

The FEAR network

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Mitosis is governed by the oscillation of cyclin dependent kinase (CDK) activity and ubiquitin-dependent proteolysis. Entry into mitosis is initiated by mitotic cyclin-CDK activation. Anaphase onset occurs upon activation of the Anaphase Promoting Complex/Cyclosome (APC/C), a ubiquitin ligase that promotes the destruction of the anaphase inhibitor Securin. Destruction of Securin initiates chromosome segregation by activation of the protease Separase, allowing it to cleave a subunit of the cohesin complexes that hold the duplicated sister chromatids together. Upon completion of nuclear division cells exit from mitosis, a process defined by the inactivation of CDKs, disassembly of the mitotic spindle, and cytokinesis. In the budding yeast *S. cerevisiae*, a signaling network known as the FEAR network is critical to ensure accurate anaphase chromosome segregation and the integration of this process with other anaphase events. Here, we summarize what is known about the regulation and function of the FEAR network in budding yeast and discuss the potential for conserved FEAR network functions in other eukaryotes.

Control of late mitotic events in budding yeast

In budding yeast, cyclin B-CDK (Clb-CDK) activity drives the entry and progression through mitosis. Inactivation of Clb-CDKs is essential for exit from mitosis. In budding yeast, Clb-CDK inactivation is controlled by the essential phosphatase Cdc14. Cdc14 promotes Clb-CDK downregulation by two primary mechanisms: (1) Cdc14 dephosphorylates the APC/C specificity factor Cdh1 thereby stimulating the APC/C-dependent destruction of Clb cyclins. (2) Cdc14 dephosphorylates the Clb-CDK inhibitor Sic1 and the transcription factor Swi5, resulting in the stabilization of Sic1 and Swi5-dependent activation of *SIC1* transcription. Furthermore, Cdc14 counteracts CDK activity by dephosphorylating a host of CDK substrates, allowing for the rapid resetting of cells to the G1 state. Cdc14 activation in late anaphase is controlled by the Mitotic Exit Network (MEN), a GTPase signaling cascade, and results in exit from mitosis (**Box 1**).

In addition to the essential role Cdc14 plays in Clb-CDK inactivation at the end of anaphase, there is growing appreciation for the ability of Cdc14 to modulate CDK activity in early anaphase. This important function of Cdc14 is mediated by the FEAR network (Cdc Fourteen Early Anaphase Release network).

Cdc14 regulation

Cdc14 activity is tightly regulated. In cell cycle stages prior to anaphase, Cdc14 is sequestered within the nucleolus as a result of its association with its nucleolar-localized inhibitor Cfi1/Net1 (**Figure 1**). Upon Separase activation and entry into anaphase, Cdc14 is released from the nucleolus and spreads throughout the nucleus and, to a significantly lesser extent, the cytoplasm. This early anaphase release of Cdc14 is mediated by the FEAR network and results in a pulse of Cdc14 activity that coordinates many of the anaphase events to be discussed below. FEAR network promoted Cdc14 release from the nucleolus is transient: in the absence of a functional MEN, Cdc14 becomes re-sequestered in the nucleolus during late anaphase and cells fail to exit from mitosis. While not essential, the FEAR network is crucial for the faithful execution of anaphase, as illustrated by the significant loss of viability of cells undergoing anaphase in the absence of FEAR network function. Cdc14 release from the nucleolus during late anaphase is promoted by the MEN (**Box 1**), which drives the sustained release of Cdc14 in both the nucleus and the cytoplasm and results in exit from mitosis.

The FEAR network

Work over the last eight years has identified a number of proteins that together function to regulate the release of Cdc14 from the nucleolus during early anaphase (**Figure 1**). These proteins, collectively known as the FEAR network, include Separase, the Separase binding protein Slk19, polo-like kinase Cdc5, Spo12, the replication fork block protein Fob1, PP2A phosphatase bound to its regulatory subunit Cdc55, Zds1 and Zds2, histone modifications such as H2B ubiquitylation and H3 methylation, and CDKs associated with the cyclins Clb1 and Clb2. We do not fully understand how these proteins function together to bring about the release of Cdc14 from the nucleolus but a mechanism is beginning to emerge.

The association between Cdc14 and its nucleolar-localized inhibitor Cfi1/Net1 appears to be regulated by phosphorylation. Prior to anaphase, Cdc14 and Cfi1/Net1 are in an unphosphorylated or hypophosphorylated state. During this stage of the cell cycle, Fob1 stabilizes the Cdc14 - Cfi1/Net1 interaction (**Figure 1**). At anaphase onset, APC/C activation results in the degradation of the Separase-inhibitor Securin, thereby resulting in the activation of the Separase – Slk19 complex. By a poorly understood mechanism that involves the proteins Zds1 and Zds2, the Separase – Slk19 complex is thought to

downregulate PP2A^{Cdc55} phosphatase activity. This downregulation of PP2A^{Cdc55} phosphatase activity allows Clb1-CDK and Clb2-CDK to phosphorylate Cfi1/Net1. Clb1-CDK and Clb2-CDK are also responsible for the phosphorylation of Spo12. It appears, however, that it is Cdc14, rather than PP2A, that restrains Spo12 phosphorylation in cell cycle stages prior to anaphase.

Cdc5 is essential for the release of Cdc14 from the nucleolus. The mechanism by which this polo-like kinase functions in the FEAR network is not yet understood and is clouded by the fact that Cdc5 acts positively in both the FEAR network and the MEN. Epistasis analysis places Cdc5 downstream of and/or in parallel to Separase - Slk19. Cdc5 promotes the phosphorylation of Cdc14 and Cfi1/Net1 *in vivo*, which is at least in part due to Cdc5 dependent degradation of the CDK inhibitory kinase Swe1. Furthermore, Cdc5 can dissociate the Cdc14 – Cfi1/Net1 complex *in vitro*. Ultimately, phosphorylation of Cfi1/Net1 is thought to decrease its affinity for Cdc14 while phosphorylation of Spo12 is thought to promote, in part, the dissociation of Fob1 from the Cdc14 - Cfi1/Net1 complex.

The FEAR network harbors two regulators of chromosome segregation, Separase and Cdc5. This allows for the coordination of FEAR network activation and chromosome segregation. Activation of Separase at the metaphase – anaphase transition both initiates chromosome segregation and FEAR network activation, thereby ensuring that Cdc14 activation does not occur prior to genome partitioning. Importantly, the essential nature of Separase in FEAR network activation also ensures that FEAR network activation is responsive to insults such as mitotic spindle damage and DNA damage, both of which stabilize Securin.

FEAR network functions

Although not essential for progression through mitosis, Cdc14 released by the FEAR network regulates multiple aspects of chromosome segregation. Interestingly, during early anaphase Cdc14 dephosphorylates its targets when overall Clb-CDK activity is high, raising the possibility that local high concentrations of active Cdc14 or locally repressed Clb-CDK activity is critical to regulate and coordinate anaphase events. FEAR network promoted Cdc14 activation ensures that the segregation of all chromosomes is initiated simultaneously, it is required for the efficient segregation of the

rDNA, positioning of the anaphase nucleus, proper regulation of mitotic spindle dynamics, localization of spindle proteins, and initiation of the MEN. In a sense, the FEAR network can be viewed as the “fine tuner of anaphase events,” bringing accuracy to the system (**Figure 2**).

Sharpening the metaphase – anaphase transition

The onset of anaphase is marked by the rapid loss of sister chromatid cohesion, resulting in the synchronous segregation of sister chromatids. This rapid loss in sister chromatid cohesion is driven, in large part, by the activation of Separase, which then cleaves a subunit of the cohesin complex. Separase activation at the metaphase-anaphase transition occurs as a result of APC/C dependent destruction of Securin. The mechanisms by which the switch-like activation of Separase is made are beginning to be understood. Securin is phosphorylated by Clb-CDKs and two of these phosphorylation events decrease the efficiency of Securin ubiquitination by the APC/C, at least *in vitro*. Cdc14 dephosphorylates these CDK marks on Securin, thus suggesting the presence of a positive feedback loop. The initial degradation of Securin activates the Separase – Slk19 complex, which then promotes the early anaphase release of Cdc14. Cdc14 in turn dephosphorylates Securin, resulting in enhanced APC/C dependent Securin degradation (**Figure 2**). When this positive feedback loop is disrupted, anaphase chromosome segregation is less abrupt and occurs with a significantly increased error rate.

Segregation of the rDNA

Activation of Separase at the metaphase-anaphase transition results in cohesin cleavage and initiates chromosome segregation. While cleavage of the cohesin complexes is sufficient for segregation of most parts of the genome, the rDNA repeats, which encode the rRNA and that nucleate the nucleolus, are late to segregate during anaphase and are linked through additional cohesin independent mechanisms. The physical nature of these cohesin independent chromosome linkages remains a mystery. What is known, however, is that the resolution of these linkages is dependent on Cdc14 activated by the FEAR network. Inactivation of either results in the defective segregation of the rDNA but not other parts of the genome. Activation of Cdc14 in early anaphase results in the silencing of transcription of rDNA by RNA PolI and the exclusion of RNA PolI subunits from the nucleolus (**Figure 2**). This anaphase-specific silencing of rDNA

transcription is thought to allow for the recruitment of condensins, cohesin-like complexes that mediate the condensation of chromosomes, to the rDNA and facilitate both the compaction and segregation of this genomic region.

Nuclear positioning

In budding yeast, the nucleus is positioned at the bud neck during metaphase. At the onset of anaphase, microtubule motors associated with the cytoplasmic microtubules emanating from the daughter bound spindle pole body (SPB; the yeast equivalent of the centrosome) pull the nucleus into the bud. Concomitantly, microtubule motor proteins associated with the mitotic spindle push the two SPBs apart. The end result of anaphase-specific microtubule associated forces is the equal distribution of the genetic material between the mother and daughter cells.

In cells lacking Separase function the cohesin complexes are not cleaved and sister chromatids fail to segregate. Interestingly, in the majority of these Separase deficient cells the undivided nucleus is pulled into the bud, the future daughter cell. The reason for this inheritance pattern is that cells that lack Separase function, while proficient in generating a daughter directed cytoplasmic microtubule pulling force, fail to generate or maintain a mother directed cytoplasmic microtubule pulling force (**Figure 2**). This asymmetric force pattern, coupled with the inability to cleave cohesin complexes and thus properly elongate the mitotic spindle, results in the undivided nucleus being pulled into the daughter cell. While the mechanism by which this mother directed pulling force is generated remains unknown, its activation is dependent on FEAR network components and Cdc14. Thus, during a normal cell cycle, Cdc14 released by the FEAR network directs the establishment or maintenance of a mother directed pulling force that, together with the daughter directed pulling force and elongating spindle, faithfully segregates the genetic material.

Spindle dynamics, stability, and midzone assembly

Microtubules, the basic components of the microtubule cytoskeleton, are highly dynamic structures. Microtubule dynamics increases as cells enter mitosis. This increase is brought about by mitotic CDK activity and is thought to promote both the formation of the mitotic spindle and the attachment of chromosomes to the assembling spindle. At the onset of anaphase, microtubule dynamics decreases dramatically and results in the

stabilization of the anaphase spindle and chromosome segregation. This is the result, at least in part, of the activation of Cdc14 by the FEAR network (**Figure 2**). Cdc14 dephosphorylates a number of microtubule binding proteins, e.g. Ask1 and Fin1, which then allows these proteins to interact with and stabilize the elongating spindle.

In addition to regulating microtubule dynamics, FEAR network mediated Cdc14 activation also controls the assembly of the spindle midzone. The spindle midzone is the site of overlap between the interpolar microtubules. Numerous proteins are recruited to this site to stabilize this fragile region of the elongating anaphase spindle. The spindle midzone also plays an important role in cytokinesis, particularly in higher eukaryotes. FEAR network driven Cdc14 activation results in the dephosphorylation of a host of proteins, including the microtubule bundling protein Ase1. Ase1 dephosphorylation then results in the proper localization of this protein to the spindle midzone (**Figure 2**). Cdc14 also dephosphorylates Sli15, which leads to the targeting of the Ipl1 - Sli15 - Bir1 complex, the conserved Aurora B – INCENP - Survivin chromosomal passenger complex in higher eukaryotes, to the spindle midzone (**Figure 2**). Together, Ase1 and Sli15 - Ipl1 - Bir1 recruit additional factors that stabilize the anaphase spindle, critical among them being the Separase – Slk19 complex. In the absence of proper targeting of these components to the spindle midzone, the elongating anaphase spindle frequently breaks.

Activation of the MEN

FEAR network mutants display a 10 - 20 minute delay in exit from mitosis (an approximately 10 - 20% increase in the length of the *S. cerevisiae* cell cycle). The reason for this delay is that the lack of FEAR network dependent Cdc14 release delays the activation of a positive feedback loop that stimulates Cdc14 release. The MEN kinase Cdc15 is phosphorylated during cell cycle stages prior to anaphase and these phosphorylation events have been shown genetically to inhibit the mitotic exit promoting role of Cdc15. The early anaphase release of Cdc14 dephosphorylates Cdc15, thereby stimulating MEN activity and further promoting Cdc14 release and mitotic exit. As all of the core MEN components are phospho-proteins, it is possible that Cdc14 has multiple targets that collectively function in this positive feedback loop.

The FEAR network - a key coordinator of the meiotic divisions

Meiosis is a specialized gamete-producing cell division in which a single round of DNA replication is followed by two sequential rounds of chromosome segregation, termed meiosis I and meiosis II. During meiosis I, homologous chromosomes are segregated from each other and during meiosis II sister chromatids segregate. The transition from meiosis I to meiosis II represents an unusual form of cell cycle exit in that there is not an intervening DNA synthesis phase.

The FEAR network and Cdc14 play the same role in meiosis as they do in mitosis, that is accomplish Clb-CDK down-regulation and exit from the chromosome segregation phase. However, while the FEAR network is important but not essential for Clb-CDK downregulation during mitosis, it is absolutely essential to bring about the proper transition from meiosis I to meiosis II. In the absence of FEAR network or Cdc14 function, meiotic cells fail to downregulate Clb-CDK activity at the end of meiosis I which leads to a severe delay in meiosis I spindle disassembly. In addition, Cdc14 and FEAR network mutants exhibit a “mixed” chromosome segregation pattern, which is characterized by some chromosomes segregating in a meiosis I-like pattern and others segregating in a meiosis II-like pattern. This unusual chromosome segregation pattern appears to result from meiotic events being uncoupled. Despite cells arresting in anaphase I, meiosis II chromosome segregation events continue to occur, leading to some chromosomes undergoing both meiotic divisions on the same anaphase I spindle. These findings suggest that Cdc14 and the FEAR network ensure that the two meiotic divisions occur on two sequentially built spindles.

It is interesting to note that unlike in mitosis, the MEN is dispensable for exit from meiosis I. The meiosis I to meiosis II transition is unique in that Clb-CDK activity needs to be lowered to a sufficient extent such that the meiosis I spindle disassembles but kept sufficiently high to prevent the formation of pre-replicative complexes (preRCs) and any subsequent DNA replication. MEN-mediated Cdc14 activation leads to complete inactivation of Clb-CDKs and resetting of the cell to the G1 state. Cdc14 released by the FEAR network lowers Clb-CDK activity, perhaps in a localized manner and/or for certain critical substrates, which may just accomplish this balancing act. Employing the FEAR network for Cdc14 activation may lower CDK activity sufficiently for meiosis I spindle

disassembly to occur but not low enough to allow assembly of preRCs onto origins of replication.

Does a FEAR network operate in higher eukaryotes?

Cdc14 homologues are found across eukaryotic species and play a conserved role in the regulation of CDK activity, although it appears that in many species this regulation may not be restricted to anaphase. It is a long-standing question as to whether or not a FEAR-like network functions in other organisms. All eukaryotes appear to have homologues of many of the budding yeast FEAR network components (Separase, polo-like kinase Cdc5, Clb-CDK, PP2A). In *S. pombe*, it was recently demonstrated that fission yeast FEAR network homologues do not regulate the localization of the Cdc14 homologue Clp1p/Flp1p. The signal transduction pathways that regulate Cdc14 homologue activation in metazoans are not yet known and there has yet to be any assessment for FEAR network contribution to Cdc14 activation in any other organism. It is possible that fission yeast and mammals do not require a FEAR network that brings about localized antagonism of mitotic CDK activity as, unlike in budding yeast, the bulk of Clb cyclin degradation and hence mitotic CDK downregulation occurs globally and abruptly at the metaphase – anaphase transition.

While a conserved role for a FEAR-like network has not yet been implicated in Cdc14 activation in other eukaryotes, it appears that at least some aspects of a FEAR network-mediated Cdc14 function, that of central spindle assembly and stability, may in fact be conserved. In *C. elegans*, the Cdc14 homolog CeCDC-14 localizes to the spindle midzone in anaphase and to the midbody in telophase. As a result of RNAi depletion of CeCDC-14, the mitotic kinesin ZEN-4 as well as other proteins fail to localize to the spindle midzone, resulting in the complete loss of the central spindle. These CeCDC-14 depleted embryos ultimately die, likely as a failure of cytokinesis. In fission yeast, the Cdc14 homolog Clp1/Flp1 localizes to the spindle midzone in anaphase and regulates the ability of Ase1 to recruit the kinesin Klp9 to the spindle midzone. Mammals contain two Cdc14 homologues, with one isoform (Cdc14B) residing in the nucleolus during interphase but not mitosis and one isoform (Cdc14A) located at centrosomes. Recent work has demonstrated that homozygous deletion of Cdc14B in tissue culture cells resulted in no defects in spindle assembly, anaphase progression, mitotic exit, and cytokinesis. siRNA depletion of the Cdc14A homologue, however, resulted in defects in

cytokinesis, perhaps as a result of defective spindle midzone assembly. Furthermore, while roles for Cdc14 homologues have not yet been implicated in species other than budding yeast, the translocation of Ipl1-Sli15 homologs Aurora B-INCENP from kinetochores to the spindle midzone appears to be a conserved feature of anaphase in eukaryotes. Abrogation of this translocation is believed to result in defects in cytokinesis.

Conclusions and Outlook

In the eight years since the discovery of the FEAR network the field has made great strides towards understanding the mechanisms by which the FEAR network operates and, particularly, about how Cdc14 activated by the FEAR network functions during anaphase. Activation of Separase at the metaphase to anaphase transition triggers a cascade of events that ultimately leads to the phosphorylation of Cfi1/Net1 as well as additional proteins, thereby promoting the release of Cdc14 from the nucleolus. This early anaphase activation of Cdc14 is critical for the coordination of multiple anaphase processes including the promotion of the switch-like transition to anaphase, rDNA segregation, positioning of the anaphase nucleus, quelling of microtubule dynamics, promotion of spindle stability and spindle midzone formation, and activation of the MEN.

While much has been learned, many important questions remain. Our molecular understanding of FEAR network driven Cdc14 release is far from complete. In the absence of MEN activity, Cdc14 becomes re-sequestered after only a brief release from the nucleolus (~ 10 minutes) indicating that FEAR network function is restricted to a brief period during early anaphase. The mechanisms by which this pulse of FEAR network function is generated are unknown. In addition, we do not yet understand the essential FEAR network role of polo-like kinase Cdc5, nor have we determined the mechanism by which Separase – Slk19 downregulates PP2A^{Cdc55}. Furthermore, recent data suggest that Cdc14 may in fact be active in the nucleolus in cell cycle phases prior to anaphase and may be a potent negative regulator of its own FEAR network and MEN dependent release. Finally, identification of additional Cdc14 substrates will reveal novel early anaphase functions for Cdc14. In conclusion, as progression through mitosis in the absence of FEAR network function is associated with a loss of genomic integrity and viability in *S. cerevisiae*, it will be important to further determine the broader significance of the findings in budding yeast in higher eukaryotes.

Box 1. The Mitotic Exit Network

The MEN resembles a Ras-like GTPase signal transduction cascade, with the G protein Tem1 functioning at the top of the pathway (Figure 1). Tem1 is positively regulated by its putative guanine nucleotide exchange factor (GEF) Lte1 and negatively regulated by its two component GTPase activating protein (GAP) Bub2-Bfa1. As a regulator of the MEN, Cdc5 phosphorylates Bfa1 which reduces Bub2-Bfa1 GAP activity. During late anaphase, Tem1-GTP is thought to bind to and activate the protein kinase Cdc15, which then activates the downstream kinase Dbf2 associated with its activating subunit Mob1. Nud1 is thought to function as a scaffold for the core MEN components Tem1, Bub2-Bfa1, Cdc15, and Dbf2-Mob1 at spindle pole bodies. Activation of Dbf2-Mob1 results, at least in part, in the phosphorylation of the Cdc14 nuclear localization sequence, thereby resulting in the retention of Cdc14 in the cytoplasm where it can act on its substrates. Activation of the MEN in late anaphase is essential for the sustained release of Cdc14 from the nucleolus and results in exit from mitosis.

Figure 1. Cdc14 regulation

Cdc14 release from the nucleolus during early anaphase is mediated by the FEAR network and results in a pulse of Cdc14 activity that coordinates multiple anaphase events. Cdc14 release from the nucleolus in late anaphase is mediated by the MEN and results in sustained Cdc14 activity and exit from mitosis.

Figure 2. FEAR network functions

Cdc14 released by the FEAR network coordinates multiple mitotic events.

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