions: it can detect protein interactions *in situ* in intact cells, and makes it possible to visualize the localization of the interacting proteins and follow their dynamics during the cell cycle.

Since the aim of my project was to investigate RANBP2 interactions with components of the nucleo-cytoplasmic transport machinery in mitosis, as a preliminary step I established the PLA method testing pairs of RAN network members that are known to interact, i.e. the RANBP2/RANGAP1 and RAN/CRM1 pairs.

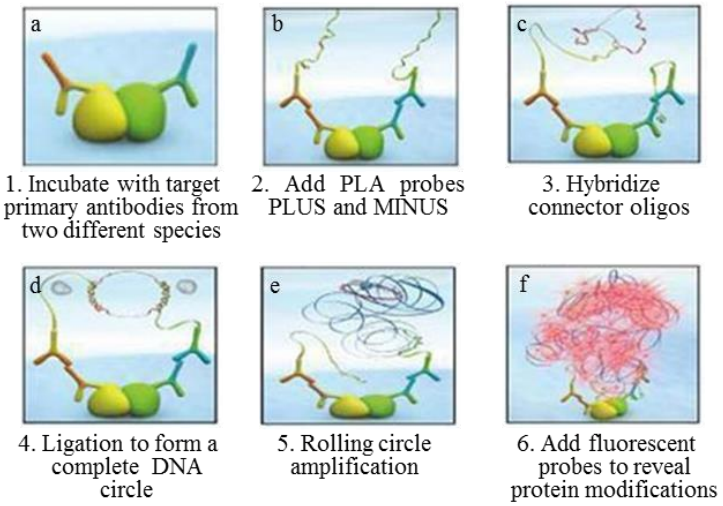

Fig. 14: **Schematic representation of Proximity Ligation Assay (PLA).** Panels (a-f) show the steps of the intermolecular *in situ* Proximity ligation protocol (images by courtesy of Duolink). See text for details.

The preliminary PLA tests show that both combinations (**Figure 15**) yield clean interaction signals in interphase, which localize around the nuclear rim (more peripheral for RANBP2/RANGAP1, left column, and more embedded within the nuclear face of the NE for RAN/CRM1, right column), as expected from the localization of the single components.
Since the PLA technique has a DNA amplification step, I also performed a time-course assay to identify the most effective amplification condition for optimal signal-to-noise ratio and established the best amplification time at around 60 minutes.
I next addressed the interactions between RANBP2 and the two transport factors: Importin beta and CRM1. PLA results show that, in interphase, both interactions localize around the nuclear rim, visualized by the Lamin B1, consistent with the

localization of each single protein, while PLA reactions

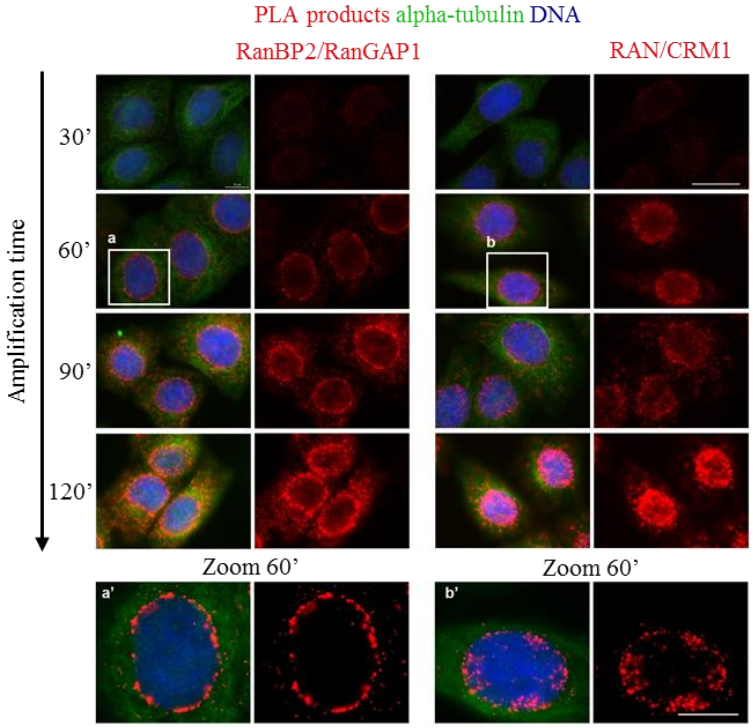

Fig. 15. Test of the PLA method on two characterized interactions Time-course assay of the amplification step on two known interactions, RANBP2/RANGAP1 (left column) and RAN/CRM1 (right column) in HeLa cells. PLA signals (red spots) localize around the nuclear envelope for both interactions. The most effective amplification condition for optimal signal-to-noise ratio is 60' amplification. Upper bar, 20 μm; lower bar, 10 μm.

between RANBP2 and a non-expressed protein, i.e. GFP, gave no signal (**Figure 16**).

I therefore can conclude that the PLA technique is specific and is a valuable tool to visualize *in situ* interactions between RAN network components in intact cells.

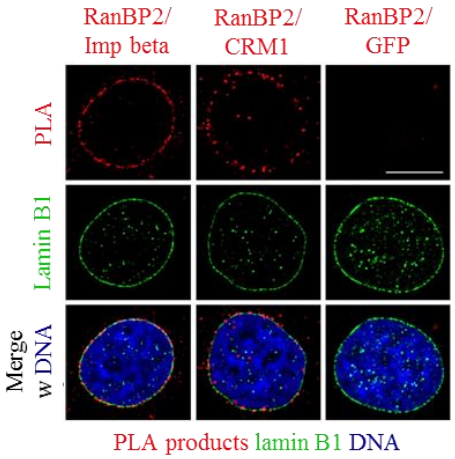

Fig. 16: RANBP2 PLA products with Importin beta (left), CRM1 (middle) and GFP (right) in interphase
PLA signals are detected at the nuclear envelope. No signals are observed in reactions with anti-RANBP2/anti-GFP antibodies in cells non expressing GFP. Bar, 10 μm.

2. Distinct complexes, involving RANBP2, are subjected to dynamic changes during mitotic progression

After nuclear envelope break down components of the nucleo-cytoplasmic transport machinery play important mitotic roles.
Here, I focused my study on the nucleoporin (NUP) RANBP2, the Importin beta nuclear import vector and the exportin CRM1. Since the localization patterns of these factors were previously investigated in different laboratories using different cell lines (*Joseph et al., 2004; Ciciarello et al., 2004; Roscioli et al., 2012*; *Wu et al., 2013*), I first developed a comprehensive immunofluorescence (IF) analysis of their localization under comparable experimental conditions in the HeLa cell line. **Figure 17** shows that, when the NE disassembles, each of these proteins re-localizes on specific structures. In particular:

- RANBP2 localizes at spindle microtubules (MTs) and, in metaphase, a fraction of protein is visible on kinetochores (KTs) of aligned chromosomes. Finally, in the late stages of mitosis, RANBP2 re-localizes around the re-forming nuclear rim of the two daughter cells (**Figure 17A**).
- Importin beta co-localizes in part with RANBP2, since it interacts with spindle poles and MTs but, differently from the NUP, it never reaches KTs. At ana-telophase, Importin beta also localizes around the re-forming NE (**Figure 17B**).
- Finally, CRM1 localizes at spindle MTs; at metaphase, a fraction also resides at KTs; at later stages of mitosis it becomes visible around the nuclear rim as the other two proteins (**Figure 17C**).

These patterns suggest that fractions of RANBP2 co-localise with transport factors, Importin beta and CRM1. I then used PLA to directly visualize their interactions during stages of mitosis. The results show that RANBP2/Importin beta interactions are abundant in early mitosis and decrease from metaphase onwards (**Figure 18A**). RANBP2/Importin beta localize exclusively on spindle MTs throughout mitosis (**Figure 18A and C, top**); in telophase they localize around the reforming NE ().

On the contrary, in parallel PLAs RANBP2/ CRM1 interactions increased in metaphase and at that time they localize abundantly in the chromosome region (**Figure 18B and 18C, bottom row**). In anaphase they still interact at kinetochores (KTs). In telophase they re-localize together around the reforming NE, concomitant with the RANBP2/Importin beta complex (**Figure 18B**). The PLA patterns are consistent with the IF localization of single components and reveal that there is not only co-localization, but a real interaction between them. So, I can conclude that the PLA technique is able to follow faithfully, in space and time, the interactions occurring between RANBP2 and the transport factors in mitotic stages.

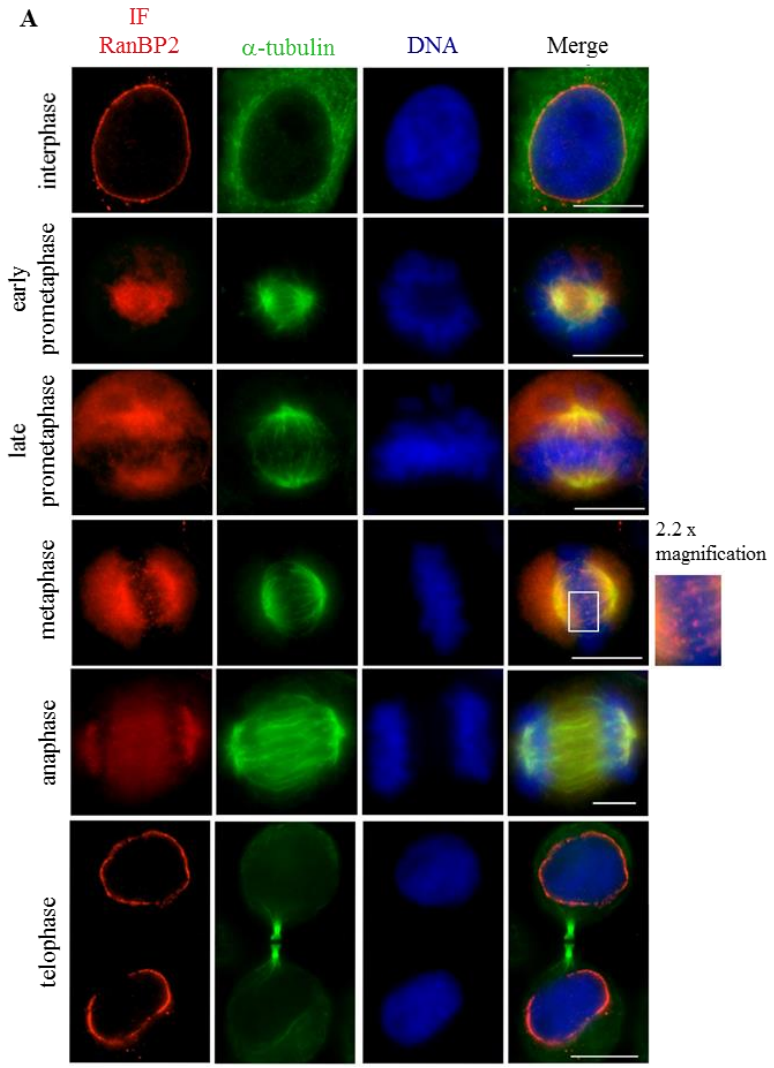

Fig. 17A: **RANBP2 localization during mitotic progression** RANBP2 (red) co-localizes with the spindle microtubules (green) and with KTs (red spots co-localizing with DNA, blue) at metaphase. In telophase RANBP2 localizes around the re-forming nuclear envelope of the two daughter cells. Scale bar, 10 μm.

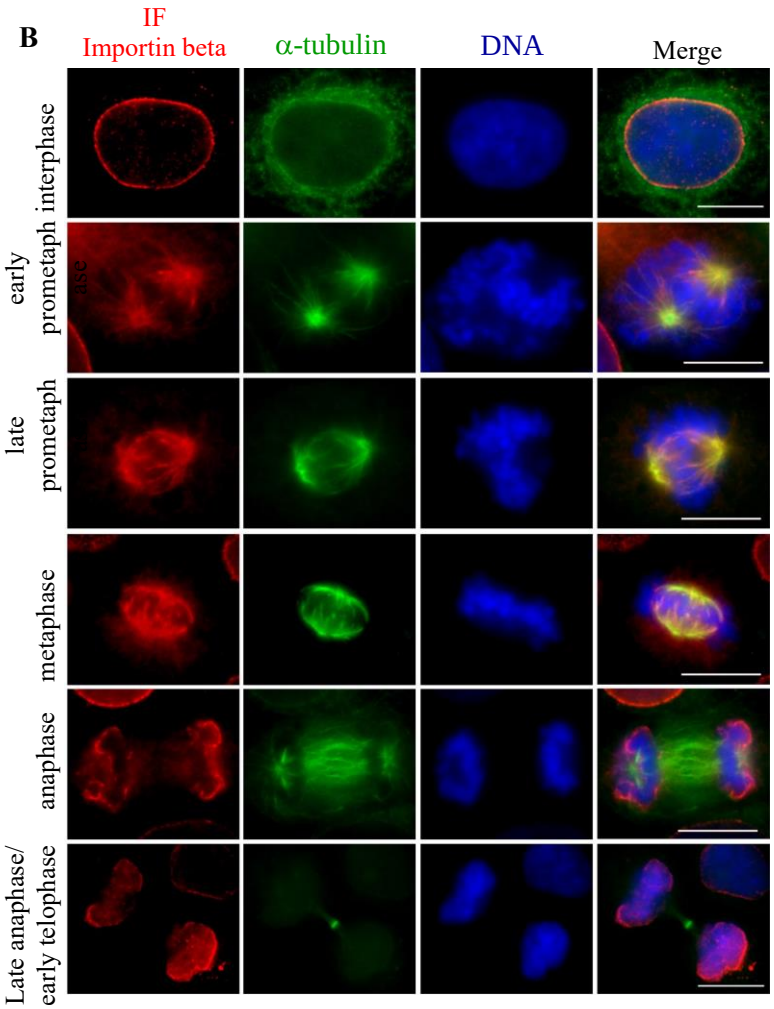

Fig. 17B. Importin beta localization by IF assay during mitotic progression. The immunofluorescence pattern of Importin beta (red) co-localizes with mitotic spindle microtubules (green) from early prometaphase to metaphase and then localizes around the re-forming nuclear envelope of the two daughter cells in ana/telophase. Scale bar: 10 μm.

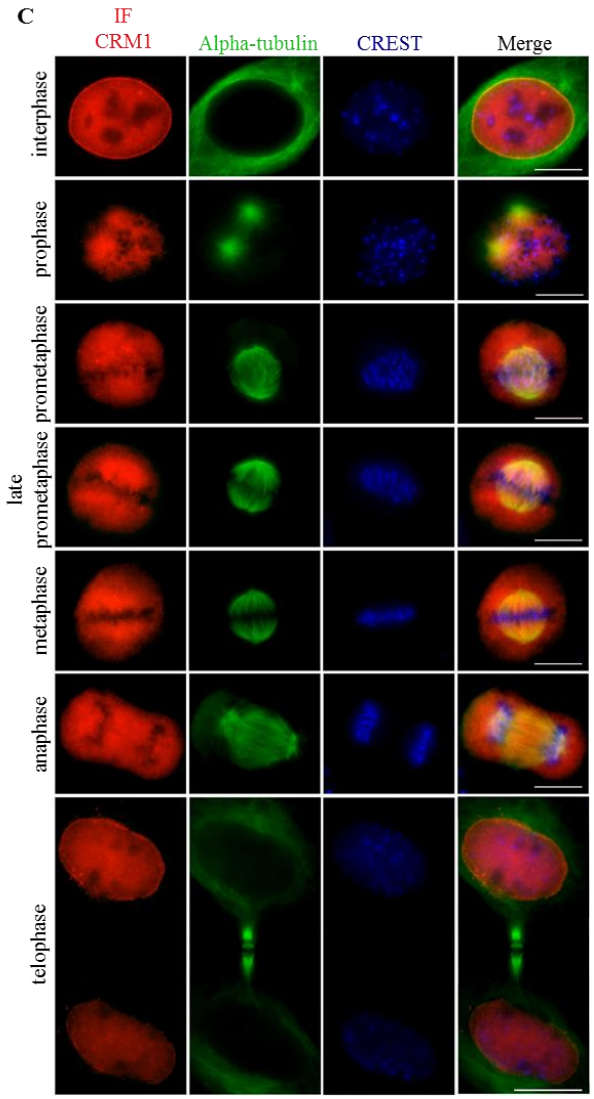

Fig. 17C. **CRM1 localization by IF assay during mitotic progression.** The immunofluorescence pattern of CRM1 (red) co-localizes with mitotic spindle microtubules (green) and with kinetochores (CREST marker, blue) in metaphase. In telophase it localizes around the re-forming nuclear envelope of the two daughter cells. Scale bar: 10 mm.

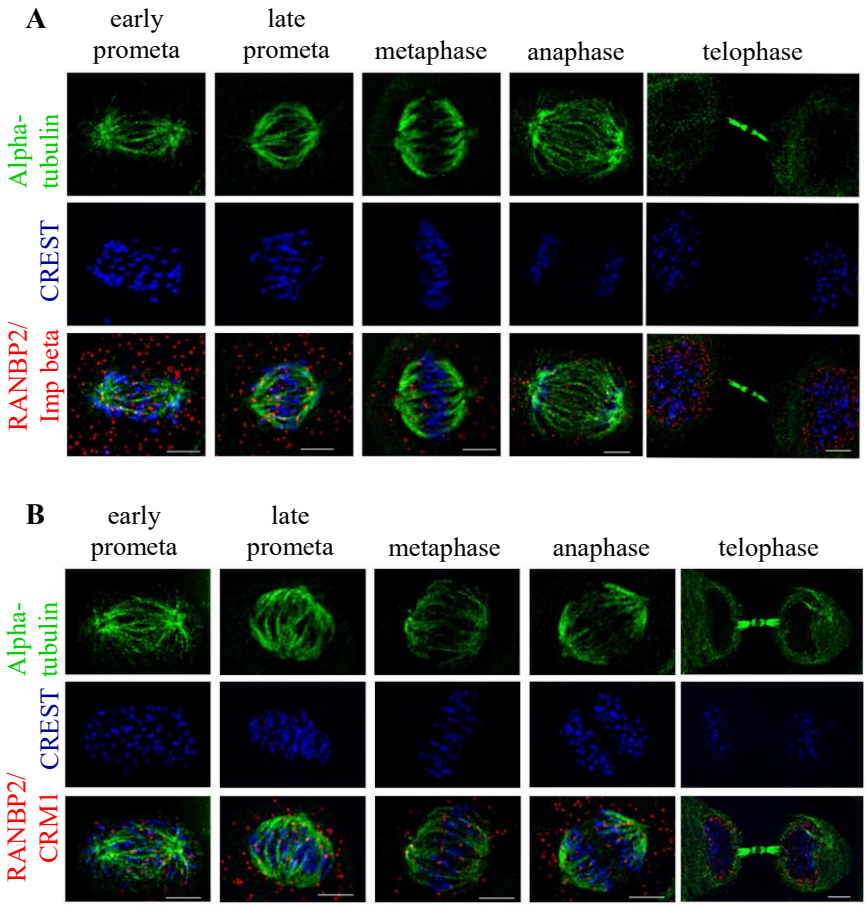

Fig. 18. **RANBP2 interactions with transport receptors during mitotic progression detected by PLA. A.** RANBP2/Importin beta PLA products are abundant in prometaphase, localize mostly at spindle MTs and decrease in metaphase. In anaphase, residual PLA signals mostly localize along polar MTs, but not at KT-bound MTs. **B.** RANBP2/CRM1 PLA signals localize at KTs and are abundant in metaphase. In anaphase, they remain associated with KTs of segregating chromosomes. Scale bar: 5 μm; telophase bar: 10 μm.

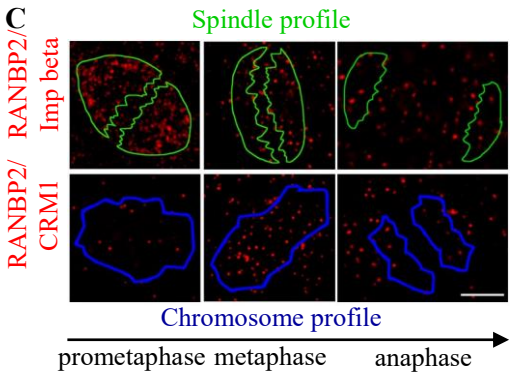

Fig. 18 (continues): **C.** PLA signals for RANBP2/Importin beta (top) localize mainly at MTs (delimited by green profile) and for RANBP2/CRM1 (bottom) mainly at chsomosomes (delimited by blue profile) Scale bar: 5 μm.

To quantify the abundance of RANBP2-centered PLA interactions, I analysed PLA signals in mitotic cells by counting them either manually or automatically (using the imaging software "object count" function, in which every single PLA signal is an object). Henceforth the automatic mode was used unless specified otherwise. After PLA spot counting, I classified the cells in discrete classes of signal abundance and calculated the percentage of cells having a number of signals falling in every class. The quantification confirms that RANBP2/Importin beta interactions along MTs significantly decrease from prometaphase to metaphase (**Figure 19A**), while RANBP2/CRM1 increase at KTs in metaphase compared to prometaphase (**Figure 19B**).Together, these data suggest that, after NE breakdown, RANBP2 establishes interactions with Importin beta and CRM1, preferentially with Importin beta along the spindle MTs before they attach to KTs, and later with CRM1 at MTs-attached KTs.

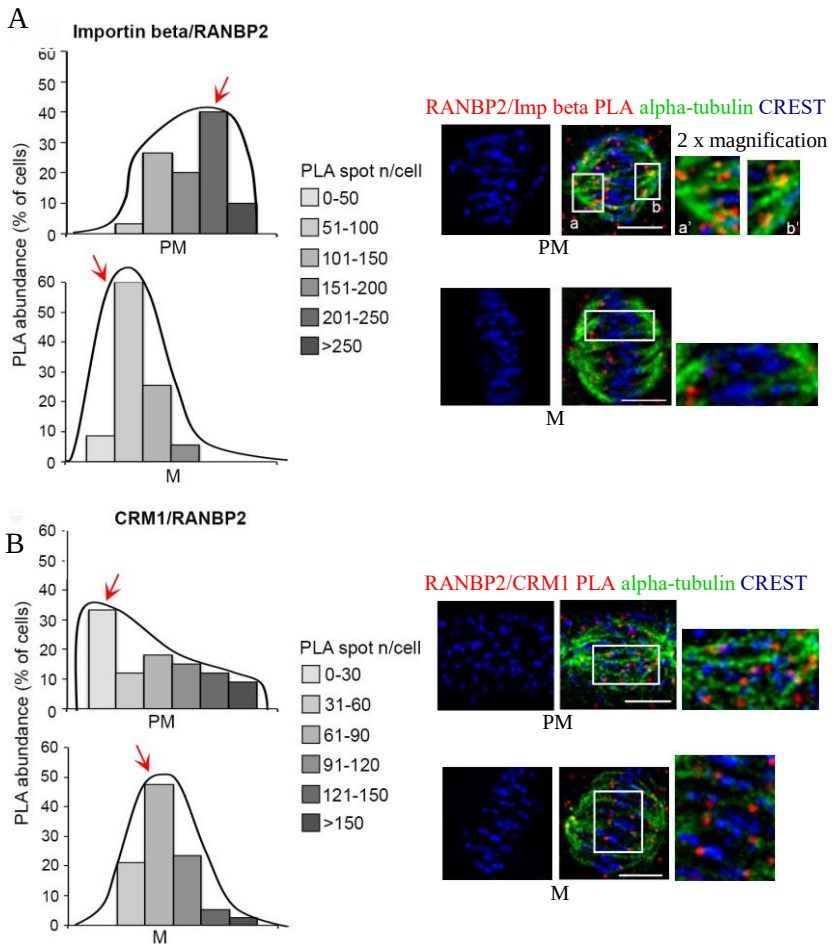

Fig. 19: **RANBP2/Importin beta and RANBP2/CRM1 PLA products are spatially and temporally regulated in mitosis A.** RANBP2/Importin beta PLA in mitotic cells. The histograms represent the frequency of PLA signals (in classes of abundance) in prometaphase (n, 180) and metaphase (n, 320) cells. Red arrows indicate modal classes. The IF panels show representative PLA product localization (insets, 2x zoom-in). The decrease in metaphase is highly significant ($p<0.0001$, X^2 test; 9 experiments). **B.** RANBP2/CRM1 PLA in mitotic cells. The histograms represent the frequency of RANBP2/CRM1 PLA signals in prometaphase (n, 140) vs. metaphase (n, 540) cells. In the IF panels, a fraction of RANBP2/CRM1 PLA products becomes KT-associated in metaphase (insets, 2x zoom-in). $p<0.0005$, X^2 test; 9 experiments). Bar: 5 μm.

3. In mitosis RANBP2 is in complex with RANGAP1 and they interact with transport factors as a single unit

SUMOylated RANGAP1, RANBP2 and Ubc9 (the E2 SUMO-ligase enzyme) form a complex, called RRSU. To verify if the PLA results obtained above using RANBP2 reflects the interactions between all components of the RRSU complex with transport factors, I performed RANGAP1 PLA assays with either Importin beta or CRM1. PLA assays using RANGAP1 show the same spatial and temporal patterns as RANBP2, with abundant interactions with Importin beta in prometaphase, preferentially on spindle MTs, which decrease in metaphase (**Figure 20A**). Conversely, RANGAP1 interacts with CRM1 mostly in metaphase in the chromosome area (**Figure 20B**). I also performed PLA reactions between RANBP2 and RANGAP1. The results show that the complex localizes differentially before and after MTs/KTs attachment (**Figure 20C**), reflecting the behaviour of RANBP2 and RANGAP1, when tested individually.

In summary, RANBP2 and RANGAP1 remain associated in mitosis in the RRSU complex, which interacts with transport factors in a spatially and temporally regulated manner: with Importin beta in early stages of mitosis at spindle MTs; in metaphase, when all KTs are attached to MTs, its association with Importin beta decreases and the RRSU complex interacts preferentially with CRM1 at MTs-attached KTs.

4. RANBP2 silencing controls the specificity and validates the mitotic PLA patterns

To validate the PLA results, I silenced RANBP2 using specific siRNAs (compared to GL2 siRNAs, against luciferase, not expressed in mammalian cells for control). HeLa cells were treated with RANBP2- (or GL2)-specific siRNA. The protein decrease was measured by both Western Blot (**Figure 21B**) and IF (**Figure 21C**).

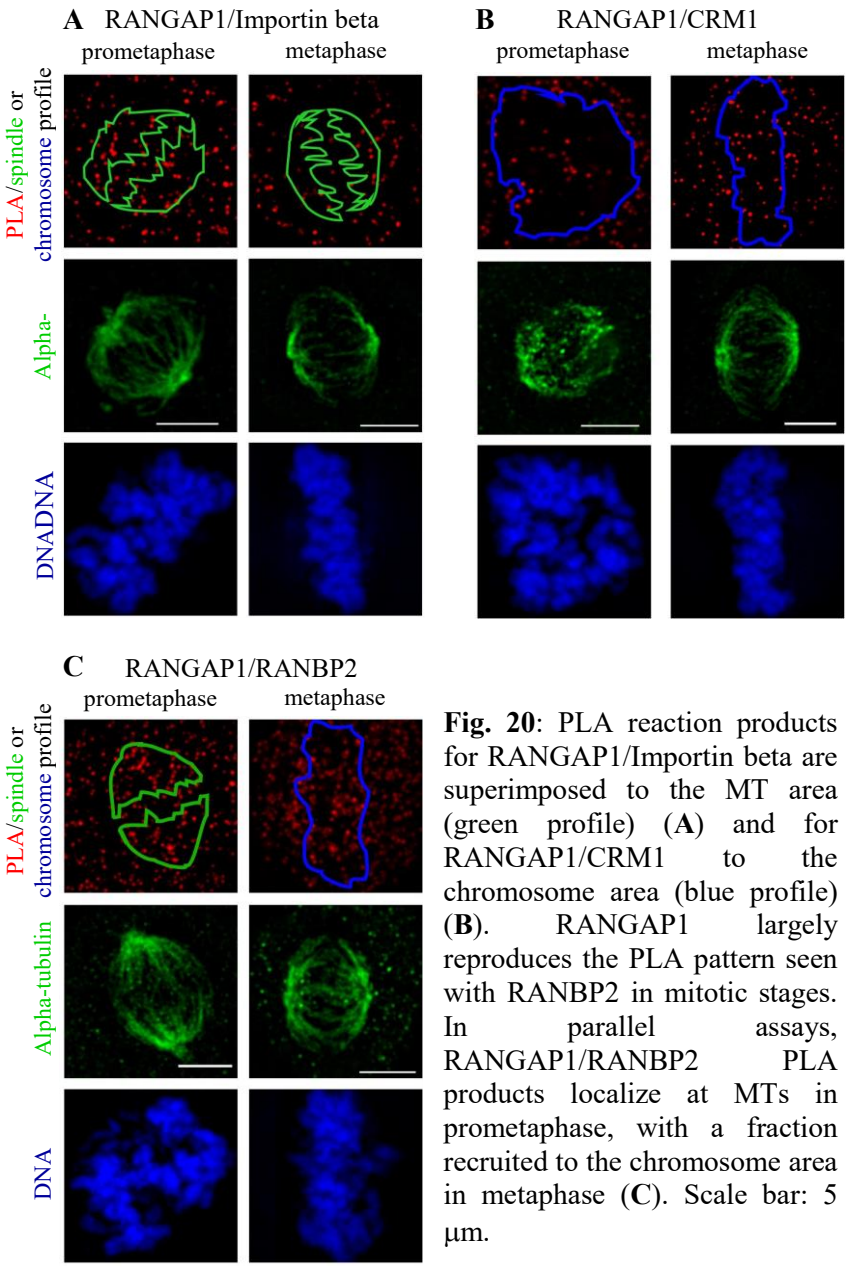

Fig. 20: PLA reaction products for RANGAP1/Importin beta are superimposed to the MT area (green profile) (**A**) and for RANGAP1/CRM1 to the chromosome area (blue profile) (**B**). RANGAP1 largely reproduces the PLA pattern seen with RANBP2 in mitotic stages. In parallel assays, RANGAP1/RANBP2 PLA products localize at MTs in prometaphase, with a fraction recruited to the chromosome area in metaphase (**C**). Scale bar: 5 µm.

As a read-out of the effectiveness of RANBP2 silencing, I observed a dramatic reduction of SUMOylated RANGAP1 by WB (**Figure 21B**) and the absence of RANGAP1 at metaphase KTs in IF images (**Figure 21D**). Moreover, RANBP2-silenced cultures display an increased mitotic index compared to control cells (**Figure 21E**), indicating lengthened mitotic duration and increased mitotic defects (**Figure 21E**), in particular multipolar and mis-aligned mitoses, consistent with previous studies (*Salina et al. 2003; Joseph et al. 2004*).
In this RANBP2-silenced background, PLA interactions were strongly reduced for both RANBP2/Importin beta (**Figure 22A**) and for RANBP2/CRM1 (**Figure 22B**). Although Importin beta abundance was reported to decrease in RANBP2-silenced cells in other experiments (*Hashizume et al. 2013*), I observed no evidence for either Importin beta or CRM1 variation by WB (**Figure 21B**): thus, the loss of PLA signals reflects exclusively the reduction of RANBP2, indicating that the PLA technique depicts genuine RANBP2 mitotic interactions.

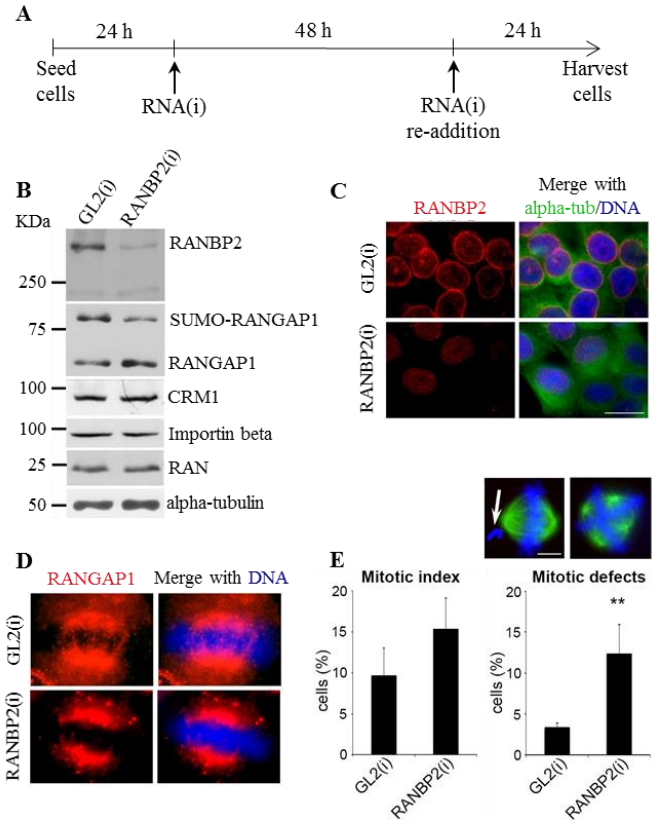

Fig. 21: **A.** Experimental protocol for RANBP2 interference. **B.** The efficiency of RANBP2 depletion after RNAi was assessed by WB analysis. Slowly migrating SUMO-conjugated RANGAP1 also decrease in RANBP2-interfered cells, while nuclear import receptors do not vary. **C.** IF panels show reduced RANBP2 abundance in RANBP2-silenced cells. Scale bar: 20 mm. **D.** RANGAP1 recruitment to KTs fails in RANBP2-interfered cells. **E.** Increased mitotic index and mitotic abnormalities (representative examples are shown, scale bar: 5 μm) in RANBP2-interfered cells compared to control (at least 660 counted mitotic cells per condition in two experiments). ** highly significant differences (X^2 test p value<0.0001)

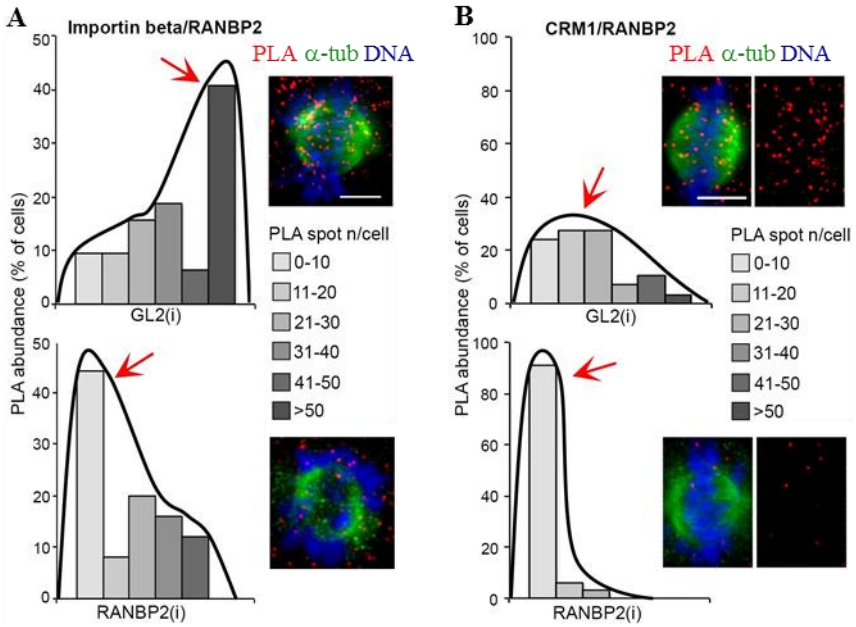

Fig. 22: RANBP2 silencing assays validate the RANBP2 "switch partners" model visualized by PLA
A. The histograms show a significant decrease of Importin beta/RANBP2 PLA signals along MTs in RANBP2-interfered compared to control prometaphase cells (at least 35 cells counted per condition in 2 experiments, $p<0.005$ in the X^2 test). B. Parallel decrease of CRM1/RANBP2 PLA signals in RANBP2-interfered compared to control metaphases (at least 35 counted cells per condition in 2 experiments, X^2 test $p<0.0005$, highly significant). Bars in IF images represent 5 μm.

5. CRM1 silencing, or functional inhibition, reduces RRSU complex deposition at KTs in metaphase

The PLA data suggest that RANBP2 is engaged in complementary, yet mutually antagonistic interactions with importin beta and with CRM1 during mitosis. To verify this, I

analysed RANBP2 localization in cells with down-regulated CRM1. Two different approaches were used to achieve this.
I first used specific siRNA to silence CRM1. Effective protein decrease was detected by WB (**Figure 23B**) and IF (**Figure 23C**); that induced significant mitotic defects, in particular multipolar mitoses, misaligned and mis-segregating mitoses (**Figure 23D**), consistent with previous reports (*Arnautov et al. 2005*). In this CRM1-silenced context I observed decreased PLA CRM1/RANBP2 signals at metaphase KTs compared to controls, consistent with expectations (**Figure 23E**).
Since the RNA interference took 72 hours to achieve effective silencing, I also devised an alternative way to reduce CRM1 activity using a less prolonged treatment to avoid possible indirect effects caused by alterations in nucleo-cytoplasmic transport. I therefore used Leptomycin B (LMB), a functional inhibitor able to abolish CRM1 function in 2 hours. CRM1 inhibition was verified by the retention of RANBP1, a NES-containing protein, within interphase nuclei (**Figure 24B**), and by increased mitotic defects, in particular misaligned and mis-segregating mitoses, compared to controls (**Figure 24D**). After 2 hours of LMB, neither RANBP2 nor RANGAP1 reach KTs in metaphase (**Figure 24C**). PLA results reveal, once again, that RANBP2 and RANGAP1 reach KTs in metaphase as a single unit and that they need functional CRM1 to do this.
Indeed, PLA signals between RANBP2 and RANGAP1 do not decrease in abundance in LMB-treated metaphases, yet fail to localise in the chromosome area while remaining visible on spindle MTs (**Figure 25A**). In parallel, RANBP2/CRM1 PLA signals strongly decrease at metaphase KTs in LMB-treated cells compared to control cells (**Figure 25B**).
Together, these results suggest that functional CRM1 is required to recruit the RRSU complex at KTs in metaphase, which, in absence of CRM1, remains on spindle MTs.

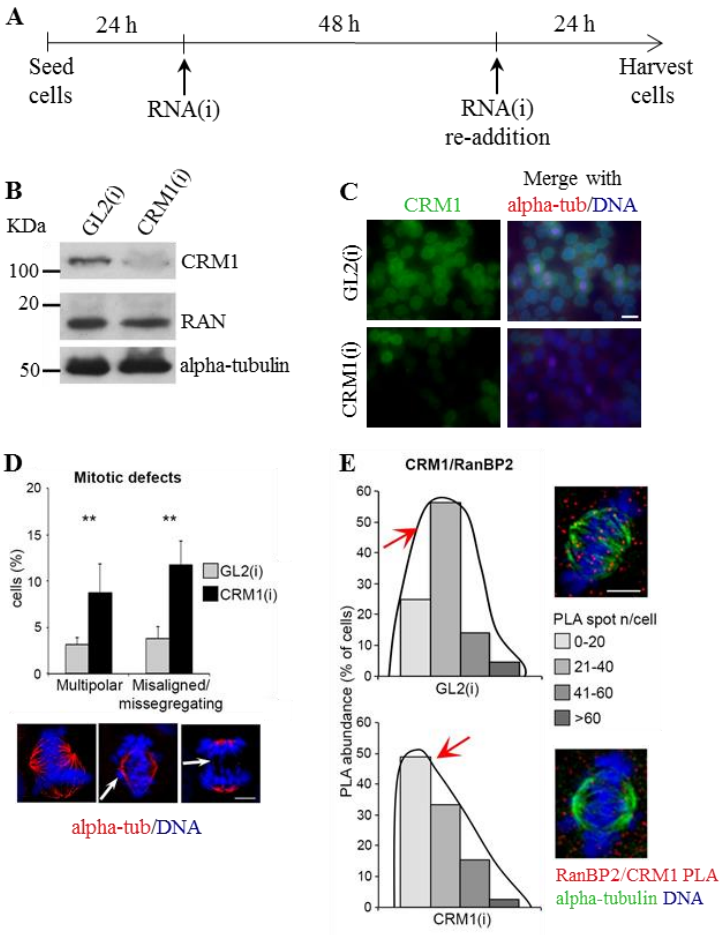

Fig. 23: CRM1 silencing by RNAi reduces RRSU complex deposition at KTs in metaphase

A. Experimental protocol for CRM1 interference. **B**. The efficiency of CRM1 depletion after RNAi was assessed by WB analysis. **C**. IF panels show reduced CRM1 abundance in CRM1-silenced cells. Scale bar: 20 μm. **D**. Frequency of mitotic abnormalities in CRM1-silenced cultures compared to GL2-interfered controls (at least 3000 counted mitotic cells in 3 experiments; ** X^2 test p value<0.0001). Most frequent defects are shown: multipolar spindles, misaligned and mis-segregating chromosomes. Scale bar, 5 μm.

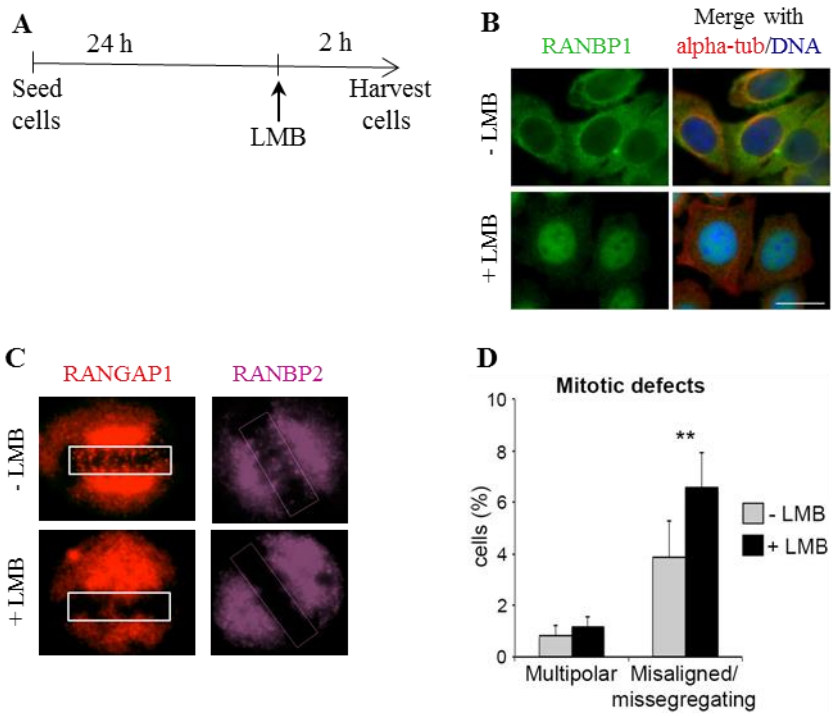

Fig. 24: CRM1 inhibition by LMB impairs recruitment of both RANGAP1 and RANBP2 at KTs in metaphase. **A**. Experimental protocol for CRM1 inhibition by LMB. **B**. LMB treatment abolishes CRM1-dependent export in interphase HeLa cells, as shown by nuclear retention of RANBP1. Bar, 20 μm. **C**. CRM1 inhibition impairs recruitment of both RANGAP1 and RANBP2 to KTs in metaphase. **D**. Frequency of LMB-dependent mitotic abnormalities (at least 3400 counted cells, 2 experiments). ** X^2 test p value<0.0001.

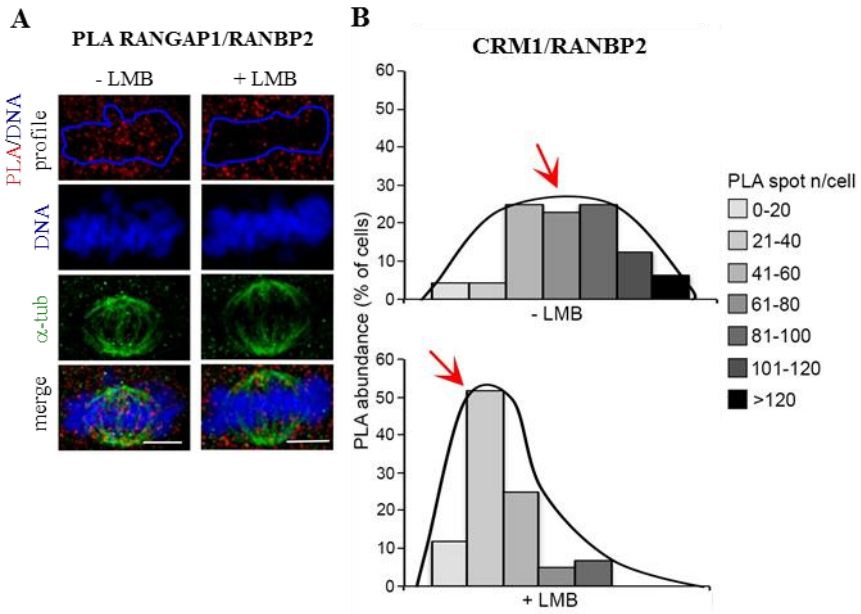

Fig. 25: CRM1 inhibition by LMB impairs RRSU recruitment to KTs in metaphase. A. RANBP2/RANGAP1 PLA signals do not vary quantitatively, but they are not recruited to the chromosome area in LMB-treated metaphases, while remaining visible on spindle MTs. Bar, 5 μm **B.** Distribution of mitotic cells in LMB-treated (+) or untreated (-) cultures grouped in classes of abundance of RANBP2/CRM1 PLA products at KTs (at least 60 cells per condition, 2 experiments, X^2 test p value<0.0001). Red arrows indicate modal classes.

6. Induction of Importin beta overexpression alters RANBP2-dependent interactions in mitotic cells

The data thus far suggest that the RRSU complex in mitosis "switches partners" between Importin beta and CRM1 in an accurately controlled manner, with the final aim to reach KTs in a specific moment of the cell cycle.

A strategy to test the model relies on unbalancing the components that permit the RRSU complex transfer to KTs and

assess whether altered abundance of one transport factor influences RANBP2 interactions with the other one. Transient Importin beta overexpression previously yielded multipolar spindles and chromosome mis-segregation as the most prominent defects (*Nachury et al., 2001; Ciciarello et al., 2004; Kalab et al., 2006*), associated with SUMO-RANGAP1 absence from KTs (*Roscioli et al., 2012*). If SUMO-RANGAP1 moves with RANBP2 in a complex, then Importin beta overexpression should influence the localization of RANBP2, too.To eliminate the variability associated with transient expression, I generated a HeLa cell line with stably integrated EGFP-tagged Importin beta, expressed under the control of a doxycycline (dox)-inducible promoter (**Figure 26A**). In time lapse imaging assays, after dox administration, cells begin to express the exogenous protein, visible via the EGFP-tag, and after 24 hours of recording, all cells display the green fluorescence. In particular, the exogenous Importin beta-EGFP is visible after 3-4 hours of dox-induction and, after 6 hours I measured an overexpression by about 1,8-fold, which increases at about 2,5-fold after 24 hours (**Figure 26B**). WB analysis confirms the presence of the exogenous protein in cells treated with dox (**Figure 26C**) and IF shows that it localizes correctly at the spindle MTs in mitosis (**Figure 26D**). Dox-inducible cell lines overexpressing importin beta displayed complex mitotic defects. 24 hours after dox induction, multipolar mitoses and cell death were recorded by time-lapse (**Figure 27A**). In fixed samples, there was a signficnat increase in mis-aligned and mis-segregating mitoses (**Figure 27B**), which could not be distinguished under the resolution of time-lapse. I used this cell line to assess whether CRM1 overexpression influences RANBP2 interactions. Given that Importin beta-EGFP is visible 6 hours after dox induction, I used this time to avoid cell death induction at later times. I found increased Importin beta-EGFP/RANBP2 PLA interactions at the spindle MTs. The interactions persisted in metaphase, whereas under normal conditions, RANBP2

dissociates from Importin beta at this time and interacts with CRM1 at KTs (**Figure 27C**). Indeed, in Importin beta-induced cells, CRM1/RANBP2 interactions decrease compared to controls (**Figure 27D**). Thus, excess Importin beta retains RANBP2 at the spindle MTs and prevent its recruitment at KTs.

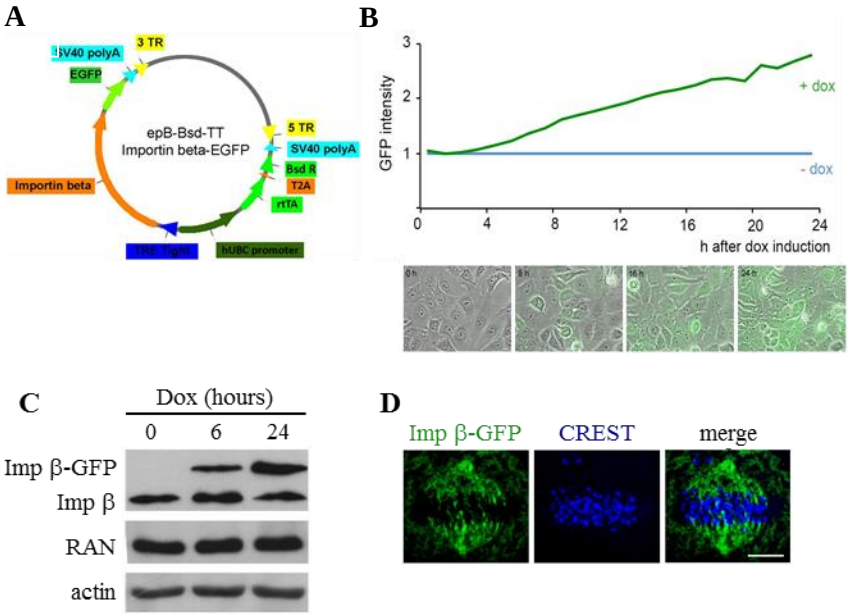

Fig. 26: A Dox-inducible Importin beta-GFP HeLa cell line. A. Enhanced piggyBac (*epB*) vector used to generate stable importin beta-EGFP integrants. **B.** Time-lapse recording of importin beta-EGFP fluorescence after dox induction. **C.** Western blot of cell extracts probed with anti-importin beta: the upper band corresponds to importin beta EGFP chimaera, the lower band is the endogenous protein. **D.** Importin beta-EGFP localizes at MTS, as the endogenous protein. Bar, 5 μm

Indeed, in Importin beta-overexpressing cells, CRM1/RANBP2 interactions decrease compared to controls (**Figure 27D**). These data suggest that excess of Importin beta retains RANBP2 on the spindle MTs in metaphase and prevent its recruitment at KTs.

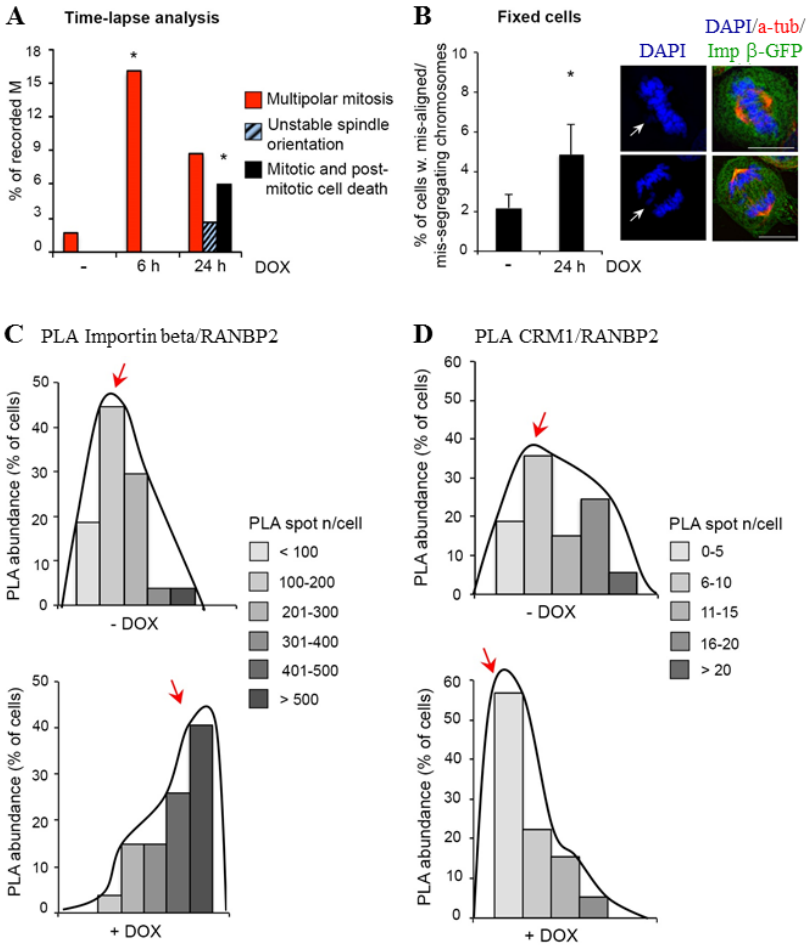

Fig. 27: Decreased RANBP2/CRM1 PLA products at KTs in importin beta-induced cells. A. Mitotic abnormalities in time-lapse recording of uninduced (-) and dox-induced (6 h, 24 h) cells. At least 115 cells per time point were analyzed in 3 experiments. **p* <0.01 (X^2 test). **B.** Frequency of mitotic abnormalities in dox-induced importin beta cultures, exemplified in the IF panels (arrowed): misaligned metaphase chromosome (top), lagging chromosome in anaphase (bottom). Bar, 10 μm. At least 500 mitotic cells per point were counted, two independent experiments, **p* <0.01 (X^2 test).

Fig. 27(continues): C. The histograms represent the distribution of metaphases according to their abundance of importin beta/RANBP2 PLA signals: the increase in dox-induced compared to non-induced cells is highly significant ($p<0.0001$, X^2 test); at least 128 cells per condition were analysed in 3 independent experiments. **D.** The histograms represent the distribution of metaphases according to the abundance of CRM1/RANBP2 PLA signals at KTs: a highly significant decrease of KT-localized PLA products was observed in dox-induced compared to non-induced metaphase cells ($p<0.005$, X^2 test); at least 215 metaphases per condition were counted in four independent experiments. Bars in E-F, 5 μm.

7. Induction of CRM1 overexpression alters the timing of RANBP2-dependent interactions in mitosis

It was interesting at this point to assess whether elevated CRM1 levels also affected RANBP2 interaction in mitosis. I generated a dox-inducible CRM1-EGFP HeLa cell line using the same vector as for Importin beta (**Figure 28A**). Also in this newly generated cell line, time-lapse imaging timed the induction of the exogenous protein within 3-4 hours of dox administration. Western blot of cell samples collected 6 hours after induction detected an increase of CRM1 by about 1,4-fold, which reached about 2-fold after 24 hours (**Figure 28B**). The exogenous protein was detected by WB (**Figure 28C**), and, by IF, it was found to localize largely at the spindle and in part at metaphase KTs, like the endogenous counterpart (**Figure 28D**).

Time-lapse analysis revealed a significant increase in mitotic defects, particularly multinucleated cells (**Figure 29A**). Indeed, fixed cells displayed a remarkable fraction of metaphases harbouring mis-aligned chromosomes and telophases with unsegregated chromosomes (**Figure 29B**).

PLA assays revealed that RANBP2/Importin beta interactions are down-regulated in CRM1-overexpressing prometaphases compared to controls (**Figure 29C**). Concomitant with this, CRM1/RANBP2 localized prematurely at KTs, and more abundant PLA signals were detected in CRM1-overexpressing

prometaphase compared to controls (**Figure 29D**). Interestingly, CRM1 overexpression did instead not increase the recruitment of RANBP2 at metaphase KTs.

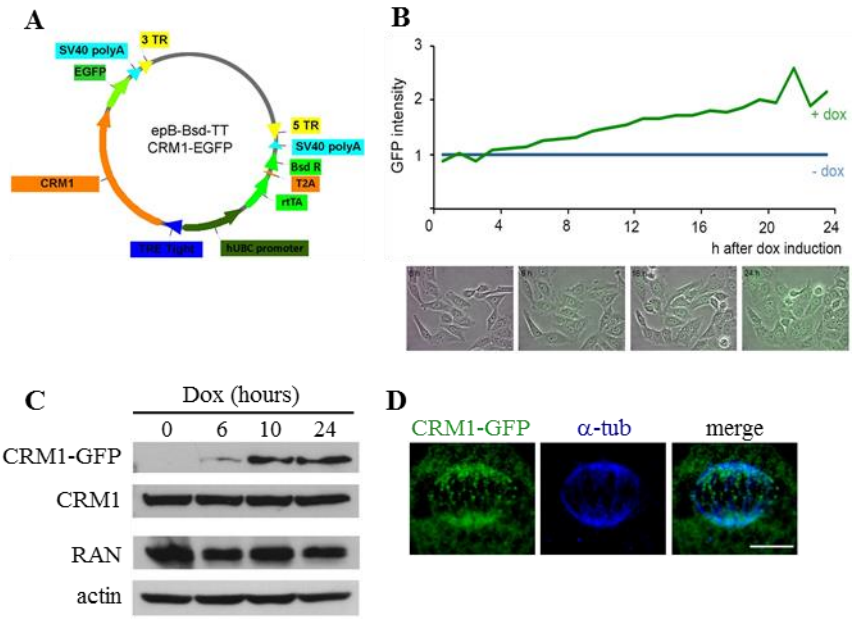

Fig. 28: A HeLa cell line with stably integrated dox-inducible CRM1-GFP. A. Enhanced *piggyBac (epB)* derived vector used to generate stable CRM1-EGFP integrants. **B.** Time-lapse recording of exogenous CRM1-EGFP fluorescence after dox induction. **C.** Western blot of cell extracts probed with anti-CRM1 antibody after dox induction. **D.** Dox-induced CRM1-EGFP reproduces the same localization as the endogenous protein at spindle MTs and at KTs. Bar, 5 μm.

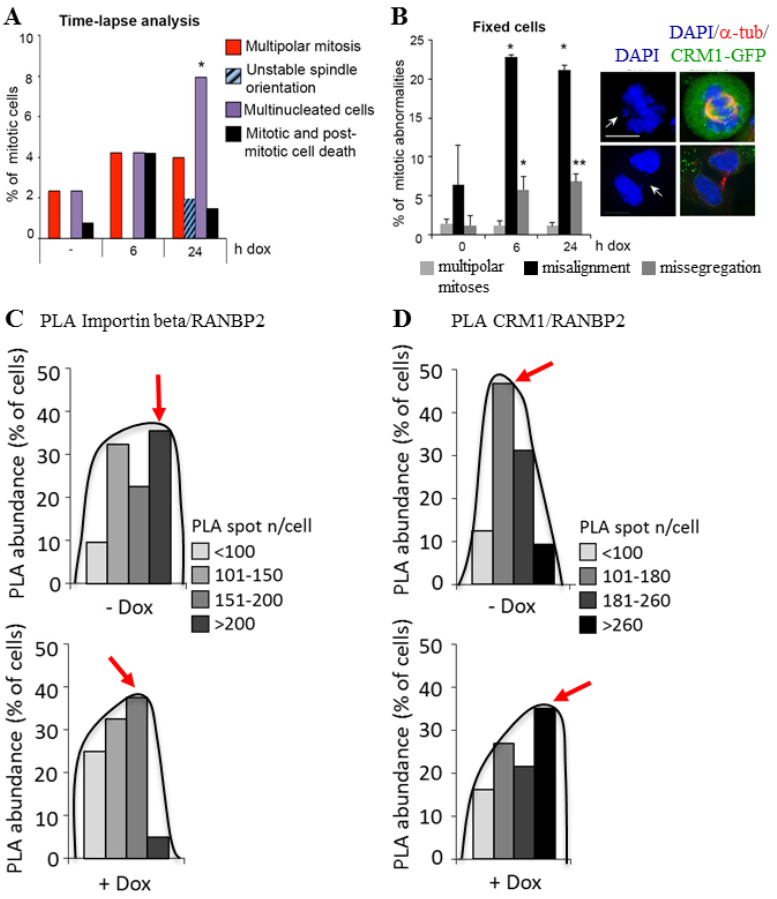

Fig. 29: In a CRM1-induced context, RANBP2 interacts prematurely with CRM1. A. Mitotic abnormalities in uninduced (-), and dox-induced (6 h, 24 h) videorecorded live cultures. At least 130 cells were analysed in 2 independent experiments. * *p* value<0.05 (X^2 test). **B.** Mitotic abnormalities in fixed and IF-stained CRM1-induced cultures: top, metaphase misaligned chromosome; bottom, failed chromosome segregation in telophase (bar, 10 μm). Pooled abnormalities (quantified in the histograms) showed significantly different frequencies in dox-induced vs. uninduced cultures (X^2 test *p* values, *<0.025 and ** <0.001; at least 300 counted mitotic cells per time point in 2 experiments).

Fig. 29(continues): C. The histograms represent the distribution of metaphase cells in classes of abundance of importin beta/RANBP2 PLA products at MTs. The X^2 test indicates a significant decrease in CRM1-induced compared to non induced cultures (X^2 test *p* value<0.01, from 40 analysed metaphases per condition in 3 independent experiments). **F.** PLA signals for CRM1/RANBP2 significantly increase at KTs in prometaphase cells in CRM1-induced vs. non-induced cultures. Histograms represent the distribution of prometaphases according to their content of RANBP2/CRM1 PLA products at KTs (at least 40 analysed prometaphases per condition in 2 independent experiments, $p<0.05$).

These data suggest that CRM1 overexpression impairs Importin beta/RANBP2 interaction in prometaphase and causes premature RANBP2 recruitment at KTs. This is associated with severe segregation abnormalities, many of which are not corrected and generate multi-nucleated cells.

In experiments designed to test the functional consequences of this altered recruitment, I found that CRM1-overexpresssing cells harbour hyperstable MTs, particularly at the level of K-fibers, as indicated by their resistance to cold-induced depolymerisation (**Figure 30C**). Furthermore, when cells are shifted from 0°C to 37°C, MTs re-grow faster in CRM1-overexpresssing cells compared to controls (**Figure 30D**). Collectively, these data suggest that increased CRM1 levels stabilize KT-MT interactions and accelerate MT-nucleation.

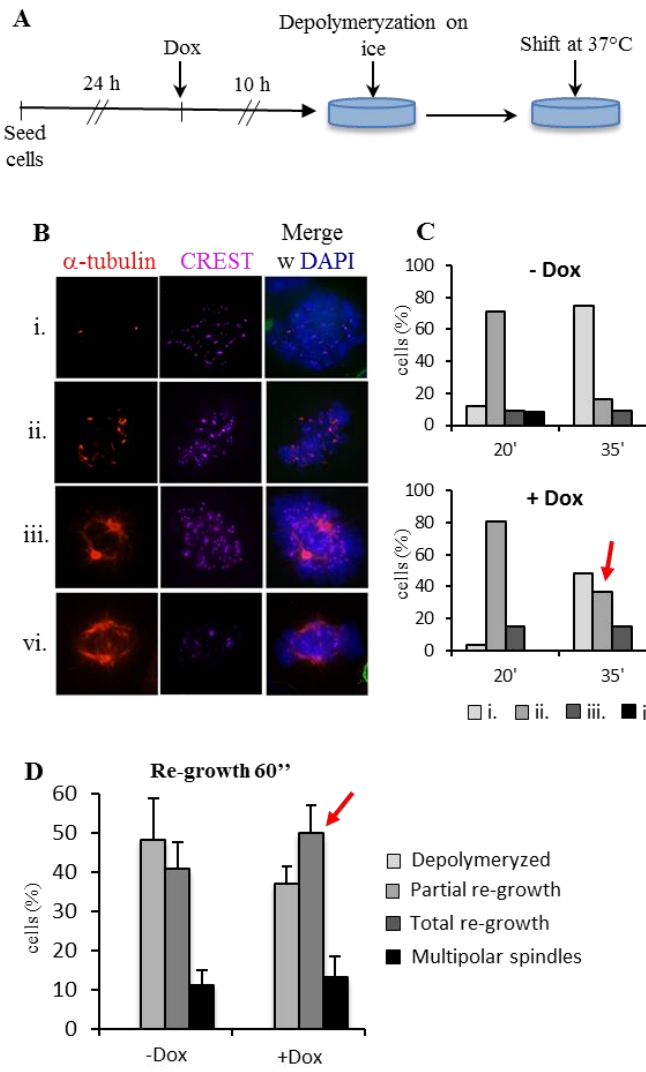

Fig. 30: CRM1 overexpression stabilizes K-fibers. A. Experimental protocol for MT depolymerization on ice and re-growth at 37°C. **B.** Different phenotypes observed during depolymerisation on ice: (i) complete depolymerisation (ii) K-fibers (iii) partial depolymerisation (vi) normal spindles.

Fig. 30(continues): C. Depolymerization on ice shows that CRM1-overexpressing cells (+dox) are more stable than non-overexpressing cells (-dox), as indicated from K-fibers (red arrows) present after 35' of depolymerization. **D**. MTs re-growth after shift from 0°C to 37°C shows that in CRM1-overexpressing context MTs re-polymerize faster than non-overexpressing context.

8. MTs are essential for RRSU complex localization in mitosis

Previous findings that RANBP2 co-immunoprecipitates with transiently transfected Importin beta, with or without MTs (*Roscioli et al., 2012*), suggest that MTs are not indispensable for their interaction to occur, at least in mitotic cell extracts. It was interesting to assess whether the absence on MTs can influence the interactions between components of the nuclear transport machinery in intact mitotic cells.

To completely inhibit MTs assembly I treated pre-synchronized HeLa cells with Nocodazole while they were synchronously progressing towards mitosis. With this protocol, treated mitoses showed complete depolymerisation of MTs (by alpha-tubulin staining) and spread chromosomes in a prometaphase-like configuration (**Figure 31D**).

I found that, under these conditions, PLA products for Importin beta and RANBP2 were spread throughout the cell, very different from the localization seen in normal cells. However, quantitative analysis showed no differences in the abundance of PLA products between NOC-treated and untreated cells (**Figure 31B, upper graph**). This finding, extending the results obtained by *Roscioli* et al. in co-immunoprecipitation assays, indicates that Importin beta and RANBP2 can interact independent on the presence of MTs, but MTs are required to localize them.

CRM1/RANBP2 also interact in NOC-treated cells (**Figure 31B, lower graph**) and, as seen for Importin beta/RANBP2, they are spread throughout the cell with no particular localization (**Figure 31D, right panels**). To examine KTs more accurately, I found that manual counting of each spot relative to

the KTs in single z-stacks was more accurate then automated counting. Indeed, using this method I can discriminate between genuine KT-localized PLA signals (**Figure 31D**, *a* square, enlarged in *a'*), which localize therein not only in the MIP image but also in every single z-stack, and PLA signals that seem to localize on KTs in the MIP image, but in fact do not do so in single z-stacks (**Figure 31D**, *c* square, enlarged in *c'*). In the "manual" mode, i.e. intentionally selecting the signals to be quantified, it is possible to observe that KT-localized CRM1/RANBP2 signals significantly decrease in NOC-treated compared with untreated cells (**Figure 31C**). As a control, I examined the BubR1 SAC kinase. Differently from CRM1/RANBP2, BubR1 localizes on all KTs in the absence of MTs, both in the MIP image and in every single z-stack (**Figure 31D**, b square, enlarged in b'). These results show that CRM1 and RANBP2 interact independent on the presence of MTs, yet their localization on KTs need MTs, as previously seen for Importin beta and RANBP2.

In summary, therefore, MTs are not required as an assembly platform for RANBP2 interactions with nuclear transport factors, but they are necessary for their localization.

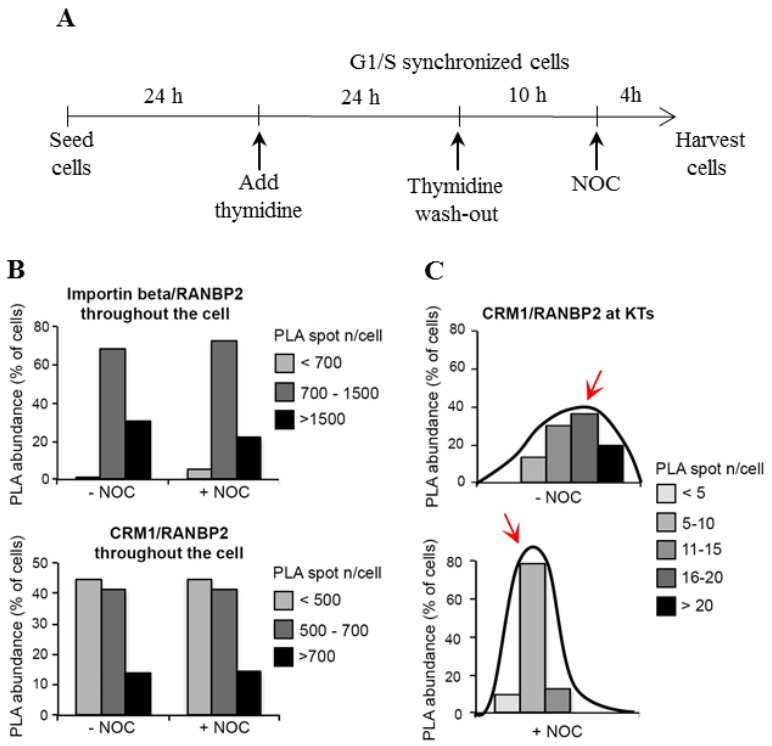

Fig. 31: NOC disrupts the localization but not the formation of RANBP2-containing PLA products. A. Experimental protocol for MT depolymerization with Nocodazole (NOC). **B.** Abundance of PLA products for either RANBP2/Importin beta, or RANBP2/CRM1, in prometaphases from control and NOC-treated cultures. No statistical difference was observed. **C.** The histogram distribution of cells according to the abundance of localized RANBP2/CRM1 PLA products at KTs shows a dramatic decrease in NOC-treated cultures (at least 65 counted cells in 2 experiments, X^2 p-value <0.0001)

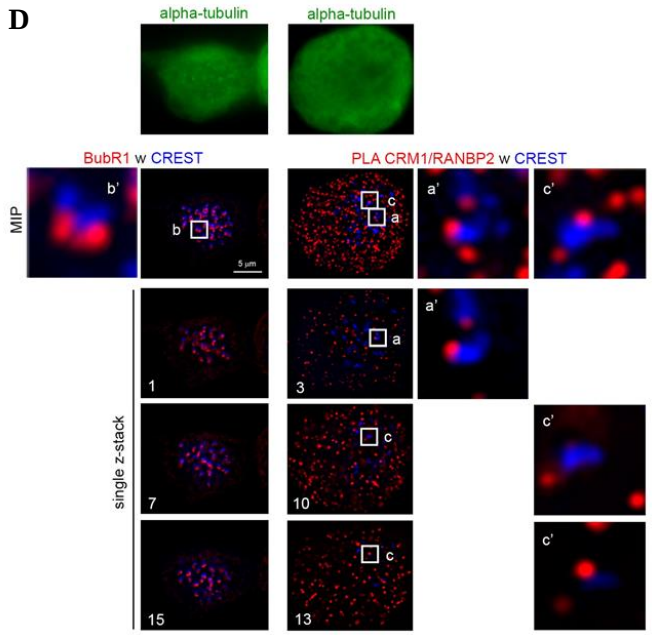

Fig. 31D. Analysis of BubR1 (left columns) and RANBP2/CRM1 PLA products (right columns) at the single z-stack level in NOC-treated cultures. NOC effectiveness was monitored by staining alpha-tubulin (green), which is totally diffuse after treatment. Individual z-stacks are shown below the MIP fields. In the left column, BubR1 signals co-localize with CREST-stained KTs in all stacks (stacks 1, 7 and 15 are shown as examples). In the right column, the MIP field shows many RANBP2/CRM1 PLA products (red) spread in the cell outside of KTs (CREST); single stack scanning shows that, of the apparent PLA-CREST associations seen in the MIP image, only some are genuine (an example is framed in the MIP field as a, enlarged in a', which shows genuine localization on the outer KT in stack 3); other PLA signals, though apparently overlapping with CREST, in fact do not associate with KTs in individual stacks, as the framed example in c in the MIP field: the c' zoom-in shows the KT at stack 10, whereas the PLA spot lies at stack 13. This dissociation was not seen in PLA experiments in cells with unperturbed MTs. Bar, 5 µm. a', b' and c' represent 8x enlargements of framed insets in a, b, c.

As a whole, the results obtained in this study indicate a) PLA is a novel informative tool to investigate dynamic interaction during mitotic progression; b) regulated interactions are operated by nuclear transport factors to achieve temporally and spatially controlled delivery of the RRSU complex to KTs at metaphase; c) alterations in the system are associated with severe mitotic abnormalities that are due, at least in part, to altered propertied of KT-originated MTs.

DISCUSSION

1. Members of the nuclear transport machinery in mitosis

It is now well established that components of the nuclear transport machinery play fundamental functions both in interphase and during mitosis (*Cavazza and Vernos, 2016*). These include the GTPase RAN, the regulators of its nucleotide-bound state (i.e. RCC1 and RANGAP1), nuclear transport receptors (e.g. Importin beta and CRM1), and several components of NPCs. At mitosis onset, after NE breakdown and NPCs disassembly, these proteins re-localize on mitotic structures, where they contribute to mitotic spindle formation and function.

As described in the introduction, fractions of CRM1 localize at centrosomes, spindle MTs and metaphase KTs. The KT-associated fraction of CRM1 was previously shown to be necessary to recruit the RANBP2/RANGAP1 complex to metaphase KTs (*Arnaoutov et al., 2005*). Once at KTs, RANGAP1 induces local RANGTP hydrolysis. Thus, the timely recruitment of RANGAP1 can introduce a local discontinuity in the RAN gradient at the level of MT-attached KTs (*Dasso, 2006*).

RANBP2 also interacts with Importin beta in mitosis (*Roscioli et al, 2012*) along mitotic spindle MTs.

RANBP2 and SUMOylated RANGAP1 remain associated throughout mitosis forming, together with Ubc9, the RRSU (RANBP2/RANGAP1-SUMO/Ubc9) complex (*Swaminathan et al. 2004*), a multisubunit SUMO E3 ligase (*Werner et al., 2012*).

Most of the findings summarised above rely on biochemical studies with whole cell extracts, and on immunofluorescence localization studies of single components, but their dynamic interplay could not be clarified using these methods.

2. The PLA approach is a new informative tool to investigate dynamic interactions between the RRSU SUMO ligase complex and transport receptors in mitosis: the "switch partners model"

To gain insight into the RRSU regulation in space and time during mitosis, here I have developed Proximity Ligation Assay with the aim of visualizing the interaction(s) between the RRSU complex and transport receptors, Importin beta and CRM1, *in situ* in intact cells. PLA results show that, in mitosis, RANBP2 engages in dynamic interactions with the two transport receptors in a temporally and spatially regulated manner.

In particular, RANBP2 interacts abundantly with Importin beta in early stages of mitosis, until metaphase. These interactions localize along spindle MTs. When KTs become attached to MTs at metaphase these interactions decrease. Conversely, RANBP2 interactions with CRM1 increase in metaphase, when they localize specifically at MT-attached KTs. In performing PLA using RANGAP1, instead of RANBP2, the same dynamic localization pattern was obtained, indicating that using an antibody against one or the component of the RRSU complex, it is possible to trace the entire complex. This is confirmed by performing direct PLA between RANBP2 and RANGAP1, whose interaction shows a parallel pattern in space and time.

The RRSU complex localization at metaphase KTs is of high importance for proper progression of mitosis. In the absence of RANBP2, RANGAP1 is not SUMOylated and does not reach KTs in metaphase (*Joseph et al., 2004*). This was previously reported to induce mitotic defects (*Salina et al., 2003; Joseph et al., 2004*). I have now confirmed that RANBP2 silencing does indeed affect the establishment of a bipolar mitotic spindle and chromosome alignment in metaphase.

Similar mitotic defects were induced when CRM1 function was inhibited, either by RNA interference or by LMB treatment.

This is known to prevent RANGAP1 localization to KTs in metaphase (*Arnaoutov et al., 2005*).
Interestingly, in the PLA assays developed in this project, that both RANBP2- and CRM1 silencing yield significantly decreased interactions at metaphase KTs if compared to control conditions. Most importantly, I found that, when CRM1 function is inhibited by LMB treatment, complexes comprising RANBP2 and RANGAP1 fail to reach KTs in metaphase and remain along the spindle MTs, indicating that the RRSU complex needs functional CRM1 to reach metaphase KTs. In retrospect, these data suggest that the mitotic defects observed in the absence of either RANBP2, or CRM1, are due - in part - to the lack of RRSU complex at KTs, and also, at least in part, to their retention along MTs, to which they could impart excessively highly dynamic functions, which fit the scopes of prometaphase ("search-and-capture" process) but require some down-modulation in metaphase.

3. Microtubules are not required for RANBP2 interactions, but play an important part in spatial control of the "switch partners" model.
Since Importin beta interacts with mitotic MTs (*Ciciarello et al., 2004*), I investigated the role of MTs in the RRSU complex interactions and localization. Using NOC to depolymerize MTs, I found that MTs are not indispensable for the RRSU complex interaction with transport receptors, but are required for its localization on mitotic structures. Indeed, after NOC treatment, RANBP2/Importin beta PLA signals are still visible but they are spread throughout the cell. Similarly, RANBP2/CRM1 PLA signals are visible in NOC-treated cells, but they are not recruited to KTs. Thus, the localization of the RANBP2/CRM1 complex require MTs as an integral part of the "switch partners" model underlying the RRSU localization in mitosis.

4. Generating inducible cell lines to unbalance transport receptors

To assess whether unbalancing the ratio between transport receptors influences the localization of the RRSU complex in mitosis, I generated two stable HeLa cell lines that overexpress either Importin beta, or CRM1, in an inducible, and regulated, manner. PLA results show that, in an Importin beta-overexpressing context, the RRSU complex is retained at mitotic MTs in metaphase, in association with Importin beta, instead of being released at KTs via CRM1 recruitment. Conversely, when CRM1 is overexpressed, RRSU interactions with Importin beta are down-regulated in prometaphase compared to control cells, when this interaction is physiologically abundant. At the same time, the RRSU/CRM1 complex recruitment to KTs is anticipated in CRM1-overexpressing prometaphases compared to controls.

These data suggest that increased CRM1 abundance induces a premature release of the RRSU complex from its interaction with Importin beta, recruiting it at KTs before all KTs are attached by MTs in a bi-oriented manner. Interestingly, KT-associated CRM1/RANBP2 complexes, which give abundant signals in physiological metaphases do not increase any further by increasing CRM1 abundance in the dox-inducible cell line.

These results together suggest that unbalancing one or the other transport receptors disrupt the temporal and spatial control of the RRSU localization in mitosis, respectively yielding a failed or a premature recruitment at KTs.

As a mean to assess the functional consequences of deregulating RRSU interactions at KTs, we have carried out MT depolymerization assays under conditions under which RANBP2 is delocalized from KTs (importin beta overexpression, CRM1 inactivation), or is recruited precociously (CRM1 overexpression), or is absent (RANBP2 silencing) (summarized in Table 2). In these assays, we measure the extent of KT-attached MTs stabilization. Indeed,

overexpressing CRM1 makes kinetochore-fibers (K-fibers) more stable to cold-induced depolymerization, while Importin beta overexpression de-stabilizes K-fibers.

Table 2. K-fibers stability in cell contexts with a complex localization

Deregulated factor	RRSU at KTs	K-fiber status after cold	Reference
CRM1 overexpression	Anticipated (prometaphase)	Stabilized	This study
Importin beta overexpression	Failed or reduced (this study)	Destabilized	Verrico A. PhD project
CRM1 inactivation/ silencing	Failed (this study)	Destabilized	Arnaoutov et al., 2005
RANBP2 silecing	Failed (this study)	Destabilized	Joseph et al., 2004

RRSU mislocalization is also associated with mitotic defects, in particular i) multipolar mitosis, significantly increased when Importin beta is overexpressed and RRSU is retained along the spindle, ii) defects in chromosome alignment in metaphase and segregation in ana/telophase in both CRM1- and importin beta-inducible cell lines, suggesting that the correct timing of RRSU recruitment at KTs is necessary for correct metaphase alignment and progression to anaphase. Finally, iii) multinucleated cells were also observed in the CRM1-overexpressing cell line; it will be interesting to ascertain whether these cells originate solely from RANBP2-dependent mis-segregating chromosomes or form some additional effect of CRM1.

5. RRSU complex activity at metaphase kinetochores is required for correct metaphase to anaphase progression

As previously described in the introduction, the RRSU complex may have two fundamental functions at metaphase KTs.

First, RANBP2 may be required to SUMOylate or stabilize KT proteins that need this modification for their interactions and/or functions at KTs. Indeed, a growing number of KT proteins, in addition to RANGAP1 itself, are known to be conjugated with SUMO. Altering the timing, and/or the amount, of KT-associated RRSU may perturb the SUMOylated status of these proteins and hence the processes in which they operate.
Second, RANGAP1 has an important function at KTs via RAN. Highly concentrated chromosomal RANGTP induces MT nucleation from KTs, as recalled (*Tulu et al, 2006; Torosantucci et al. 2008*), which contribute to the formation of a functional spindle in prometaphase (*Cavazza and Vernos, 2016*). When metaphase is reached, nucleation of new MTs from KTs must stop, while those MTs that have attached to KTs must be stabilized to allow chromosome segregation at anaphase. RANGAP1 deposited to KTs in metaphase may function to locally hydrolyse RANGTP, thus inducing the cessation of MT nucleation from KTs.
The findings in this Thesis suggest that the delivery of the RRSU complex at KTs is crucial for correct progression of mitosis. I have shown that transport receptors are crucial to ensure timely and spatial regulation to the RRSU complex. The PLA studies that I performed suggest that the RRSU complex "switches partner" from prometaphase to metaphase: it first interacts with Importin beta along the spindle MTs in prometaphase. In metaphase, after all KTs are MT-attached in a bioriented manner, CRM1 recruits the RRSU complex at KTs. At this moment, RANBP2 and RANGAP1 can play their functions and mitotic progression can move on from metaphase to anaphase without errors.
The defects observed in this study are compatible with unscheduled (in the CRM1-overexpressing cell line, in which the RRSU is prematurely recruited at KTs), or failed (in the importin beta-overexpressing cell line) RANGTP hydrolysis at KTs, affecting both RAN-directed MT nucleation from KTs

(*Tulu et al., 2006; Torosantucci et al., 2008; Cavazza and Vernos, 2016*), and possibly, also maturation of MT/KT attachments (*Dasso et al., 2006; Roscioli et al., 2012*).
Finally, the RRSU complex acts as a disassembly machinery for CRM1-dependent complexes in interphase nuclear transport (*Ritterhoff et al., 2016*). Premature RRSU complex deposition at mitotic KTs may therefore cause the early release of NES-containing proteins, for example Survivin (*Knauer et al., 2006*). Anticipating these events at a stage in which MT/KT attachments are not yet fully established may cause defects on chromosome segregation, as recorded in the CRM1-inducible cell line.
In conclusion, in my PhD project I have characterized a finely regulated mechanism through which the RRSU complex localizes at specific mitotic structures in a temporally and spatially regulated manner. Importin beta and CRM1 play an antagonistic role in the regulation of the RRSU complex localization. I found that unbalancing their mutual ratio yields the loss of regulated localization of the RRSU complex in mitotic cells, leading to severe defects in mitotic chromosome segregation. This can have relevant implications, since several cancer types overexpress these karyopherins (*Rensen et al., 2008; van der Watt et al., 2009*) and inhibitors of nuclear transport factors are being developed with therapeutic purposes (*Stelma et al., 2016; Mahipal and Malafa, 2016*).

MATERIALS AND METHODS

Cell culture, synchronization and treatments

Human HeLa epithelial cells (American Tissue Culture Collection, CCL-2) were grown in DMEM supplemented with 10% fetal bovine serum, 2% l-glutamine, 2.5% HEPES and 2% penicillin/streptomycin at 37°C in 5% CO2. Cells were synchronized in 2 mM thymidine for 20-24 hours to induce G1/S arrest, then released in medium containing 30 μM deoxycytidine to progress synchronously towards M phase. Cells were treated with 400 ng/ml NOC (Sigma-Aldrich) 10 h after thymidine release and harvested 4 h later. For MT stability studies cells were incubated for 20 min on ice; in MT regrowth assays after depolymerization, cells were incubated for 35 min on ice, prewarmed media was then added, and cells were incubated at 37°C for 1 min. LMB (Enzo Life Sciences) was used 20 nM in asynchronous cultures for 2 h.

Generation of stable cell lines for importin beta and CRM1

Inducible expression Vectors for importin beta-EGFP and CRM1-EGFP were derived from the enhanced piggyBac (ePiggyBac) vector. The vector carries a tetracycline-responsive promoter element followed by a multicloning site. To generate epB-Bsd-TT-importin beta-EGFP, the importin beta-EGFP sequence was PCR-amplified from the pIB-GFP construct (Ciciarello et al., 2004) using the oligos pEGFP-N1_Fw_ClaI (GGCATCGATAGCGCTACCGGACTC) and pEGFP-N1_Rv (ACCTCTACAAATGTGGTATGGC). The PCR fragment was digested and cloned between the ClaI and NotI sites in the epB-Bsd-TT plasmid, in which the Puromycin resistance gene in the original epB-Puro-TT (Rosa et al., 2014) with a Blasticidin resistance gene. The epB-Bsd-TT-CRM1-EGFP vector was generated by subcloning the CRM1-EGFP sequence (Roscioli et al., 2012) between the BamHI and NotI sites of epB-Bsd-TT. HeLa cells were co-trasfected with vector and hypb7 (encoding the transposase gene) using Lipofectamine (Invitrogen). 24 h after

transfection, the medium was replaced with Tet-free DMEM supplemented with 3 μg/ml blasticidine-S hydrochloride (Sigma). Blasticidine-S-resistant foci were expanded and tested for expression after administration of 1 μg/ml doxycyline hyclate (dox, Santa Cruz Biotechnology).

RNA interference

The following small RNA oligonucleotides were used: 5'-GGACAGUGGGAUUGUAGUGTT-3' targeting RanBP2 (Ambion) and 5'-CGUACGCGGAAUACUUCGA TT-3' (GL2, Ambion) targeting the luciferase gene. For CRM1 a pool of three siRNAs was used (sc-35116, Santa Cruz Biotechnology). Final concentrations of siRNAs were 150 nM for RanBP2 and 20 nM for CRM1 and GL2. siRNA duplexes were diluted in serum-free OptiMem and transfected using Oligofectamine (Invitrogen).

Immunofluorescence

Cells were fixed in 3.7% paraformaldehyde/30 mM sucrose, permeabilised with 0.1% Triton X-100 and incubated with antibodies (Table). Blocking and incubation (in PBS, 0.05% Tween-20, 3% bovine serum albumin) were at room T°. Secondary antibodies were conjugated to fluorescein isothiocyanate (FITC), Cy3 or 7-amino-4-methylcoumarin-3-acetic acid (AMCA) (Jackson Immunoresearch Laboratories), or Texas Red (Vector Laboratories). DNA was stained with 0.1 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma Aldrich) and coverslips were mounted in Vectashield (Vector Laboratories).

Proximity ligation assay (PLA)

Duolink PLA kits were used following the Olink Bioscience protocol. Cells were blocked and incubated with primary antibody (Table); anti-mouse MINUS and anti-rabbit PLUS PLA probes (diluted 1:5 in PBS, 0.05% Tween-20, 3% bovine serum albumin) were added and incubated in a pre-heated humidity chamber (1 hour, 37°). Hybridizations, ligations and detection were performed

following the Duolink Detection protocol.

Table 3. Primary antibodies.

Protein	Host	Source	Catalog	Dilution (IF)	Dilution (PLA)
alpha-tubulin	chicken	Abcam	ab89984	1:50	
alpha-tubulin	mouse	Sigma	T5168	1:3000	
BUBR1	rabbit	Bethyl	A300-995A	1:1000	
CREST	human	Antibodies Inc.	15-234-0001	1:20	
CRM1	rabbit	Santa Cruz	sc-5595	1:50	1:50
CRM1	mouse	Santa Cruz	sc-74455		1:100
Importin beta	mouse	Abcam	ab2811	1:3000	1:3000
RANBP1	goat	Santa Cruz	sc-1160	1:25	
RANBP2	mouse	Santa Cruz	sc-74518	1:50	1:50
RANBP2	rabbit	Abcam	ab64276	1:2000	1:2000
RANGAP1	rabbit	Santa Cruz	sc-25630	1:200	1:200
RANGAP1	mouse	Abcam	ab28322		1:100
GFP	rabbit	Abcam	ab6556		1:1000

Microscopy

Samples were analyzed under a Nikon Eclipse 90i microscope equipped with a Qicam Fast 1394 CCD camera (Qimaging). Single-cell images were taken using an immersion oil 100x objective (NA 1.3) and entire fields under a 40x objective (NA 0.75) using NIS-Elements AR 3.2 and 4.0 softwares (Nikon); three-dimensional deconvolution of 0.3-0.4 μm z-serial optical sections was performed using the "AutoQuant" deconvolution module of NIS-Element AR 3.2/4.0. Creation of image projections from z-stacks was performed using the Maximum Intensity Projection (MIP, for quantitative analyses), and Extended Depth of Focus (EDF) functions of NIS-Element AR 3.2/4.0. IF signals were quantitatively analysed using NIS-Element AR 3.2/4.0 (nd2

file format); external background correction was applied and the sum intensity of signals on indicated selected areas was measured. PLA spots were counted on images acquired on three dimensions. In the manual count mode, PLA spots were counted in each individual z-stack. In the automatic mode, images were processed using the MIP method (therefore loosing quantitative information for each separate z-stack) and activating the "spot detection" and “count objects” tools of NIS-Element AR 3.2/4.0. All figures shown in this work represent MIP images unless specified otherwise. Images were processed with Adobe Photoshop CS 8.0.

Time-lapse imaging

Cells were seeded in 4/8 wells µ-Slide (chambered coverslip, 80426/ 80821, IbiTreat; Ibidi). During recording, cultures were kept at 37°C in a T°- and CO2-controlled stage incubator (Okolab). Cultures were recorded under a Ti Eclipse automated inverted microscope (Nikon) equipped with a DS-Qi1MC camera, an Intensilight C-HGFIE lamp, and the NIS-Elements 3.1 software (Nikon). Images were taken using a 60x, 0.7 NA objective: phase every 15 min, GFP-fluorescence every 60 min.

Western immunoblotting

HeLa cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP40, 1 mM EGTA, 1 mM EDTA, 0.1% SDS, 0.25% sodium deoxycholate) supplemented with protease (05892791001, Roche) and phosphatase (PhoSTOP, 04906837001, Roche) inhibitors. 40 µg extract per lane were separated through SDS-PAGE and transferred to nitrocellulose filters (Protran BA83, Whatman) in a semi-dry system (BIO-RAD). Blocking and antibody incubations were in TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 0.1%Tween 20 and 5% low fat milk (1 h, room T°). HRP-conjugated antibodies (Santa Cruz Biotechnology) were revealed using the ECL system (GE Healthcare) on Hyperfilm-ECL films (GE Healthcare).

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