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A fine balancing act

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A fine balancing act: a delicate kinase-phosphatase equilibrium that protects against chromosomal instability and cancer.

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Cancer cells rewire signalling networks to acquire specific hallmarks needed for their proliferation, survival, and dissemination throughout the body. Although this is often associated with the constitutive activation or inactivation of protein phosphorylation networks, there are other contexts when the dysregulation must be much milder. For example, chromosomal instability is a widespread cancer hallmark that relies on subtle defects in chromosome replication and/or division, such that these processes remain functional, but nevertheless error-prone. In this article, we will discuss how perturbations to the delicate kinase-phosphatase balance could lie at the heart of this type of dysregulation. In particular, we will explain how the two principle mechanisms that safeguard the chromosome segregation process rely on an equilibrium between at least two kinases and two phosphatases to function correctly. This balance is set during mitosis by a central complex that has also been implicated in chromosomal instability - the BUB1/BUBR1/BUB3 complex – and we will put forward a hypothesis that could link these two findings. This could be relevant for cancer treatment because most tumours have evolved by pushing the boundaries of chromosomal instability to the limit. If this involves subtle changes to the kinase-phosphatase equilibrium, then it may be possible to exacerbate these defects and tip tumour cells over the edge, whilst still maintaining the viability of healthy cells.

Running title: Balancing kinases and phosphatases at the kinetochore.

Introduction

Protein phosphorylation is critical for regulating protein function, propagating intracellular signals, and maintaining cell and tissue homeostasis (Day et al., 2016). It is not surprising, therefore, that dysregulated phosphorylation is a major cause of several human diseases, including cancer (Creixell et al., 2015; Fleuren et al., 2016). In the context of cancer, mutations within key oncogenes or tumour suppressors enable tumour cells to acquire characteristic traits needed for their growth, survival, and metastasis (Hanahan and Weinberg, 2011). These mutations are frequently associated with the constitutive activation, inactivation, or rewiring, of protein phosphorylation networks (Creixell et al., 2015; Fleuren et al., 2016; Julien et al., 2011; Reimand et al., 2013). For example, the ability to sustain uncontrolled proliferation can result from the hyperactivation of tyrosine kinase receptors, such as EGFR (Henson et al., 2017), the inactivation of other receptor types, such as TGF-beta (Huang and Blobe, 2016), or the constitutive activation of downstream signalling intermediates, such as Ras (Stephen et al., 2014), Raf (Holderfield et al., 2014) or PI3-Kinase (Lim et al., 2015). In addition, this can be supported by the inactivation of tumour suppressor phosphatases, such as PTEN (Lim et al., 2015) or PP2A (Grech et al., 2016), or the activation of oncogenic phosphatases, including many members of the Protein Tyrosine Phosphatase (PTP) superfamily (Hardy et al., 2012; Julien et al., 2011). In this way, tumour cells are able to grossly alter their 'phosphatome' in a manner that best befits their continued proliferation and survival.

In addition to these individual gene mutations, the genomic landscape of cancer cells can be radically reshuffled by the continual gain, loss and/or rearrangement of whole chromosomes or parts of chromosomes. This is referred to as chromosomal instability, or CIN, and it represents perhaps the

most dangerous of all cancer hallmarks, because it provides a rich source of genetic variability that tumours can then use to evolve (Funk et al., 2016; Greaves, 2015; McGranahan and Swanton, 2017; Roschke and Rozenblum, 2013). The more heterogeneous the tumour, the greater the chance that it can positively select lethal subclones that can survive the hostile tumour microenvironment, escape to colonise new areas, and ultimately resist chemotherapy treatments (Greaves, 2015; Lee et al., 2011; McGranahan and Swanton, 2017). That is why CIN is such a good predictor of poor patient outcome (Jamal-Hanjani et al., 2017) and it is also why there is an urgent need to uncover the underlying mechanistic causes.

Beneath the seemingly chaotic exterior of CIN cells, lie some surprisingly mild perturbations to the processes of chromosome replication and/or division (Bastians, 2015; Funk et al., 2016). If one were to visualise mitosis in these cells, for example, it would still look surprisingly well organised, save for the odd one or two chromosomes that occasionally lag in the division plane. Delve a little deeper, however, and there is a perfectly good explanation for this relatively mild dysregulation: these processes are essential for survival, even for a resilient tumour cell, and they cannot tolerate gross perturbations because excessive aneuploidy is detrimental to tumour fitness (Duijf and Benezra, 2013; Roschke and Rozenblum, 2013; Silk et al., 2013; Tang and Amon, 2013; Weaver et al., 2007). Instead, tumours rely on the fact that perpetual low-grade errors can eventually build up a vast bank of different karyotypes, that crucially, *are* still viable.

In this article, we will discuss how dysregulated protein phosphorylation could still lie at the heart of these defects. However, rather than the sledgehammer-type of dysregulation that is typically associated with cancer, CIN may instead rely on subtle alterations to the kinase-phosphatase equilibrium to allow essential processes to become mildly dysfunctional. We will focus on chromosome segregation, because this process is known to fail in a wide variety of CIN cells and, as we will come on to discuss, it also critically depends on a fine balance of activities between multiple kinases and phosphatases to function correctly. In particular, we will highlight how a key node in this network that has well established links to CIN - the BUB1/BUBR1/BUB3 complex – is critical for setting the correct kinase-phosphatase balance during mitosis. The signalling output of this complex, and its ability to promote or protect against CIN, is well known to depend on the expression levels of its constituent proteins (Baker et al., 2013; Baker et al., 2009; Jeganathan et al., 2007; Ricke et al., 2011; Ricke and van Deursen, 2011; Suijkerbuijk et al., 2010). Therefore aneuploidy itself, which alters gene dosage and introduces protein imbalances, could drive the type of mild errors that generate further aneuploidy in an evolutionary cycle that pushes the boundaries of CIN to the limit (Giam and Rancati, 2015).

Chromosome segregation errors as a source of CIN

There are two main regulatory processes that safeguard the chromosome segregation process: the spindle assembly checkpoint (SAC) and kinetochore-microtubule error-correction (Joglekar, 2016; Krenn and Musacchio, 2015; Musacchio, 2015). The SAC delays mitosis until each and every chromosome has attached to microtubules via the kinetochore (a large protein complex assembled at the centromere of chromosomes). The error-correction network monitors this process and removes any faulty microtubule attachments that may form. The main kinases that regulate these processes, MPS1 and Aurora B, are both essential for viability because efficient inhibition of either one causes catastrophic chromosome segregation errors and cell death (Kwiatkowski et al., 2010; Sliedrecht et al., 2010; Wilkinson et al., 2007; Yang et al., 2007). Not surprisingly, therefore, inactivating mutations within these kinases have never been found in tumours, and in fact, MPS1 and Aurora B inhibitors are currently in clinical trials as anti-cancer agents (Bavetsias and Linardopoulos, 2015; Dominguez-Brauer et al., 2015; Falchook et al., 2015; Mason et al., 2017; Tang et al., 2017; Wengner et al., 2016).

Although the kinases themselves are not inactivated in cancer, the SAC and error-correction networks that they control may, however, still be defective. Partial inhibition of MPS1 or Aurora B causes premature mitotic exit with kinetochore-microtubule attachment defects (Cimini et al., 2006; Santaguida et al., 2010). This results in chromosome gains, losses and/or translocations, as cells divide with unattached or merotelically attached kinetochores (i.e. a single kinetochore attached to both spindle poles). These merotelic attachments cause chromosomes to lag in the division plane during anaphase, where they can be damaged during cytokinesis and then repaired incorrectly by non-homologous end joining in G1 (Janssen et al., 2011), or incorporated into micronuclei and damaged during subsequent DNA replication (Crasta et al., 2012; Zhang et al., 2015). If cells continue to divide in this manner, then they develop the type of karyotype heterogeneity that is typical of tumours with CIN. So, do these defects cause or contribute to CIN in tumour cells? The direct evidence implies that they do, and you only have to visualise these cells dividing to begin to understand why.

CIN cells display an abnormally high proportion of lagging chromosomes during anaphase due to merotelic kinetochore-microtubule attachments (Bakhoum et al., 2014; Gascoigne and Taylor, 2008; Godek et al., 2015; Thompson and Compton, 2008). Furthermore, artificially destabilising these incorrect attachments is sufficient to reduce chromosome segregation errors and supress CIN (Bakhoum et al., 2009). Therefore, CIN cells are unable to fully correct their kinetochore-microtubule attachment defects, which implies that the error-correction network is partially defective. There does not, however, appear to be a similar defect in the SAC, because the same panel of CIN cells that displayed elevated merotelic attachments, showed no evidence of unattached chromosomes at anaphase (i.e. the SAC is able to halt division until all kinetochores become attached) (Gascoigne and Taylor, 2008; Thompson and Compton, 2008). It should be noted, however, that others have observed divisions with unattached chromosomes in a breast cancer CIN line (Ryan et al., 2012). Furthermore, it is very difficult to identify these unattached chromosomes at anaphase, at least in comparison to lagging chromosomes which are clearly visible in the division plane. Therefore, a definitive answer as to whether the SAC is impaired will require an accurate quantification of SAC strength (i.e. the amount of inhibitory signal generated by each kinetochore) in a wide variety of cancer cell lines. In summary, weakening of the error-correction network likely contributes to CIN, and although the current evidence implies that SAC weakening does not, it is still too premature to rule this out.

There have been various mechanisms put forward to explain the mitotic errors in CIN cells (Abe et al., 2016; Bastians, 2015; Burrell et al., 2013; Ertych et al., 2014; Ganem et al., 2009; Manning et al., 2014; Solomon et al., 2011; Tanno et al., 2015; Yu et al., 2016), and many of these have the potential to impact, directly or indirectly, on the error-correction network and its main regulator Aurora B. For example, CIN has been associated with defects in centromeric cohesion (Manning et al., 2014; Solomon et al., 2011), the inner centromeric shugoshin network (Tanno et al., 2015), and heterochromatin binding protein 1 (HP1) (Abe et al., 2016), which can all impact on Aurora B localisation and activation (Abe et al., 2016; Carmena et al., 2012; Kleyman et al., 2014). In addition, the tumour suppressor DAP2IP affects kinetochore-microtubule attachments and the SAC, perhaps by regulating the phosphatase pathway that antagonises Aurora B at kinetochores (PP2A-B56) (Yu et al., 2016). Furthermore, as we will come on to discuss, there are also a variety of expression level alterations that can modulate kinetochore Aurora B/PP2A activity to drive CIN and tumourigenesis in animal models (Dai et al., 2004; Jeganathan et al., 2007; Rao et al., 2005; Ricke et al., 2011).

In summary, the phenotypes widely observed in CIN cells are reminiscent of partial Aurora B inhibition, and many of the established links to CIN may also impact on Aurora B activity. These two facts alone, reinforce the need to understand whether the Aurora B network is frequently perturbed in CIN cells. This could be achieved by accurately quantifying kinetochore Aurora B activity, however, the relatively

mild chromosome segregation errors observed in CIN cells imply that any defects will be subtle. It is therefore important to use sensitive assays that can distinguish small changes in Aurora B activity, specifically at the outer kinetochore, where it is needed to regulate microtubule attachments.

We will now discuss the Aurora B network in more detail to highlight how it depends on a fine balance of kinase and phosphatase activities to function correctly. This is important, because many of the defects discussed above could destabilise this balance to cause kinetochore-microtubule attachment defects and CIN. In fact, this particular equilibrium is determined by two kinases and two phosphatases, which work together in a single network to regulate both kinetochore-microtubule attachments and the SAC. Therefore, by rewiring the same underlying circuitry, cancer cells could achieve a 'double hit' that weakens both of these key mitotic processes.

The role of the KMN network in chromosome segregation

The KMN network (for KNL1, Mis12 and NDC80 complexes) is a major signalling centre at kinetochores that regulates both microtubule attachments and the SAC (Musacchio and Desai, 2017) (figure 1). The principle microtubule binding component is the NDC80 complex, which has a coiled coil structure that reaches out from kinetochores to hold on to microtubules via interactions with the CH domains and a N-terminal tail. Aurora B, which is the catalytic component of the Chromosomal Passenger Complex (CPC), phosphorylates this tail region of NDC80 to electrostatically interfere with microtubule binding (Krenn and Musacchio, 2015). NDC80 also signals to the SAC because it binds the kinase MPS1 and allows it to phosphorylate KNL1 on what are known as 'MELT repeats'. These phosphorylations recruit the BUB1/BUB3/BUBR1 complex to kinetochores, which subsequently helps to recruit a variety of other proteins needed for SAC signalling, including MAD1 and MAD2. That is why KNL1 is viewed as a platform for SAC signalling at the kinetochore (Joglekar, 2016; Musacchio, 2015) (figure 1).

In addition to recruiting SAC proteins, KNL1 also recruits two phosphatases - PP1 and PP2A-B56 – which are needed to antagonise MPS1 and Aurora B signalling at kinetochores. This is important, because otherwise microtubule attachments would never form and the SAC would be constitutively engaged (Espert et al., 2014; Kruse et al., 2013; Nijenhuis et al., 2014; Suijkerbuijk et al., 2010; Xu et al., 2013). Instead, PP2A-B56 dampens Aurora B signalling to allow NDC80 to bind to microtubules and to enable KNL1 to bind to PP1 via an 'RVSF' motif in its extreme N-terminus. This PP1-KNL1 complex can subsequently dephosphorylate the MELTs to antagonise the SAC (figure 1).

There are therefore various phosphorylation sites on the KMN network that are in dynamic equilibrium during prometaphase; the stage of mitosis when kinetochore-microtubule attachments form. This equilibrium is critical for the SAC and kinetochore-microtubule attachment processes to function correctly, and to explain why, we will now focus on three of these sites in particular, which are all highlighted in figure 1: p-NDC80, p-MELT and p-RVSF. Although these phosphorylation sites are by no means the only critical sites on the outer kinetochore, they are some of the best characterised, and they may in fact act as surrogates for other key phosphorylations that regulate the SAC or microtubule attachment (i.e. other Aurora B and MPS1 targets, as highlighted below).

A kinase-phosphatase equilibrium that controls chromosome segregation

We will begin by discussing each of these key sites independently to highlight how their phosphorylation is delicately balanced and why that is important (points 1-3 below refer to the scales in figure 2a). We will then move on to discuss how their interconnected nature means that they all function together within a single network that, if unbalanced, can lead to defects in both kinetochore-microtubule attachments and the SAC (figure 2b).

1) NDC80 tail phosphorylation. As mentioned previously, Aurora B phosphorylates substrates at the kinetochore to inhibit microtubule attachment (Biggins et al., 1999; Cheeseman et al., 2002; Hauf et al., 2003; Lampson et al., 2004; Tanaka et al., 2002). Although many different substrates are targeted by Aurora B (Krenn and Musacchio, 2015), these are symbolised in figure 2 by NDC80, which is the principle microtubule attachment complex at kinetochores (Cheeseman et al., 2006; Ciferri et al., 2008; DeLuca et al., 2006; Guimaraes et al., 2008; Miller et al., 2008; Wei et al., 2007). Phosphorylation of NDC80 needs to be counterbalanced by the phosphatase PP2A-B56 to allow initial microtubule attachments to form (Foley et al., 2011; Kruse et al., 2013; Suijkerbuijk et al., 2012; Xu et al., 2013). If these attachments are correct (i.e. they generate tension), then the phosphatase wins out and they are rapidly stabilised. If not, then Aurora B activity predominates and the microtubules become quickly detached. This ability to switch the kinase-phosphatase equilibrium in the presence or absence of tension is the underlying basis of error-correction (Krenn and Musacchio, 2015).

2) KNL1-MELT phosphorylation. MPS1 phosphorylates proteins at the kinetochore to initiate SAC signalling; this is also likely to involve many different substrates (Faesen et al., 2017; Ji et al., 2017; Maciejowski et al., 2017), but these are symbolised in figure 2 by the 'MELT repeats' in KNL1, which as mentioned previously, is the major SAC signalling scaffold at kinetochores (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). These MELT phosphorylations also require counterbalancing, but this time by a phosphatase relay involving both PP2A-B56 and PP1 (Espert et al., 2014; Nijenhuis et al., 2014). When the SAC signal is on, the MELT repeats are rapidly phosphorylated and dephosphorylated by the cooperative action of kinase (MPS1) and phosphatase (PP1). Although this may seem like an excessive waste of energy, at least one benefit is that the signal is rapidly responsive to microtubule occupancy, such that when it needs to shut down it can do so incredibly quickly (Nijenhuis et al., 2014).

3) KNL1-RVSF phosphorylation. A third important balancing act involves the phosphatase PP1, which is recruited to an 'RVSF' motif in the N-terminus of KNL1 to shut down the SAC (Espeut et al., 2012; Liu et al., 2010; Meadows et al., 2011; Rosenberg et al., 2011). Phosphorylation of the serine within this motif inhibits PP1 binding, and this phosphorylation site is controlled by the antagonistic actions of Aurora B and PP2A-B56 (Liu et al., 2010; Nijenhuis et al., 2014). During mitosis, whenever the SAC is on, this motif is also rapidly phosphorylated and dephosphorylated to ensure that KNL1-MELT phosphorylation remains dynamic. When microtubules attach correctly and generate tension, Aurora B is shut down, the RVSF motif is dephosphorylated, and PP1 predominates at kinetochores to silence the SAC (Espeut et al., 2012; Meadows et al., 2011; Nijenhuis et al., 2014; Pinsky et al., 2009; Rosenberg et al., 2011; Vanoosthuyse and Hardwick, 2009). Aurora B also inhibits PP1 binding to other proteins, which can impact on Aurora B activity at the centromere (Kumar et al., 2016; Qian et al., 2013) and kinetochore (Kim et al., 2010). Whilst these PP1 complexes are clearly important, they have never been linked to the SAC directly and therefore are omitted from figure 2, which focusses exclusively on the KMN network.

A striking feature of all three of these phosphorylation sites is their interdependence. The RVSF motif controls the MELT motifs (via PP1), the MELTs control both the RVSF motif and NDC80 (via Aurora B and PP2A-B56), and NDC80 controls microtubule attachment status which regulates MPS1 and thus the MELTs ((Aravamudhan et al., 2015; Hiruma et al., 2015; Ji et al., 2015); not depicted in figure 2). Therefore, it is not possible to modulate either one site without producing knock-on effects on all the others. It will be important in future to determine exactly what these interconnections mean, but it is tempting to speculate that they may help to coordinate microtubule stabilisation with SAC silencing.

The best evidence that kinase and phosphatase inputs are delicately balanced, is that if either of these substrates are phosphorylated too much or too little, then the SAC and kinetochore-microtubule

attachment processes become defective (figure 2b). For example, if kinetochore PP2A-B56 activity is reduced then Aurora B becomes dominant, microtubule attachments are destabilised, and SAC silencing is delayed (Espert et al., 2014; Foley et al., 2011; Kruse et al., 2013; Nijenhuis et al., 2014; Suijkerbuijk et al., 2012; Xu et al., 2013). Conversely, if the phosphatases become dominant, the SAC is weakened, microtubule attachments become hyperstable, and the error-correction process fails (Ditchfield et al., 2003; Hauf et al., 2003; Santaguida et al., 2011; Saurin et al., 2011; Vader et al., 2007). This 'phosphatase dominant' situation leads to exactly the type of errors predicted to cause CIN. Therefore, it is important to consider whether cancer cells could rewire their underlying circuitry to skew this equilibrium towards the phosphatases?

To answer this question, it is important to first ask what aspects of the underlying circuitry help to ensure the system remains balanced in the first place. This is a complicated question, that will no doubt require systems biology to help solve, however, the simple diagram in figure 2 already eludes to a critical node in this network: the phosphorylated MELT motif. Figure 2a, shows how this motif jointly stimulates both kinase and phosphatase arms of the pathway, and importantly, the skewed equilibriums in figure 2b stems from an imbalance in kinase or phosphatase stimulation emanating from this MELT motif. We will now discuss the mechanistic basis for this co-stimulation because it involves a protein complex that has already been implicated in CIN: the BUB1/BUBR1/BUB3 complex (figure 3).

The BUB1/BUBR1/BUB3 complex integrates kinase and phosphatase signalling at kinetochores

As mentioned previously, an important event in SAC signalling is the phosphorylation of KNL1 on MELT repeats by MPS1 (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). This recruits the pseudo-symmetric BUB1-BUB3:BUB3-BUBR1 complex to kinetochores by virtue of interaction between BUB1-BUB3 and the phosphorylated MELT motif (figure 3) (Overlack et al., 2015; Primorac et al., 2013). One of the best characterised functions of BUB1 at kinetochores is the stimulation of Aurora B activity: BUB1 phosphorylates Histone-H2A tails adjacent to the kinetochore to recruit shugoshin, which is important for the co-localisation and activation of the Aurora B (Kawashima et al., 2007; Kawashima et al., 2010; Kitajima et al., 2005; Tsukahara et al., 2010; Wang et al., 2011; Yamagishi et al., 2010) (figure 1). BUBR1, on the other hand, recruits PP2A-B56 to kinetochores via direct interaction with a phosphorylated 'LSPI' motif in BUBR1 (Hertz et al., 2016; Kruse et al., 2013; Suijkerbuijk et al., 2012; Wang, J. et al., 2016; Wang, X. et al., 2016; Xu et al., 2013). This is important to counteract Aurora B activity at kinetochores and stabilise microtubule attachments. Therefore, the BUB1-BUB3:BUB3-BUBR1 heterotetramer has two well-characterised functions at the kinetochore: 1) to elevate Aurora B activity, and 2) to elevate PP2A-B56 activity (figure 3a). This complex is therefore the key signal integrator that activates both arms of the kinase-phosphatase network outlined in figure 2. This alone, suggests that it may be a particularly vulnerable node in the network, since protein imbalances and/or mutations that interfere with either BUB1 or BUBR1 signalling would be predicted to disrupt the kinase-phosphatase balance at kinetochores. We will now discuss the pre-existing links between the BUB complex and chromosomal instability, since there is already good evidence that the balance of BUB1/BUBR1 expression is critical for regulating chromosome segregation, CIN and tumourigenesis.

The BUB1/BUBR1/BUB3 complex and chromosomal instability

Modulating the levels of the BUB proteins has dramatic consequences for chromosomal stability and tumorigenesis. In mice, loss of BUB1 is embryonic lethal, however, reductions in BUB1 expression cause chromosome segregation defects, aneuploidy, and elevated rates of spontaneous tumorigenesis (Jeganathan et al., 2007). These defects, which are associated with reduced

centromeric Sgo1, are reminiscent of partial Aurora B inhibition (i.e. misaligned and/or lagging chromosomes during anaphase (Cimini et al., 2006)). In contrast, elevating BUB1 expression also has dramatic effects, but this time due to Aurora B hyperactivation. Transgenic mice that overexpress BUB1 exhibit chromosome segregation defects, near-diploid aneuploidies, and an elevated incidence of spontaneous tumours (Ricke et al., 2011). These defects are associated with enhanced BUB1 and Aurora B activities, and importantly, Aurora B hyperactivation is the principle cause, because partial Aurora B inhibition can completely rescue both the mitotic errors and the resulting increase in aneuploidy (Ricke et al., 2011). Together, these data suggest that manipulating BUB1 levels affects chromosome segregation and tumourigenesis, principally by modulating kinetochore Aurora B activity.

There are at least two ways in which BUB1 expression levels could impact on kinetochore Aurora B activity: 1) by controlling the recruitment and activation of Aurora B at centromeres, and 2) by modulating the activity of kinetochore PP2A-B56 (figure 3a). Interestingly, BUB1 overexpression causes a dramatic increase in kinetochore BUB1 levels, but crucially, this does not cause a reciprocal increase in kinetochore BUBR1 (Ricke et al., 2011). The result is that the kinase-phosphatase balance is tipped in favour of Aurora B, and kinetochore substrate phosphorylation is enhanced. It is unclear why the extra BUB1 at kinetochores does not also bind BUBR1, but this may reflect an imbalance in BUB1-BUB3:BUB3-BUBR1 complex formation, such that in the absence of sufficiently over-expressed BUBR1, BUB1-BUB3 heterodimers are left free to act at kinetochores instead (figure 3b). In this regard, it is perhaps particularly revealing that co-overexpression of BUBR1 can completely rescue the mitotic errors and aneuploidy in BUB1 overexpressing cells (Baker et al., 2013; Ricke et al., 2011). In this situation, the high kinetochore BUB1 levels are unaffected, but now BUBR1 is also enhanced and kinetochore substrate phosphorylation is reduced; presumably due to the extra PP2A-B56 that is co-recruited by BUBR1, although this was never directly tested. It should be noted, however, that BUBR1 is also able to protect against aneuploidy in different tumour models, and it has been proposed to have pleotropic effects that are independent on PP2A-B56, but maybe related to its ability to engage the SAC (Baker et al., 2013; Weaver et al., 2016).

If the relative expression of BUB1 and BUBR1 is critical to preserve chromosomal stability, then BUBR1 depletion should have similar effects to BUB1 overexpression. BUBR1 is an essential component of the SAC, and efficient knockdown causes a SAC override and kinetochore-microtubule attachment defects, which combine to cause catastrophic chromosome segregation errors and cell death (Chan et al., 1999; Fang, 2002; Kops et al., 2004; Lampson and Kapoor, 2005; Sudakin et al., 2001; Tang et al., 2001). The microtubule attachment defects in this case are caused by elevated Aurora B activity because chromosomal alignment can be rescued by Aurora B inhibition (Lampson and Kapoor, 2005). If BUBR1 expression levels are partially reduced, then cells can survive and proliferate, but they do so with mild defects in the SAC and chromosome alignment that lead to aneuploidy (Bohers et al., 2008; Suijkerbuijk et al., 2010). In fact, bi-allelic mutations in BUBR1, which reduce BUBR1 expression levels, are a major cause of Mosaic Variegated Aneuploidy (MVA) and are associated with susceptibility to gastrointestinal neoplasia (Bohers et al., 2008; Burum-Auensen et al., 2008; Hanks et al., 2004; Rao et al., 2005; Rio Frio et al., 2010; Suijkerbuijk et al., 2010; Wijshake et al., 2012). In addition, MVA patients suffer from a variety of aneuploidy-related conditions, including an increased susceptibility to a wide range of early-onset childhood cancers (Kajii et al., 2001). This provides good evidence that aneuploidy itself can drive cancer, and in this case, a major contributing factor is an imbalance in kinase-phosphatase signalling at kinetochores, because the associated microtubule attachment defects can be rescued by wild type BUBR1, but not by BUBR1 that is incapable of binding to PP2A-B56 (Xu et al., 2013). Furthermore, this imbalance is caused only by a reduction in kinetochore phosphatase activity because recruiting PP2A-B56 alone to kinetochores can also rescue the chromosome alignment

defects (Xu et al., 2013). Therefore, in summary, reduced BUBR1 expression causes a shift in the equilibrium towards Aurora B at kinetochores by reducing PP2A-B56 (figure 3b), which produces similar phenotypes to BUB1 overexpression; in terms of the chromosome segregation defects, aneuploidy and cancer predisposition (Ricke et al., 2011; Ricke and van Deursen, 2011). It will be interesting to test in future whether BUB1 inhibition, using recently developed small molecules (Baron et al., 2016), can help to restore this balance and perhaps limit aneuploidy in MVA patients.

Alternative ways to upset the kinase-phosphatase balance at kinetochores

Altering BUB1:BUBR1 expression levels is not the only way to perturb the kinase-phosphatase balance at kinetochores. The BUB1:BUBR1 kinetochore equilibrium may also be regulated by other proteins, because knockdown of the BUB3 binding protein, BuGZ, selectively reduces kinetochore BUB1, but not BUBR1, and causes reciprocal reductions in Histone H2A-T120 phosphorylation and kinetochore Aurora B activity (Toledo et al., 2014). Alternatively, the Aurora B or PP2A-B56 pathways could be selectively perturbed by direct changes to either their expression levels or their respective regulatory pathways.

Aberrant expression of Aurora B has been previously linked to CIN, and knockout of the tumour suppressor ARF causes mitotic defects and CIN by elevating Aurora B levels (Britigan et al., 2014; Hindriksen et al., 2015). Altering the expression of proteins within the CPC, or within the larger centromeric Aurora B recruitment pathway, could similarly affect Aurora B localisation and activity (Hindriksen et al., 2015). For example, the interaction with the centromeric chromatin regulator, HP1, appears to be disturbed in a variety of cancer lines, which missegregate chromosomes due to reduced Aurora B activity (Abe et al., 2016). Other well-established Aurora B regulators, such as Sgo1 and cohesin, have been independently linked to CIN (Manning et al., 2014; Solomon et al., 2011; Tanno et al., 2015), and at least part of these effects could be mediated by an imbalance in Aurora B:PP2A-B56 activity (Gutierrez-Caballero et al., 2012; Kleyman et al., 2014). Sgo1, for example, localises Aurora B to centromeres (Kawashima et al., 2007; Kawashima et al., 2010; Tsukahara et al., 2010; Wang et al., 2011; Yamagishi et al., 2010), but also recruits PP2A-B56 to limit Aurora B activity (Meppelink et al., 2015; Tang et al., 2006).

Kinetochore PP2A-B56 activity could also be dependent on expression levels, and in this case, the relative expression of individual B56 isoforms may be particularly important, because only a subset of these isoforms bind the outer kinetochore (Nijenhuis et al., 2014). This kinetochore binding depends on a variety of different kinase inputs (MPS1, PLK1, CDK1 (Kruse et al., 2013; London et al., 2012; Shepperd et al., 2012; Suijkerbuijk et al., 2012; Yamagishi et al., 2012)), which could each be selectively perturbed in CIN cells. Finally, PP2A-B56 activity is directly regulated by at least one inhibitory protein at kinetochores (Porter et al., 2013), and the extent of this inhibition may depend on protein expression levels and/or additional regulation.

Conclusions

We have outlined here how maintaining the correct kinase-phosphatase balance at kinetochores is important to protect against aneuploidy and cancer. Changing the relative protein expression levels within the BUB1/BUBR1/BUB3 complex can clearly upset this balance, however, there are likely to be many alternative ways to achieve the same end result. The genetic changes that support this kind of dysregulation may be very difficult to pinpoint within individual tumours, especially if they produce only subtle changes in protein expression, and even harder to detect within the context of a population, when each tumour can select different ways to mildly perturb the same network. However, if the Aurora B-PP2A network is a central hub onto which many of these genetic changes

converge, then it may be possible to screen components of this network to categorise CIN cells, and potentially, to direct treatment.

As has been pointed out previously by others, many tumours have evolved by pushing the boundaries of CIN to the limit (Giam and Rancati, 2015; Greaves, 2015; Lee et al., 2011; McGranahan and Swanton, 2017; Sansregret et al., 2017). A key aspect of this is the weakening of otherwise reliable networks involved in DNA replication and/or cell division (Bastians, 2015; Funk et al., 2016). To fuel tumour evolution, this weakening needs to be sufficient to allow rapid diversification, but not too extensive, otherwise viability may be adversely affected. In other words, tumours live life on the edge. If that edge is defined by a fine balance of kinase and phosphatase activities, then it may be possible to use small molecule inhibitors to either exacerbate these defects and kill tumour cells, or limit the errors to restrict CIN and tumour evolution (Bakhoum and Compton, 2012; Janssen et al., 2009; McGranahan et al., 2012; Roschke and Kirsch, 2010).

Figure legends

Figure 1. Regulation of the SAC and microtubule attachments at the KMN network. Schematic to show how the various kinases, phosphatases and SAC proteins discussed in this review cross-talk at the KMN network.

Figure 2. A kinase-phosphatase equilibrium at kinetochores that regulates microtubule attachments and the SAC. A. Schematic to show how three key phosphorylation sites on the KMN network are in a dynamic equilibrium during prometaphase. The main function of these phosphorylation sites is to control kinetochore-microtubule (KT-MT) attachments and the SAC. **B.** How changing the kinase-phosphatase balance at kinetochores can lead to defects in KT-MT attachments and the SAC.

Figure 3. How the BUB complex stimulates kinase and phosphatase signalling at kinetochores. A. BUB1 phosphorylates Histone H2A-T120 to recruit Shugoshin (SGO) to centromeres. This helps to recruit and activate the CPC; containing Survivin (Sur), Borealin (Bor), Inner Centromere Protein (INCENP) and the catalytic subunit Aurora B (AurB). Aurora B is able to phosphorylate outer kinetochore substrates, such as NDC80, to destabilise kinetochore-microtubule (KT-MT) attachments. BUBR1, on the other hand, recruits PP2A-B56 which is able to antagonise Aurora B and stabilise KT-MT attachments. **B.** How changes to BUB1/BUBR1 expression can disturb the kinase-phosphatase equilibrium at kinetochores. BUB1 overexpression elevates SGO recruitment and kinetochore Aurora B activity, without increasing kinetochore BUBR1/PP2A-B56. Conversely, BUBR1 knockdown (or MVA patient lines with diminished BUBR1 expression) reduces kinetochore BUBR1, whilst BUB1 recruitment remains unchanged. The net effect in both cases, is that the kinase-phosphatase equilibrium is skewed in favour of the kinase Aurora B and KT-MT attachments are destabilised.

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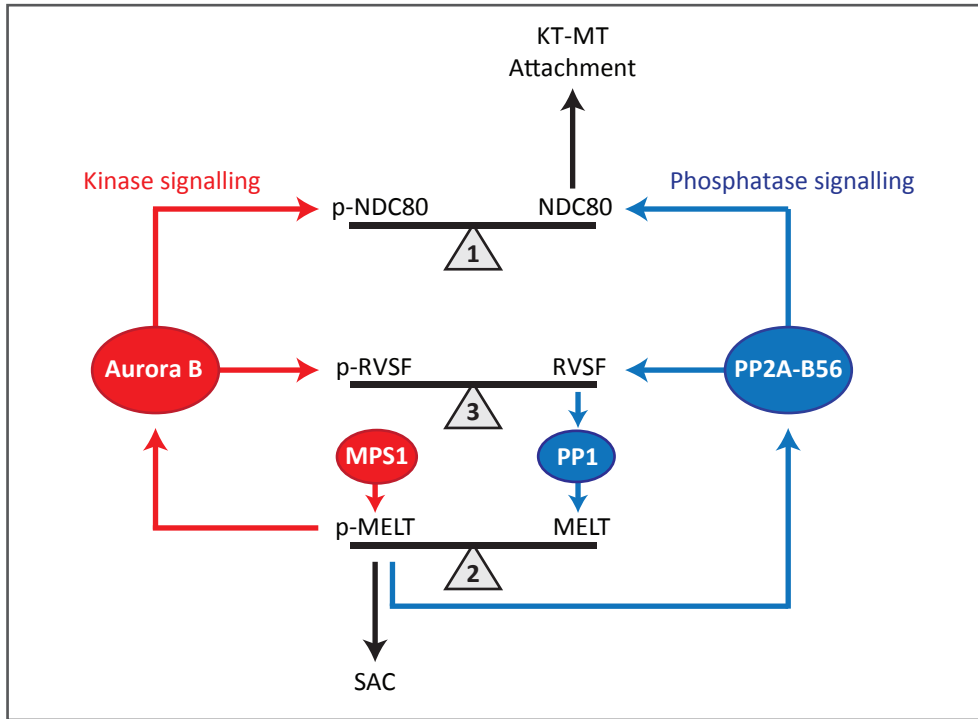
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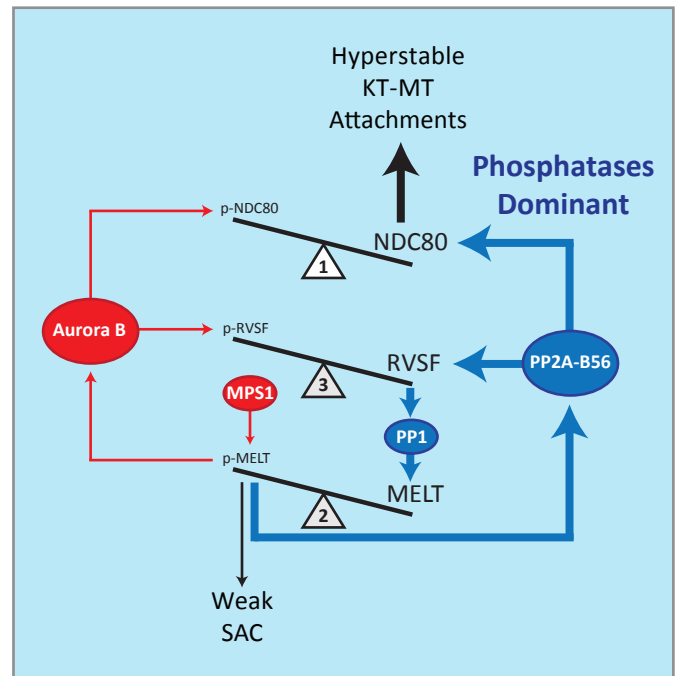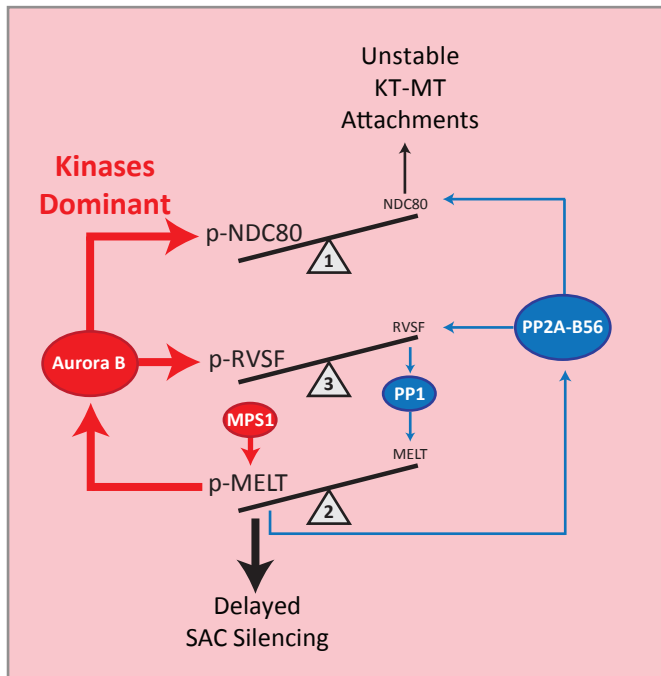
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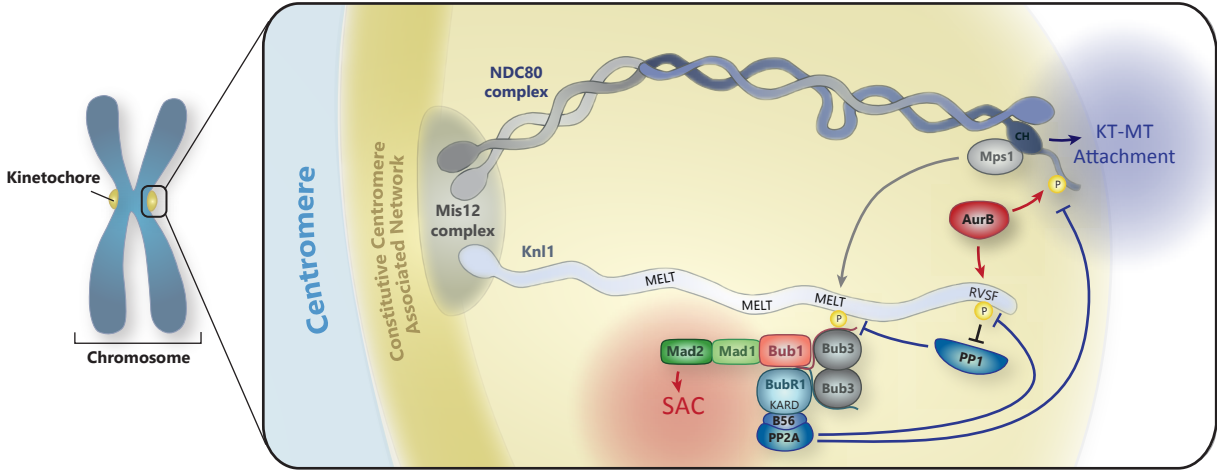
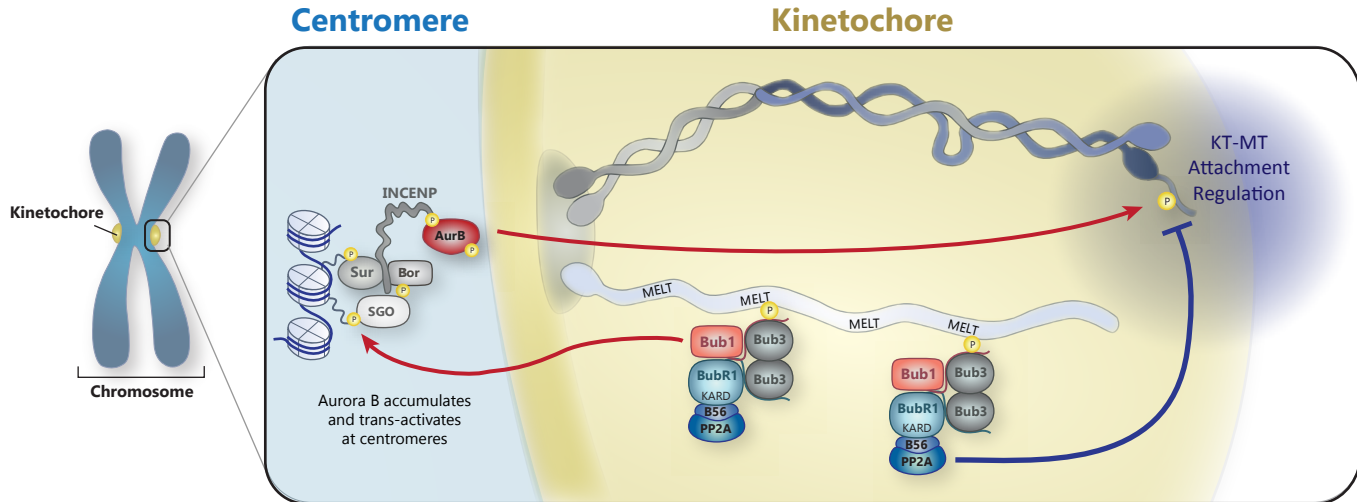**A. Kinases and phosphatases in dynamic equilibrium (during prometaphase)**

B. Kinase and phosphatase activities unbalanced

A

Centromere

Kinetochore

Kinetochore

Chromosome

INCENP

Sur / Bor / SGO

AurB

KT-MT Attachment Regulation

Aurora B accumulates and trans-activates at centromeres

MELT MELT MELT MELT

Bub1 / Bub3 / BubR1 / Bub3 / KARD / B56 / PP2A

Bub1 / Bub3 / BubR1 / Bub3 / KARD / B56 / PP2A

B

Centromere

Kinetochore

BubR1

Bub1

High Bub1 expression
Low BubR1 expression
(Mouse models/MVA patients)

INCENP

Sur / Bor / SGO

AurB

Unstable KT-MT Attachments

MELT MELT MELT MELT

Bub1 / Bub3 / Bub1 / Bub3 / Bub1 / Bub3 / Bub1 / Bub3

BubR1 / KARD / B56 / PP2A / Bub3