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Review

Deciphering the spatio-temporal regulation of entry and progression through mitosis

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Mitosis has been studied since the early 1880s as a key event of the cell division cycle where remarkable changes in cellular architecture take place and ultimately lead to an equal segregation of duplicated chromosomes into two daughter cells. A detailed description of the complex and highly ordered cellular events taking place is now available. Many regulators involved in key steps including entry into mitosis, nuclear envelope breakdown, microtubule (MT) spindle formation, and chromosome attachment, as well as mitotic exit and cytokinesis, have also been identified. However, understanding the precise spatio-temporal contribution of each regulator in the cell reorganization process has been technically challenging. This review will focus on a number of recent advances in our understanding of the spatial distribution of protein activities and the temporal regulation of their activation and inactivation during entry and progression through mitosis by the use of intramolecular Förster resonance energy transfer (FRET)-based biosensors.

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1 Introduction

A human body performs about 10 000 trillion cell divisions in a lifetime. Cell division is a fascinating and highly regulated process which leads to the formation of two daughter cells with an equal partitioning of the genetic material, ensuring cell proliferation and tissue regeneration during development and adulthood. It requires in a very short time (~1 h) a complete reorganization of the cell in a highly ordered manner; reorganization of the cytoskeleton, condensation of chromatin, nuclear envelope breakdown, stable attachment of chromosomes to opposite spindle poles, chromosome segregation, and cytokinesis. Because it is technically not possible to synchronize cells at each step of mitotic progression, biochemical approaches have been limited to understanding the spatio-temporal regulation and roles of key regulators in this process, including kinases, phosphatases, and small GTPases. In this review, we will provide an overview of recent progress made in our understanding of spatio-temporal regulation of mitotic events following the development of intramolecular genetically encoded Förster resonance energy transfer (FRET) biosensors which give access for the first time to quantitative data on the regulation of protein activities in real time. Emergence of this approach has been for some time limited by technical difficulties and time-consuming design of a specific biosensor for a given protein activity with sufficient FRET dynamic range. Recent optimization of FRET-compatible fluorescent proteins with increased fluorescence quantum yield and molar extinction coefficient, photostability, PH insensivity, and simpler fluorescence decay as well as the recent development of FRET-designed plasmid libraries are expected to greatly facilitate future development [1–6]. Protein kinase biosensors based on a "sandwich" design that consists of a fusion between a cyan fluorescent protein (CFP), a phosphorylation sequence for the relevant kinase, a phosphorylation binding domain able to recognize this site in its phosphorylated state, and yellow fluorescent protein (YFP) have been the most popular tools [7]. However, it should be kept

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Abbreviations: **FRET**, Förster resonance energy transfer; **MTs**, microtubules; **Plk1**, polo-like kinase 1

in mind that in most cases, these biosensors will report changes in the equilibrium between opposite activities of a given kinase and phosphatase(s) rather than modification of kinase activity by itself. This suffers from our weak knowledge of regulation of phosphatase activities during mitotic progression which remains a major concern (for review [8]). In other words, using a phosphorylation site of a given protein to design a biosensor will in fact report when the phosphorylation of this substrate becomes effective. As discussed in Violin et al. [9], an alternative approach to follow kinase activation by itself could be to use a phosphorylation binding domain with a low dissociation constant which will protect to a certain extent the phosphorylated site from phosphatase activities.

2 Design of a biosensor

Production of a genetically encoded biosensor requires fulfilling several conditions. First, fluorescent pairs used should be compatible for a non-radioactive transfer of energy (FRET) between them and several variants have been recently optimized for FRET applications (for reviews: [1, 5, 6]). Second, energy transfer efficiency is dependent as a function of inverse sixth power of the distance and relative orientation of the fluorescent proteins. Realization of biosensors in few weeks with a sufficient dynamic range has been recently facilitated by the development of FRET-designed plasmid libraries containing short linkers of different sizes to modulate the distance between fluorescent proteins and circularly permutated forms of the donor and/or acceptor fluorescent proteins to modify their relative orientation [3, 4]. In practice, a dynamic range above 10% is generally sufficient for accurate FRET quantifications in the presence of fluorescence intensity fluctuations due to cell morphology changes and/or to the recording system used. The different FRET microscopy and measurement approaches have been well described in Sipieter [5]. Third, concerning kinase biosensors, the choice of the phosphorylation site used is the most critical point in order to obtain a specific biosensor for a given kinase. We empirically defined several parameters:

(i) the phosphorylation site used to design a kinase biosensor should have been observed to be phosphorylated in conditions where the kinase and the substrate protein are not overexpressed, i.e. in physiological conditions.
(ii) phosphorylation of this site should be strictly dependent on the activity of the kinase of interest as experimentally tested using chemical inhibitor and/or RNA interference approaches and a corresponding phosphospecific antibody.

Finally, the kinetics of the site's phosphorylation, as determined using a phosphospecific antibody and western blot or immunofluorescence approaches, should fit with the data available concerning the regulation of the kinase of interest.

The molecular properties of a kinase biosensor based on a "sandwich" design should then be evaluated using a non-phosphorylatable version where the phosphorylated residue is replaced by an alanine and/or a mutated version of the biosensor containing an inactive phosphorylation binding domain. Its specificity for a given kinase should be tested using chemical inhibitor and/or RNA interference approaches. For example, an Aurora-B biosensor should be sensitive to Aurora-B (ZM447439) but not Aurora-A inhibitors (MLN8054). The choice of the phosphorylation-binding domain is also a critical point as depending on its affinity for the phosphorylation site used this will strongly affect the reversibility of the phosphorylation of the biosensor (for a recent review on the properties of phosphor-ser/thr binding domains, see [10]). Moreover, this phosphorylation-binding domain could compete with phosphorylation-binding domains of endogenous proteins. In fact, we previously used the Polo-box domain of Polo-like kinase 1 (Plk1) to design a Cyclin B1-dk1 biosensor and noticed that at high expression levels this domain competed with that of endogenous Plk1 and affected Plk1 subcellular localization and cell cycle progression. To solve this problem, one possibility is to select a stable cell line expressing constitutively the biosensor of interest at a level that is compatible with cell viability and normal cell cycle progression (which can be evaluated by FACS analyses). Most biosensors developed so far are freely diffusible in the nucleus and cytoplasm as determined by fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) experiments and thus accessible to the kinase of interest. If the kinase of interest is concentrated at specific locations in cell, targeting domains could be added in the design of the biosensor to improve its efficient phosphorylation assuming that this will not affect molecular properties of the biosensor. In fact, different centrosome targeted biosensors have failed to reveal any local change in protein kinase activity, possibly because the conformational switch of the biosensor was inhibited when targeted to the pericentriolar matrix (our unpublished results).

3 Entry into mitosis

Deciphering how the "decision" to enter into mitosis during each cell cycle is precisely controlled is still a major challenge in cell biology. From yeast to mammals, entry into mitosis requires the activation of a master kinase Cyclin B-Cdk1 (or Cyclin B-p34cdc2, also named MPF for "mitosis promoting factor") (for review [11]). In agreement with its functions, Cyclin B1 and Cdk1 are essential rodent genes [12–14]. The first regulatory mechanism of Cyclin B1-Cdk1 activity is the degradation of the regulatory subunit Cyclin B1 in metaphase, starting as soon as

Table 1. Composition of the biosensors mentioned in the text

Protein kinase	Donor/acceptor fluorescent proteins	Phosphorylation site	Phosphorylation binding domain	Refs.
Cyclin B1-Cdk1	mcerulean/YPet	Ser 126 of HsCyclin B1	Polo-box domain of HsPlk1	[19]
Aurora-B	CyPet/YPet or mTFP1/YPet	Thr 67 of HsKif2	FHA2 of ScRad53	[56, 61]
Plk1	CyPet/YPet	Ser 426 of HsMyt1	FHA2 of ScRad53	[56]
Plk1	CyPet/YPet or mTFP1/YPet	Ser 17 of c-jun	FHA2 of ScRad53	[59]
Protein	**Name of the biosensor**	**Donor/acceptor fluorescent proteins**	**Other domains**	**Refs.**
ran	Rango	Cerulean/EYFP	Importin-β binding domain of Hs snurportin1	[40]
Stathmin/Op18	COPY	ECFP/citrine	Full length Stathmin/Op18	[54]
RhoA	Raichu-RhoA	SEYFP/SECFP	Truncated RhoA and RhoA binding domain of PKN	[73]

the last chromosome aligns on the metaphase plate [15]. Cyclin B1 is then progressively synthesized during the next cell cycle from late S phase. In mammals, Cyclin B1-Cdk1 activation is further controlled by several regulatory factors, the activators Cdc25A, B, C phosphatases and the inhibitors Wee1 and Myt1 kinases. Moreover, these regulatory factors are the target of several upstream kinases, including the checkpoint kinases Chk1, Chk2, and p38 as well as Polo-like kinase 1 and Aurora-A which will modulate the equilibrium between their opposite activities during G2/M progression (for reviews [16, 17]). To determine if Cyclin B1-Cdk1 activation is a sudden event or if its activity level increases progressively in G2 phase until a certain threshold that will induce entry into mitosis, we previously developed a Cyclin B1-Cdk1 activity biosensor using an auto-phosphorylation site on Cyclin B1 [18, 19] (Table 1). We first established that this biosensor is not a target of other Cyclin–Cdk complexes, in particular Cyclin E–Cdk2 and Cyclin A2–Cdk2 during G1/S progression or Cyclin A2/Cdk1 during G2/M. We observed that Cyclin B–Cdk1 activation is a very late G2 phase event taking place a few minutes (~20 min) before nuclear envelope breakdown. Its activity level then progressively increased until early prometaphase. We took advantage of this biosensor to investigate the relationship between Cyclin B1-Cdk1 activation and its nuclear accumulation in prophase which is required for nuclear envelope breakdown and irreversible entry into mitosis. We found that initial Cyclin B1-Cdk1 activation and nuclear import are concurrent events. Moreover, using a Cdk1 chemical inhibitor we demonstrated that its nuclear accumulation is Cdk1 dependent. Cytoplasmic and nuclear targeted versions of this biosensor using NES and H2B fusion protein respectively revealed that activation kinetics of Cyclin B1-Cdk1 were similar in both compartments. Thus, activation of Cyclin B1-Cdk1 in the cytoplasm associated with the immediate nuclear translocation of a fraction of the active form is an elegant mechanism to synchronize cytoplasmic and nuclear events during entry into mitosis [20]. To better understand the different timing between Cyclin B1-Cdk1 initial activation and nuclear envelope breakdown which is a Cdk1 dependent event, we artificially modulated Cyclin B1 activity level expressing a constitutive active form consisting of Cyclin B1 fused to the non-phosphorylatable Cdk1AF in cells co-expressing the CyclinB1-Cdk1 biosensor. This revealed that a low Cyclin B1-Cdk1 activity level is sufficient to trigger its nuclear accumulation in prophase as well as activation of the anaphase promoting complex/C (APC) whereas a higher threshold of Cyclin B1-Cdk1 activity is required for nuclear envelope breakdown suggesting that lower affinity substrates are involved or that Cyclin B1-Cdk1 phosphorylation is counterbalanced by antagonistic nuclear phosphatases. Altogether, it revealed how different CyclinB1-Cdk1 kinase activity levels can coordinate different cellular events at mitotic entry.

Polo-like kinase 1 (Plk1) has been involved in several processes during mitosis, including centrosome maturation, sister chromatid resolution, spindle attachment of chromosomes and cleavage furrow formation [21–23]. Plk1 activity is also required for Cyclin B1-Cdk1 activation and entry into mitosis even if the underlining mechanisms are not fully understood [23–25]. In fact, several regulators of Cyclin B1-Cdk1 have been proposed to be phosphorylated and regulated by Plk1 in vertebrates including Wee1, Myt1, Cdc25B, and Cdc25C [26–32]. The activation kinetics of Plk1 during cell cycle progression has been investigated using both a phosphospecific antibody directed against the Aurora-A phosphorylation site Thr210 located in the activation loop of Plk1 kinase domain and a Plk1 biosensor using a previously identified Plk1 dependent Myt1 phosphorylation site [30, 33]. Immunostaining revealed that Thr210 phosphorylation, reporting Plk1 activation, was taking place mostly at centrosomes from late G2 cells, peaked in metaphase and

progressively declined from anaphase onset. Surprisingly, the FRET biosensor exhibited a very different dynamics with an almost linear FRET change, induced by its progressive phosphorylation, starting ~5 h before onset of mitosis. RNA interference and Plk1 chemical inhibitor experiments confirmed that FRET change during S–G2 progression was Plk1 dependent whereas during mitotic entry the biosensor was phosphorylated by another kinase, possibly Mps1 which share similar consensus phosphorylation sites [34]. Also, FRET change during S–G2 progression was mostly taking place in the nucleus. As discussed in Bruinsma et al. [35], if a cytoplasmic phosphatase is responsible for dephosphorylation of this Plk1 biosensor, this could lead to an enrichment of phosphorylated biosensor in the nucleus. Nevertheless, this does not explain the discrepancy in Plk1 activation kinetics during cell cycle progression between the two different approaches. As a tentative explanation, we noticed that the Myt1 phosphorylation site used in this biosensor is phosphorylated in cells overexpressing Plk1 [30]. However, whether this phosphorylation event is taking place in physiological conditions has not been determined. Thus, the Myt1 phosphorylation site used could be a low affinity substrate of Plk1. Consequently, the phosphorylation of overexpressed Plk1 biosensor could mostly reflect the progressive accumulation of Plk1 kinase itself during S/G2 progression exhibiting a basal activity rather than its activation process during G2 to mitosis progression. Nevertheless, it should be noticed that the activation kinetics reported by the Plk1 biosensor agree with recent publications suggesting a role of Plk1 during DNA replication (reviewed in [36]). Further investigations will be required to determine precisely when and where Plk1 activation by Aurora-A is taking place during G2 phase progression. Interestingly, activation of Plk1 could require different upstream kinase activities between G2 and mitosis. In fact, activation of Plk1 at kinetochores during mitosis was shown to be primarily dependent on Aurora-B but not Aurora-A activity in Drosophila cells [37].

4 Regulation of spindle assembly during mitosis

Mitotic and meiotic spindles are built by centrosomes and chromatin driven microtubule (MT) assembly (for review [38]). The chromatin driven MT assembly process has been proposed to be regulated by a ranGTP gradient around chromosomes. The small GTPase ran exists in two nucleotide bound states, GTP and GDP bound, which are established by the guanine exchange factor RCC1 and RanBP1/RanGAP1 (GTPase activating protein), respectively. During mitosis, ran in its GTP bound state interacts with importin β and this results in the disassembly of sequestering complexes formed between importin α/β dimers and spindle assembly factors such as TPX2, Cdk11, and lamin B (for review [39]). The release and activation of such factors is essential for spindle assembly and functions. Because RCC1 is chromatin associated and ranGAP1 and its associated protein RanBP1 are cytoplasmic, this suggested the existence of a ranGTP gradient around chromosomes during mitosis. To directly visualize the spatial distribution of ranGTP during mitosis in living cells, different FRET-based biosensors have been developed including one named Rango which contains the importin-β binding domain of human snurportin 1 flanked by CFP and YFP fluorescent proteins [40–42]. In vitro, its FRET activity was shown to be downregulated by importin-β binding and stimulated by its subsequent release induced by ranGTP. Rango biosensor revealed the existence of a ranGTP gradient around chromosomes in mitotic HeLa cells (3–4 μm broad) and meiotic *Xenopus laevis* egg extracts (15–20 μm) extending until spindle poles. A broad linear ranGTP gradient decreasing linearly from the metaphase plate has also been observed in MII mouse oocytes [43]. Interestingly, the FRET value recorded around mitotic chromosomes fit with the one observed in mitotic *Xenopus* egg extracts when MT polymerization is induced by the addition of the constitutively active form ranQ69L, suggesting that the ranGTP gradient is sufficient to promote local MT stabilization during mitosis. Experiments performed by Caudron et al. [44] in *Xenopus* metaphase egg extracts showed that high ranQ69L concentrations are required for MT nucleation whereas MT plus end stabilization increased linearly in a lower range of ranQ69L concentrations, suggesting that the ranGTP gradient could have spatially differential effects on MTs during spindle formation. Perturbation experiments in prophase (before spindle assembly) or metaphase (when a functional spindle is established) cells using ranQ69L or conversely a dominant negative form of importin-β revealed that the ranGTP gradient facilitates spindle assembly in early mitosis but seemed dispensable for spindle integrity [40]. This conclusion was challenged by further experiments using a Chinese hamster cell line tsBN2 harboring a temperature sensitive mutation in the ran GEF RCC1 [45]. Inactivation of RCC1 at the non-permissive temperature induced a decrease of the ranGTP gradient around metaphase chromosomes, visualized using the Rango biosensor, and was associated with progressive chromosome misalignments from the metaphase plate. This was also associated with increased Aurora-B activity at kinetochores, which promotes destabilization of MT-kinetochore attachments (see below) and with the reactivation of the spindle assembly checkpoint, illustrating the importance of the ranGTP gradient for mitotic spindle stability.

Stathmin/Op18 is a microtubule destabilizing factor through tubulin sequestration in a trimeric T_2S complex (two α-β tubulin heterodimers bound to one stathmin molecule) [46, 47]. An alternative model proposes that stathmin could exhibit a MT catastrophe-promoting

activity [48, 49]. Its MT destabilizing activity is downregulated during mitosis through a complex phosphorylation pattern by different kinases [50]. In *Xenopus* egg extracts, its hyperphosphorylation state is dependent on the presence of chromatin and MTs and chemical inhibition and depletion experiments revealed that it was mediated by Aurora-B kinase activity [51–53]. To investigate the possible existence of a stathmin phosphorylation gradient around mitotic chromosomes in somatic cells, a diffusible FRET-based biosensor (named COPY; CFP-OP18-YFP) was developed that consists of a fusion stathmin protein with CFP and YFP proteins at both extremities, taking advantage that free Op18 can fold into a flexible α-helix conformation and adopt a more rigid conformation when bound to tubulin heterodimers [49, 54]. In vitro, its FRET signal decreased with increased tubulin association. Imaging in *Xenopus* somatic cells revealed a high FRET gradient around mitotic chromosomes which was not observed using a non-phosphorylatable version of the biosensor. This suggested that the local stathmin phosphorylation could release tubulin and provide a favorable MT assembly environment around chromosomes. However, the precise contribution of this stathmin phosphorylation gradient for spindle assembly still needs to be further investigated. Moreover, its contribution for the spindle assembly process could be more pronounced in cells without centrosomes where chromosomes provides the dominant spindle generating force than in centrosomes containing cells [55].

5 Regulation of kinetochore–microtubule interaction during mitotic progression

5.1 Prometaphase

The kinetochore complexes assemble on centromeres, a particular chromosome structure, and link the sister chromatids to MTs originating from opposite poles of the mitotic spindle. Thus, kinetochores are essential for sister chromatid segregation during anaphase. FRET-based biosensor approaches have been particularly useful to understand how kinetochore–MT dynamics are regulated during prometaphase to anaphase progression and to decipher the role of two opposite kinases Polo-like kinase 1 and Aurora-B in this process. These sensors which contain an FHA2 phospho-thr binding domain from the yeast protein ScRad53p and mTFP1/YPet or CyPet/YPet as donor and acceptor pairs, respectively were generated from a previously developed PKC sensor that nicely reported calcium oscillations and PKC activation [9, 56]. Several studies demonstrate that Aurora-B phosphorylates multiple kinetochore substrates in the absence of tension and destabilizes kinetochore–MT interactions, this regulatory process being essential for the correction of incorrect chromosome attachments during spindle assembly (for review [57]). Because Aurora-B is located on kinetochores until anaphase onset, it has been unclear how stable MT attachments can be initiated during prometaphase in absence of kinetochore tensions.

Plk1 kinase partially localizes to the outer kinetochore during prometaphase through interactions mediated by its Polo-box domain (PBD) [58]. However, Plk1 levels at kinetochores dramatically decrease from prometaphase to metaphase when chromosomes align [21]. To investigate the role of Plk1 in the regulation of kinetochore–MT attachment, a second generation Plk1 biosensor was developed using a c-Jun phosphorylation site [59]. This sensor exhibits a differential FRET signal between interphase and mitosis which is dependent on Plk1 activity, as experimentally tested using siRNA approaches and chemical inhibitors. A kinetochore targeted fusion version with the kinetochore component Hec1 revealed that the Plk1 sensor was progressively dephosphorylated as chromosomes aligned at metaphase which correlates with the removal of Plk1. Furthermore, dephosphorylation of this sensor appears to be dependent on the kinetochore recruitment of PP1 phosphatase during metaphase. Further experiments revealed that perturbation of Plk1 recruitment to kinetochores by competition experiments using the PBD was associated with a destabilization of initial kinetochore–MT interactions during prometaphase. Conversely, artificial targeting of constitutively active Plk1 form (T210D) at metaphase kinetochores maintained the phosphorylation of Plk1 sensor suggesting that maintaining Plk1 localization at kinetochores will also maintain efficient phosphorylation of Plk1 substrates during metaphase. In these conditions, MT dynamics at metaphase kinetochores decreased resulting in a lack of tension. As a result, spindle checkpoint activation was maintained through Aurora-B activity (see below) and cells were delayed to enter in anaphase. Altogether, the combinational use of Plk1 sensor and perturbation experiments revealed the importance of Plk1 dynamics at kinetochores during prometaphase to metaphase to first establish initial kinetochore–MT attachments and then for the silencing of the spindle assembly checkpoint.

5.2 Metaphase

Aurora-B localizes to the inner centromere as a component of the chromosome passenger complex (CPC) also comprising the inner centromere protein (INCENP), Borealin (also known as Dasra) and Survivin (reviewed in [60]). The CPC remains at centromeres until anaphase onset and then redistributes to the midzone of the anaphase spindle. Since Aurora-B phosphorylates several kinetochore substrates in the absence of tension and destabilizes kinetochore–MT interactions, how tension originating from MTs attached to opposite spindle poles at metaphase is coupled to kinetochore–MT stabilization was investigated in a series of elegant experiments. To evaluate a model in

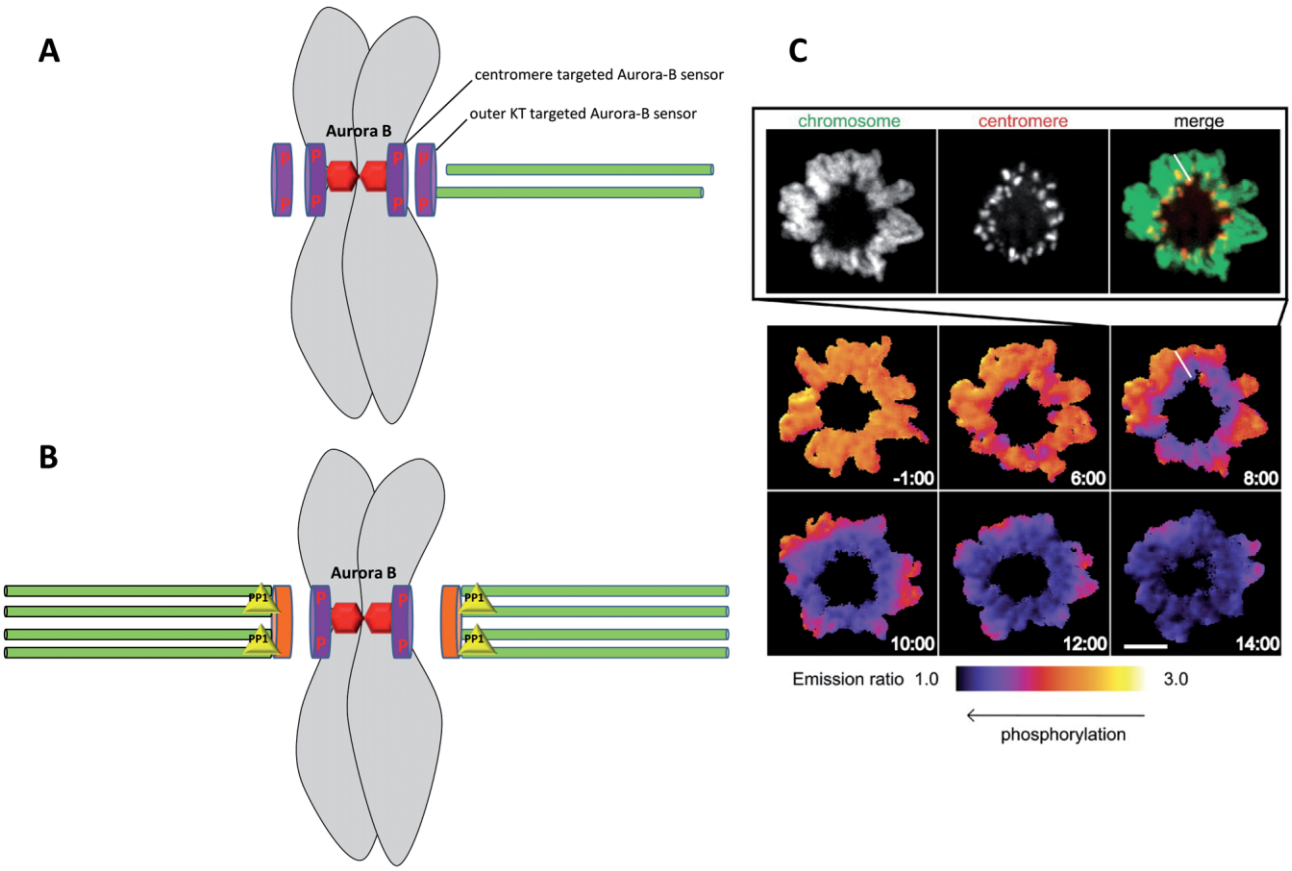

Figure 1. Mechanical tension of MTs originating from opposite spindle poles induce intra-kinetochore stretching during prometaphase (**A**) to metaphase (**B**) transition. Centromere targeted Aurora-B sensor is phosphorylated to a common extent in prometaphase and metaphase. In contrast, the outer kinetochore targeted sensor is only phosphorylated during prometaphase and displaced farther from Aurora-B kinase during metaphase. Its subsequent dephosphorylation is dependent on the recruitment of a PP1 phosphatase to metaphase kinetochores. (**C**) Live cell imaging of the kinetics of chromatin-targeted Aurora-B sensor phosphorylation after removal of the Aurora-B inhibitor ZM 447439. Cells were treated with monastrol (an Eg5 inhibitor) to create monopolar spindles. Centromeres were visualized with a CB (DNA-binding domain of CENP-B) mcherry fusion protein. After drug removal, Aurora-B biosensor becomes progressively phosphorylated from kinetochores to chromosome arms as visualized by color-coded YFP/CFP emission ratios suggesting that Aurora-B kinase can diffuse from the inner centromere. Reproduced with permission from Wang et al. [83].

which MT tension will cause intra-kinetochore stretching thereby spatially separating Aurora-B at centromeres from its kinetochore substrates, an Aurora-B biosensor was targeted either to the centromere or to the outer kinetochore by fusion with a centromere targeting domain or with the kinetochore protein Mis-12 [61]. In the absence of tension when MTs are artificially depolymerized by the drug nocodazole, both biosensors were phosphorylated to a similar extent (Fig. 1A). In contrast, when cells were arrested at metaphase with chromosomes pulled to opposite spindle poles, only the centromere targeted biosensor was phosphorylated suggesting that MT tension will separate Aurora-B from its kinetochore substrates (Fig. 1B). To more directly test this spatial separation model, Aurora-B kinase was relocated to outer kinetochores. In these conditions, kinetochore targeted Aurora-B biosensor phosphorylation was maintained at metaphase. This was associated with destabilization of kinetochore–MT attachments, activation of the spindle assembly checkpoint and delay in anaphase onset.

Aurora-B phosphorylates numerous substrates from chromatin (histone H3), to outer kinetochores (Ndc80 complex) to cytoplasmic proteins (stathmin) raising the question of how centromere associated Aurora-B can contact its different substrates on the micrometer scale. A chromatin-targeted Aurora-B sensor fused to histone protein H2B was used to beautifully demonstrate that after reversible chemical inhibition of Aurora-B kinase a progressive gradient of sensor phosphorylation was taking place, starting around the centromere and extending until chromosome arms, suggesting that Aurora-B kinase can diffuse from centromeres to reach its substrates (Fig. 1C). This was further illustrated by manipulating turnover rates of Aurora-B at centromeres. A slow exchange rate of Aurora-B induced mostly sensor phosphorylation in the vicinity of centromeres whereas a more

dynamic association spread phosphorylation over chromosomes. In spite of these impressive results, it is still unclear how outer kinetochore Aurora-B targets can be dephosphorylated under tension whereas other Aurora-B substrates are maintained phosphorylated in a diffusion-based gradient of kinase activity over micrometer distances. Specific recruitment of phosphatases at kinetochores, in particular PP1, provides an explanation for this discrepancy [62, 63]. It will be worth testing this model by looking at dephosphorylation of Aurora-B sensors targeted either to kinetochores or at a specific chromosome locus in the same cells.

5.3 Anaphase

At anaphase onset, the loss of chromatid cohesion reduces the mechanical tension on kinetochores, yet kinetochores remain attached to shortening MTs. The chromosome passenger complex including active Aurora-B relocates to the spindle midzone. To examine spatial patterns of Aurora-B substrates phosphorylation during anaphase progression, centromere and chromatid targeted sensor versions were used, which revealed that their dephosphorylation was primarily dependent on their relative position along the cell division axis. This suggests the existence of a diffusion-based gradient of Aurora-B kinase activity originating from the central spindle that now awaits further investigation. The chromatin-targeted Aurora-B sensor was used to investigate whether a phosphatase(s) counterbalances Aurora-B dependent phosphorylation and regulates kinetochore–MT stability during the poleward migration of chromosomes in anaphase [64]. A siRNA screen was performed against 225 human phosphatases (catalytic, regulatory, and scaffold subunits) which identified PP1 and its chromatin and kinetochore targeting subunits Repo-Man and Sds22, respectively, as required for the dephosphorylation of the Aurora-B sensor during anaphase [63–65]. In agreement, their inhibition was also associated with an increased phosphorylation level of the kinetochore Aurora-B substrate hDsn1, a subunit of the kinetochore Mis12 complex. In these conditions, irregular poleward chromosome movements associated with chromosome segregation defects were observed during anaphase progression [64, 66]. These results illustrated the importance of Aurora-B counteracting PP1 phosphatases for unperturbated anaphase and faithful chromosome segregation.

6 Activity of Rho-family GTPases during cytokinesis

In anaphase, the mitotic spindle dictates the site of contractile ring formation through signals originating from the central spindle and spindle asters (for review [67]). Assembly and subsequent ingression of the contractile ring involves activation of myosin-II and actin polymerization. The importance of the small GTPase RhoA in this process is supported by the observation that Rho and its effectors ROCK and citron kinases accumulate at the cleavage furrow and that cytokinesis is affected by Rho inhibition [68–71]. To investigate the regulation of RhoA activity as well as Rho-family GTPases Rac1 and Cdc42 during mitotic exit, FRET based biosensors have been developed [72, 73]. As a prototype, the Raichu-RhoA biosensor consists of truncated RhoA, the RhoA-binding domain of its effector PKN and CFP and YFP fluorescent proteins fused with the plasma membrane targeting carboxy terminus of K-ras4B (for extensive details, see the Phogemon datasheet from Matsuda's lab). In somatic cells, this biosensor exhibit a FRET increase with GTP loading which was experimentally tested by controlling the expression of either exogenous p115 Rho GEF or the Rho family GAP Grit [72]. It should be noticed that when expressed in cells, the GTP/GDP bound ratio of Raichu-RhoA biosensor is superior to the one from Flag-tagged RhoA (33% vs. 8%) indicating that the RhoA-binding domain to a certain extent protects the biosensor from GAP activity which will result in an overestimation of RhoA activity. Investigation of spatio-temporal changes of FRET signals during mitotic progression revealed that on entry into prophase, RhoA activity decreased rapidly until anaphase and then increased at early telophase at the plasma membrane including the cleavage furrow. In contrast, the activity of Rac1 and Cdc42 started to increase in late telophase and were excluded from the cleavage furrow. A different RhoA biosensor containing the Rho binding domain of the effector Rhotekin was used to investigate the role of the two RhoA GEF proteins GEF-H1 and Ect2 in the regulation of RhoA activity during mitotic exit [74–76]. Upon depletion of Ect2 by RNA interference, RhoA activity was no longer concentrated at the cleavage furrow but instead randomly distributed along the cell periphery. In contrast, cells depleted of GEF-H1 exhibited a global decrease of RhoA activity during mitotic exit. This suggested a differential role for GEF-H1 and Ect2 in the regulation and targeting of RhoA GTPase respectively. However, it should be point out that precise quantification of FRET signals at the plasma membrane was missing in these set of experiments. Finally, the time course of RhoA activity at the cell periphery during mitotic exit as well as its importance for this cellular process has been proposed to be cell type specific [77].

7 Concluding remarks

In summary, as illustrated above genetically encoded FRET-based biosensor approaches have been extremely powerful to better understand complex regulatory mechanisms controlling entry and progression through mitosis

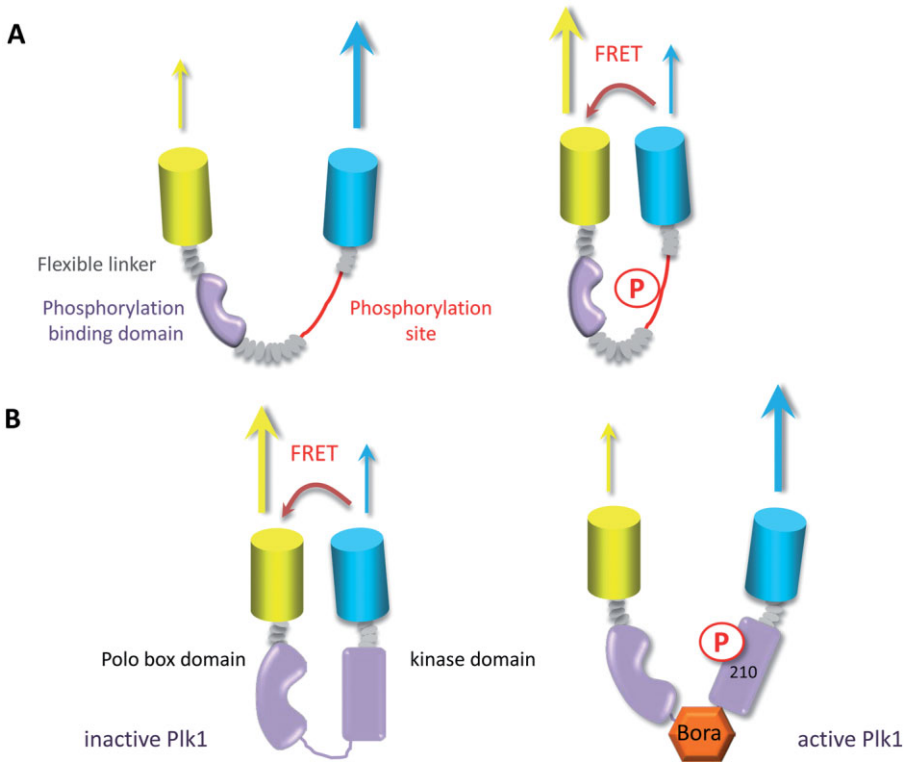

Figure 2. (**A**) Schematic representation of a "sandwich" based kinase biosensor comprising FRET compatibles fluorescent proteins CFP and YFP, a phosphorylation binding domain and a specific phosphorylation site for a given kinase. Following its phosphorylation, the phosphorylated site is recognized by the phosphorylation binding domain that induces a conformational change of the molecule associated with a FRET increase or decrease (not represented). (**B**) Schematic representation of a putative biosensor comprising the full length kinase Polo-like kinase 1 (Plk1) and FRET compatibles fluorescent proteins CFP and YFP. Activation of Plk1 kinase through Bora binding and phosphorylation of Thr210 by Aurora-A induces a conformational change between regulatory and catalytic domains and a FRET decrease.

in space and time and unlighted the importance of several regulators. With the increasing number of biosensors available, it will soon be possible to decipher how different regulators can exert cooperative or opposite activities to finely tune any step of cellular reorganization during mitotic progression as well as how activation and inactivation processes of different regulators are interconnected. For example, Polo-like kinase 1 activation during G2/M has been shown to be mediated by Aurora-A kinase [25, 33]. Conversely, some activation cofactors of Aurora-A kinase, like TPX2, are potential Plk1 targets [78]. Moreover, the recent development of multicolor FRET approaches will allow investigation of several regulators functions in a single cell at the same time [79].

Kinase biosensors have been so far the most popular ones and are very promising tools for the screening as well as evaluation of new chemical inhibitors before preclinical trials [80]. These biosensors are mostly based on a "sandwich" design which requires the identification of a specific phosphorylation site for a given kinase, a potential technical limitation depending on the data already available in the literature (see Box 1) (Fig. 2A). As an alternative approach, it could be possible in some cases to design a kinase biosensor based on a conformational change of the kinase molecule itself during its activation and inactivation processes when physiological substrates are still unknown. For example, activation of Polo-like kinase 1 is associated with a conformational switch between its N-terminal catalytic and C-terminal regulatory domains (Fig. 2B) [25]. This last strategy could be particularly interesting to develop phosphatase biosensors which are still pending in order to better understand the roles and regulations of several counteracting phosphatases during mitotic progression [81].

Finally, the generation of transgenic mice expressing biosensors, which is still at the beginning, will be very powerful tools to compare protein activities and regulation in different physiological tissue contexts as well as for the direct evaluation of drugs efficiencies in animals. This has been for some time technically challenging in particular due to homologous recombination between fluorescent protein sequences [82].

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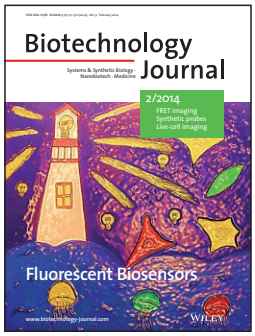

The Fluorescent Biosensor special issue of *Biotechnology Journal* is edited by Dr. May Morris and Prof. Marc Blondel. The cover image is an artistic interpretation of how fluorescent biosensors function as molecular beacons for scientists navigating a sea of molecules. Image courtesy of L. Divita and R. Wintergerst.

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