

## EMBO Gold Medal Review 2006

### What is your assay for sister-chromatid cohesion?

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#### Night skies and biochemistry

Long hikes, for which my father took us, made me appreciate the wonderful nature that surrounded us in southern Germany. Nocturnal stargazing walks, together with popular science books that my aunt supplied, started my excitement for particle physics and the origin of the universe, a fashionable topic at the time. Unfortunately, our high school in Bad Windsheim did not offer a physics A-level course that year, so instead I enrolled for chemistry. The last half a year of the course was dedicated to biochemistry and raised another fascinating question, how does chemistry explain processes of life? A big question, but thus came my interest in the subject.

I wanted to enrol for the biochemistry course at the University of Tübingen, which was highly praised but as highly oversubscribed. There was no rush, as it was at the height of the cold war. Joining the army was not the right response, so there were 2 years of civilian service for me to do instead, just the right waiting time to get a place in Tübingen. The course was designed for 6 years, leading to a Diploma in Biochemistry and Physiological Chemistry. This seems long by current ideas about university education, but I enjoyed every subject, from inorganic and physical chemistry, to botany and human anatomy. All of the subjects became important later, one way or the other, while doing research or listening to others' research. I am therefore not in favour of shorter university degrees. We all expect to live and work for longer, so why the rush early in life when this is the best time to learn? The ability to take advantage of what Tübingen had to offer largely depended on the German university system's commitment, at the time, to free education. I certainly don't

regret the diversions that courses in geology and music history provided.

Another place that taught me a lot was an exchange year at the Max Planck Institute for Biochemistry in Martinsried near Munich. Six two-month placements in groups at the institute replaced some of the practical courses back in Tübingen. This was a great opportunity to get hands on experience with many techniques, and gave a fantastic flavour of what research and researchers are like. There were places for six students from Tübingen every semester, distributed in a raffle, so it was sheer luck I got one of these places.

#### SV40 replication

Then it came to choosing a topic for my diploma work. As I had been away from Tübingen, I was less familiar with the research there. Just before half-heartedly starting on a project to optimise *in vitro* growth conditions for trypanosomes, I met Hans Probst. I felt attracted by his topic, the study of viral DNA replication. Probst's group had found that cellular replication initiation halted if oxygen levels in the growth medium dropped below a certain threshold. Cellular metabolism was unaffected, but the reactive oxygen in the ribonucleotide reductase active site was lost. This effect also stopped replication initiation of the simian virus 40 (SV40) in infected cells. Replication origin unwinding was prevented, but the underlying mechanism was mysterious (Riedinger *et al.*, 1999). To facilitate molecular analysis, my task was to see whether an effect of oxygen tension or deoxynucleotide pools on origin firing could be reproduced *in vitro*, using the powerful SV40 *in vitro* replication reaction. The experiments to reconstitute DNA replication reactions in an Eppendorf tube were exciting, but unfortunately SV40 replication proceeded just fine under anaerobic conditions and a wide range of deoxynucleotide levels. While doing these experiments I read the literature on SV40 *in vitro* replication, and the publications by Peter Bullock, Jerard Hurwitz and Bruce Stillman made a big impression on me (Murakami *et al.*, 1992; Denis and Bullock, 1993; Waga and Stillman, 1994). It was clear, I wanted to do more of these replication reactions, and study the molecular mechanisms involved. So the next logic step was to find a PhD project in the area.

I took the courage to write to these three laboratories, not really expecting a reply. Amazingly, all three did reply, letters in the mail at the time, personally signed. Both Peter and Jerry had space, and the decision between the two was not difficult. If I went to the USA I would want to go to New York where Jerry's laboratory was based at Memorial Sloan-Kettering Cancer Center. But here came a new hurdle. It would be good, Jerry wrote, if I found a fellowship to support me. So I started to look into what fellowships were, and how one could get one. The German Academic Exchange Service (DAAD) offered funding for PhD projects abroad. I applied,

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and wrote as 'research plan' how excited I was to work on one of the many interesting projects that were ongoing in Jerry's laboratory. Of course the application was rejected.

Meanwhile I had been working as a research assistant, first with Pascal Haffter at the Max Planck Institute for Developmental Biology in Tübingen comparing zebrafish *no tail* alleles that had come up in the Tübingen screen (Odenthal *et al.*, 1996), and then with Josef Johannes at the Institute for Cell Biology and Immunology at the University of Stuttgart. Having already arranged a start date with Jerry, I dreaded to inform him of the rejection. The fastest way was to send a fax from the campus copy shop, and I promised to apply again for the next round of fellowships in 6 months time. Disappointed, and without better ideas, I continued with phage library screens to fish out potential regulatory 5' UTRs of genes of interest to the Stuttgart institute (Klein *et al.*, 1998). What I didn't realise was that the same day Jerry received the fax, he replied I should come right away as he would cover my stipend until a fellowship came through. Jerry sent this message by return fax, to the campus copy shop. Only 2 months later I received a copy of the fax by mail, via one of my referees from Munich, Walter Neupert, who had been contacted by Jerry and who had forwarded it to my home address in Bad Windsheim. From there my father finally sent the letter to me in Stuttgart. Within days I had packed up, bought a plane ticket to New York and arrived at Jerry's laboratory.

## Loading a PCNA ring onto DNA

Replication factor C (RFC) was a mysteriously fascinating protein complex. The picture at the time was that RFC recognises DNA primers synthesised by the DNA polymerase  $\alpha$ /primase complex, upon which it loads the ring-shaped PCNA trimer onto DNA and initiates a switch to polymerases  $\delta$  or  $\epsilon$  that then together with PCNA carry out processive primer elongation (Tsurimoto and Stillman, 1991; Pan *et al.*, 1993; Krishna *et al.*, 1994). How RFC performed these multiple tasks was largely unknown. RFC was tricky to purify, and its subunit composition remained to be confirmed. Therefore, I joined another graduate student, Jinsong Cai, in trying to purify recombinant RFC subunits with the aim to reconstitute and study the complex. This soon led to frustration, as overexpression of each of the known five subunits in bacteria yielded rarely more than aggregated, poorly behaved preparations. The baculovirus expression system, which was just becoming available, offered new hope. Jinsong and I started to construct the necessary viruses, but this took time. I was looking for another way to express the RFC subunits, and decided to try *in vitro* translation in rabbit reticulocyte lysates. The instructions recommended not to express more than two proteins at a time, but luckily this did not prevent me from adding templates for all five subunits to the lysate. After adjusting their relative concentrations we obtained a stoichiometric complex that could be purified out of the reaction mix. The yields were not great, but sufficient for Jerry to assay in his *in vitro* replication reactions that he ran on an almost daily basis. This was the first moment of success, we had reconstituted RFC activity from its recombinant subunits (Uhlmann *et al.*, 1996).

It followed confirmation of the reconstitution using baculovirus-expressed RFC (Cai *et al.*, 1996), and both systems

allowed us to probe the architecture and mechanism of RFC action in unprecedented detail (Uhlmann *et al.*, 1997; Zhang *et al.*, 1999). Despite our advancing biochemical understanding, what we were really waiting for were crystallographic pictures of RFC's interactions with DNA, PCNA and DNA polymerases. A full appreciation of these reactions has still to wait, but a spectacular start has meanwhile been made (Bowman *et al.*, 2004).

One curious observation that I made using *in vitro* translated RFC would change my perspective for the future. The largest RFC subunit contains an evolutionarily conserved N-terminal 'ligase homology domain', thought to provide an important DNA contact of the complex. To our surprise, when I deleted this domain, RFC became more, instead of less, active in loading PCNA onto DNA. What could therefore the role for such a conserved DNA-binding domain be? Would it direct RFC to sites of action different from replication forks, for examples sites of DNA repair (Aboussekhra *et al.*, 1995)? Was it required for RFC function *in vivo*? The way to address this was to create a yeast strain that lacked the domain. Hernan Flores-Rozas had just left Jerry's laboratory for a postdoctorate with Richard Kolodner, and with his new experience in budding yeast genetics, we created such a strain. The cells grew and replicated their genome indistinguishably from wild type, and they did not show any difference in mutation or recombination rates. Art Lustig even looked at telomeres for us, that maintained their normal length. The function of the ligase homology domain remains enigmatic until today (Gomes *et al.*, 2000), but I had tasted the potential of yeast genetics to analyse molecular function in living cells.

While I was performing these experiments, Kim Nasmyth visited the institute to present a seminar on the identification of the first yeast 'cohesins'. Everybody around me was excitedly anticipating his visit, and I started to read his publications on the yeast cell cycle and asymmetric cell determination (Jansen *et al.*, 1996; Piatti *et al.*, 1996). These were packed with conceptual elegance, experimental choreography and rigour. This was a way to do science that I would love to participate in. When I met Kim afterwards, I was delighted to get invited for a postdoctoral interview to Vienna.

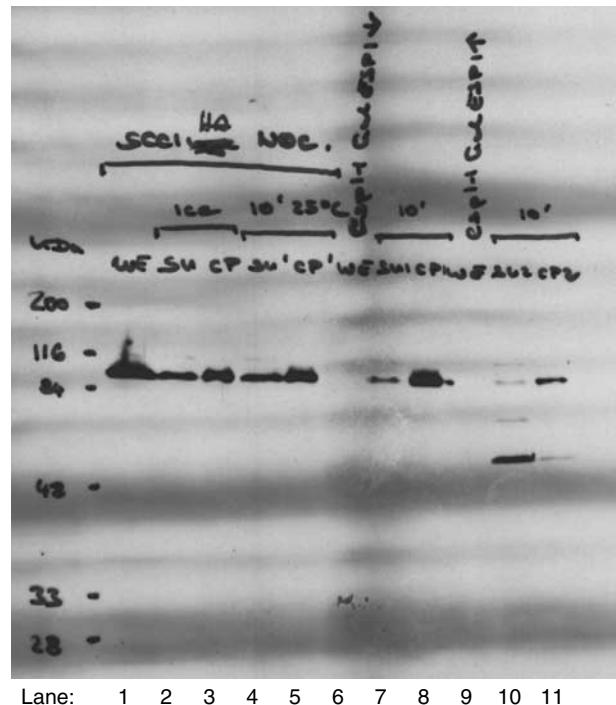
## Sister-chromatid pairing and splitting

It was time again to apply for a fellowship to join Kim's laboratory at the IMP in Vienna. A protein complex made up of cohesins, now simply called 'cohesin', was required to prevent sister chromatids from prematurely separating after DNA replication and maintaining them paired until mitosis (Guacci *et al.*, 1997; Michaelis *et al.*, 1997). Was the cohesin complex the 'glue' for sister chromatids cohesion? If so, I suggested, one could insert a protease recognition site into one of the subunits, and upon induced cleavage sister-chromatid cohesion should be lost. Kim rightly pointed out that such an experiment would be hard to do and even harder to interpret, so it did not make it into the final version of the proposal. Instead, I started to map the execution point for cohesin. Cohesin came onto chromosomes just before DNA replication, but was this important? What if it bound just afterwards? These were my first experiments at Kim's laboratory, and the answer was clear: only if cohesin was on

chromosomes at the time of DNA replication would the replication products remain paired (Uhlmann and Nasmyth, 1998). This was the first hint that cohesin is performing an important reaction right at the moment of DNA replication to link up the replication products, so that they can later be recognised as pairs in mitosis.

Cohesin stayed on chromosomes until metaphase, but disappeared from them in anaphase. Rafal Ciosk in the laboratory was re-analysing a nuclear division mutant, called *esp1* (McGrew *et al.*, 1992; Ciosk *et al.*, 1998). In this mutant, cohesin remained on chromosomes at the time of anaphase and sister chromatids failed to split. This provided a strong correlation between cohesin's presence on chromosomes, and sister-chromatid cohesion. It also suggested a role for Esp1 in cohesin removal in anaphase. But what was Esp1 doing to cohesin? Genetics was my initial approach, and I was preparing for a high-copy suppressor screen of the *esp1* mutant. If there were a protein whose increased dosage would allow chromosome segregation even in an *esp1* mutant, it would certainly be an important player. I was not convinced. A genetic screen has the power to identify more new players, but my feeling was that we had already too many players in hand that we didn't know what they do. This is where biochemistry becomes powerful, and we decided to find out how Esp1 causes cohesin removal from chromosomes. At this moment Kim left the laboratory for 6 weeks for a mountaineering expedition in the Himalayas.

Here I was with the problem, and recalled Jerry. 'What is your assay?' would be his first question. I realised, I needed an assay for cohesin removal from chromosomes. A recent paper from Bruce Stillman's laboratory described a yeast cell fractionation protocol, with which they studied association of the replication initiation machinery with budding yeast chromatin (Liang and Stillman, 1997). I decided to see whether cohesin could be recovered in such chromatin fractions, and whether they could provide a substrate to study its removal. A substantial amount of cohesin, detected by an epitope-tagged Scc1 subunit, was indeed in these chromatin fractions. Disappointingly, incubation of chromatin from metaphase cells in extracts from G1 cells, in which cohesin should come off the chromosomes, showed little effect. In retrospect, this assay did in fact recapitulate cohesin removal from chromosomes, but without knowing what to look for the efficiency of this setup was too low. I looked at these results with Wolfgang Zachariae, and he pointed out that if I really wanted to know what Esp1 does to cohesin I should use extracts from cells overexpressing Esp1. In preparation for the suppressor screen, I had constructed a strain in which Esp1 could be switched on or off under control of a strong galactose-inducible promoter. I incubated metaphase chromatin in extracts with overexpressed Esp1 and after 2 weeks of optimisation, in the tenth experiment, the effect was remarkable (Figure 1). In control incubations cohesin remained chromatin bound, but extracts containing overexpressed Esp1 led to almost complete disappearance of cohesin from chromosomes. Instead of appearing in the supernatant, a smaller Scc1 band became visible there. The result met with a lot of scepticism in the laboratory, the danger of abundant vacuolar proteases that make their way into yeast extracts was too well known. But the more often I repeated the assay, the clearer the pattern became. Then



**Figure 1** An assay for cohesin removal from chromosomes that revealed evidence for Scc1 cleavage by separase. Yeast whole-cell extract from metaphase-arrested cells (WE, lane 1) was separated into a supernatant (SU, lane 2) and chromatin fraction (CP, lane 3), which were kept on ice for the duration of the experiment. Scc1 was C-terminally tagged with an HA epitope to facilitate its detection by western blotting. An aliquot of supernatant and chromatin each were incubated for 10 min at 25°C to monitor unspecific Scc1 degradation under these conditions (lanes 4 and 5). Extracts were also prepared from an *esp1-1* mutant yeast strain in which *GAL1* promoter-driven overexpression of wild-type Esp1 had not (lane 6) or had been induced (lane 9). Note that Scc1 in these extracts did not carry an epitope tag. Aliquots of the Scc1-HA chromatin fraction were incubated in these extracts for 10 min at 25°C. After incubation in the *esp1-1* extract, a small amount of full-length Scc1 leaked into the supernatant (lane 7), while most Scc1 remained associated with chromatin (lane 8). In contrast, after incubation in the Esp1-overexpressing extract, a significant portion of Scc1 was released from chromatin (lane 11) and was seen cleaved in the supernatant (lane 10).

Kim returned, and the message was clear. Find a way to prevent Scc1 degradation in this assay, or scrap it.

The idea that Scc1 cleavage may not be unspecific, but could in fact have something to do with its dissociation from chromosomes, was tempting. On the other hand, Esp1 looked nothing like a protease, which are often recognisable in their amino acid sequence. Kim suggested that if Esp1 really caused Scc1 cleavage then Pds1, an anaphase inhibitor and binding partner of Esp1, should prevent it (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996; Ciosk *et al.*, 1998). Jan-Michael Peters laboratory at the IMP were using *in vitro* translated yeast Pds1 as a substrate in ubiquitination assays, and an aliquot of it was very effective in preventing both Scc1 cleavage and its dissociation from chromosomes in the assay. This would very unlikely be the case if Scc1 cleavage was due to unspecific proteases. In addition, I found a way to synchronise yeast cells tightly at the metaphase-to-anaphase transition, which revealed that a similar Scc1 cleavage product appeared *in vivo* when cohesin dissociates from

chromosomes. Immunopurification from a large synchronised culture provided enough material for Friedrich Lottspeich in Martinsried to identify the cleavage site by Edman sequencing. This allowed the most important prediction to be tested: if Scc1 cleavage is important for cohesin dissociation from chromosomes, and if cohesin dissociation from chromosomes is important for sister-chromatid separation, then preventing Scc1 cleavage by mutation of the cleavage site should prevent anaphase.

I changed the arginine preceding the cleavage site into aspartate, a drastic change at a position that is often crucial for protease substrate recognition, and introduced the mutant under inducible control into yeast. We expected expression of an 'uncleavable' Scc1 to have a detrimental effect but, after induction, cell proliferation continued just fine. Close to conceding that the whole idea of cohesin cleavage may have been wrong after all, I performed the western blot to see whether the cleavage site mutation had indeed blocked Scc1 cleavage. It had, but a second cleavage fragment that had been weakly visible before (Figure 1), was now the prominent band. Sequence inspection revealed a second very similar cleavage site further upstream in Scc1. After mutation of both sites the effect was striking. Scc1 was no longer cleaved, failed to dissociate from chromosomes and blocked anaphase (Uhlmann *et al*, 1999).

## A new protease, and the trigger of anaphase

The resulting model that Esp1-dependent Scc1 cleavage promotes sister-chromatid splitting at anaphase onset received a lukewarm response in the community. After all we did not know which the Scc1 protease was, or what role Esp1 played in the cleavage reaction. Also, we knew that Scc1 cleavage was required for anaphase, but not whether cleaving Scc1 by itself was the trigger for anaphase, or whether it was one of many things that are required at this time. To address the first issue, I started to purify Esp1 from yeast extracts and designed a potential peptide inhibitor for the Scc1 protease based on the recognition motif fused to a reactive group that would covalently bind to and label the protease. This had been successfully carried out with caspases (Faleiro *et al*, 1997), and inhibitor studies suggested that the Scc1 protease, like caspases, was a cysteine protease. Purified Esp1 fractions did cleave recombinant Scc1, and the band that the inhibitor labelled was of the size of Esp1. This suggested a simple solution in which Esp1 itself was the Scc1 protease. Around this time, Eugene Koonin came to visit the IMP, and Kim explained him our puzzle that Esp1 did not look like a protease. It was within a few days that Kim received an e-mail back from Eugene with an alignment of the Esp1 C-terminus to the CD clan of cysteine proteases, a superfamily that includes the caspases.

The second question was would Scc1 cleavage be sufficient to trigger anaphase? This led us back to the idea of placing an ectopic protease recognition site into Scc1. Who would have then thought that this approach would be validated by the cell's own mechanism for treating cohesin in anaphase. We replaced one of two separase cleavage sites for the recognition sequence of tobacco etch virus (TEV) protease. TEV protease recognises a seven-amino-acid consensus sequence, which it cleaves at the penultimate position, just

like separase, only with different sequence specificity. Luckily TEV protease specificity is so tight that even high levels of expression did not affect the proliferation of wild-type cells. Its effect on cells harbouring Scc1 with the TEV-recognition site was dramatic. Upon TEV protease induction in metaphase, Scc1 was cleaved, sister chromatids split and started to move to opposite cell poles (Uhlmann *et al*, 2000). Therefore, only cohesin maintains sister-chromatid cohesion in budding yeast metaphase, and its cleavage triggers anaphase. Meanwhile, Irene Waizenegger in Jan-Michael's laboratory had purified human Esp1, and shown that it cleaved human Scc1. Furthermore, cohesin cleavage was required for sister-chromatid separation in fission yeast, and also loss of sister-chromatid arm cohesion in the budding yeast first meiotic division depended on Esp1 and on cleavage of meiotic cohesin (Buonomo *et al*, 2000; Tomonaga *et al*, 2000; Uhlmann *et al*, 2000; Waizenegger *et al*, 2000). It was time to give Esp1 its new name, separase.

## Separase, more than a protease

As often with simple models, reality is more complex. This does not mean the simple model is wrong, but it means that additional levels of regulation fine-tune the process. It was now time to start my own small research group, and I was lucky to have been offered a position at what was then the ICRF (Imperial Cancer Research Fund) in London. The institute's promise that I could arrive with my lab coat on was true, in the sense that within a week of my arrival we were growing the first yeast cells. This was immensely aided by Chris Lehane, Matt Sullivan, Stefan Weitzer and Nadine Hornig who started the laboratory together with me. One of the first questions that we addressed looked back at anaphase onset triggered by TEV-mediated cohesin cleavage. Chromosomes efficiently segregated, but anaphase spindles looked frail and broken (Uhlmann *et al*, 2000). We realised that in most cells, spindles collapsed before spanning the full length of the cell. In contrast, if separase was expressed in place of TEV protease, anaphase spindles remained stable and elongated all the way to the cell poles. Thus, while cohesin cleavage triggered anaphase onset, separase was needed for successful anaphase spindle elongation.

Our first idea was that, being a protease, separase might cleave additional proteins that stabilise the spindle at anaphase onset. Separase cleavage sites, similar to the ones in Scc1, could be spotted in the sequences of several proteins. One of them was Slk19, known to be required for spindle stability (Zeng *et al*, 1999; Rao *et al*, 2001). Slk19 cleavage at the predicted site should have cut one-third off the protein's N-terminus. But in cells synchronised at anaphase onset, we only saw a small mobility shift, typical for protein dephosphorylation rather than cleavage. I suggested to stop considering Slk19 any further, but Matt, as a proof of principle, phosphatase treated the samples. This resolved the bands not into one, but clearly two. Separase did cleave Slk19, not at the predicted site, but at a related peptide motif close to the protein's N-terminus. Our initial excitement about a second separase cleavage target was muted when it turned out that Slk19 cleavage makes, at best, a small contribution to anaphase spindle stability (Sullivan *et al*, 2001). So Matt went on to refine experimentally the separase recognition consensus, and tested 63 more candidate proteins containing it,

without success. Up to date Slk19 is the only known cleavage target of separase apart from cohesin (Sullivan *et al.*, 2004b). In higher eukaryotes, separase cleaves itself, in addition to cohesin. While self-cleavage does not immediately affect separase activity, it might regulate its turnover (Waizenegger *et al.*, 2000; Herzig *et al.*, 2002; Zou *et al.*, 2002).

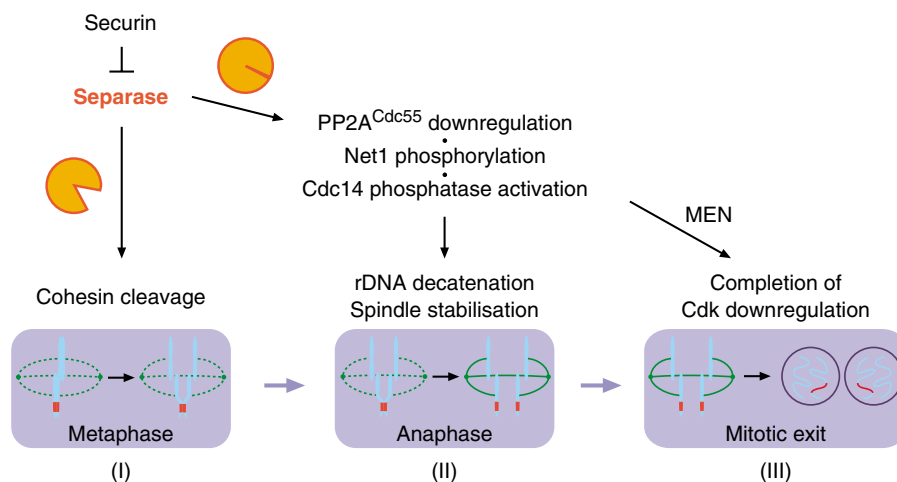
A hint as to a second separase function came when we noticed that after TEV protease expression in metaphase, cells remained stably arrested in mitosis. In contrast, similar expression of separase led to DNA re-replication and new bud formation after anaphase. This suggested that separase promoted exit from mitosis. Maybe this, rather than separase directly, caused spindle stabilisation. Mitotic exit in budding yeast is under control of the Cdc14 phosphatase, which removes phosphates from proteins that are phosphorylated by cyclin-dependent kinase (Cdk) during mitotic entry (Visintin *et al.*, 1998). Cdc14 is regulated in a curious manner, being sequestered and kept inactive in the nucleolus for much of the cell cycle, and released from the nucleolus just at the time of mitotic exit (Shou *et al.*, 1999; Visintin *et al.*, 1999). Strikingly, Cdc14 was released from the nucleolus when separase was expressed to trigger anaphase, but not after TEV protease expression. This suggested a role of separase in Cdc14 phosphatase activation, consistent with findings at Angelika Amon's laboratory at the same time, that separase mutants fail to release Cdc14 on time (Stegmeier *et al.*, 2002; Sullivan and Uhlmann, 2003). Separase harbouring a point mutation in the catalytic cysteine residue of the protease active site was perfectly proficient in activating Cdc14. Thus separase, once activated after destruction of its inhibitor securin, cleaves cohesin to trigger anaphase and uses a second non-proteolytic mechanism to activate Cdc14 and promote mitotic progression (Figure 2).

At this time Toru Higuchi came to the laboratory, and he quickly confirmed that it was Cdc14 activation that was responsible for separase-mediated spindle stabilisation. He

showed that a dramatic change in the dynamic behaviour of microtubules at anaphase onset, that had been seen in many organisms (Maddox *et al.*, 2000), was under control of Cdc14. He also pinpointed at least one protein that is dephosphorylated by Cdc14 in early anaphase to contribute to spindle stabilisation, the kinetochore component Ask1 (Higuchi and Uhlmann, 2005). We now know that Cdc14 also dephosphorylates Sli15, Fin1 and Ase1, which all contribute to the formation of a stable anaphase spindle (Pereira and Schiebel, 2003; Khmelinskii *et al.*, 2007; Woodbury and Morgan, 2007). Likewise, *Caenorhabditis elegans* CDC-14 shapes the anaphase spindle by dephosphorylation of the kinesin ZEN-4 (Mishima *et al.*, 2004).

## A quantitative model for mitotic exit

How does separase activate Cdc14? This is a question that still occupies us a great deal in the laboratory. It was unclear how important separase really was for mitotic exit, and we also needed to clarify its relationship with an essential signalling cascade, the mitotic exit network (MEN), that regulates Cdc14 (Surana *et al.*, 1993; Jaspersen *et al.*, 1998; Cohen-Fix and Koshland, 1999; Stegmeier *et al.*, 2002). None of the available separase mutants were likely to reflect a true null mutant, so Chris took on the challenge to construct a tight conditional allele based on a temperature degenon strategy (Sanchez-Diaz *et al.*, 2004). This showed that if separase was efficiently inactivated, Cdc14 was no longer released from the nucleolus at the time of anaphase. Thus, separase was crucial for initiating mitotic exit. From work in Ray Deshaies' laboratory we knew that Cdk-dependent phosphorylation of Cdc14's nucleolar inhibitor Net1 was instrumental to Cdc14 activation (Azzam *et al.*, 2004). Ethel Queralt, who had joined us, put one and one together and showed that separase promoted Net1 phosphorylation.



**Figure 2** Dual role of separase in triggering anaphase onset and mitotic progression. (I) After degradation of its inhibitor securin, separase is free to cleave cohesin, thereby triggering anaphase onset. Cohesin cleavage allows most sister sequences to separate, but not the budding yeast rDNA locus that remains interlinked by DNA catenation. (II) At the same time, separase causes downregulation of the PP2A<sup>Cdc55</sup> phosphatase. The mechanism for this is not yet understood, but involves an activity of separase independent of its proteolytic activity. This allows Cdk-dependent phosphorylation of Net1, the nucleolar inhibitor of the Cdc14 phosphatase, releasing active Cdc14. Initially, Cdc14 dephosphorylates targets important for anaphase spindle stabilisation and rDNA decatenation. (III) As Cdk activity declines, Cdc14 activates a positive feedback loop, the MEN, that sustains Cdc14 release. Cdc14 activity at low Cdk levels leads to completion of mitotic exit and cytokinesis.

This generated a paradox. How could separase promote Net1 phosphorylation, at a time when the anaphase-promoting complex (APC) not only causes securin degradation, but also starts to downregulate mitotic kinase activity by targeting cyclins? Ethel's solution was that Net1 phosphorylation in metaphase is counteracted by the PP2A<sup>Cdc55</sup> phosphatase. At anaphase onset, PP2A<sup>Cdc55</sup> activity is downregulated by separase, thus allowing Net1 phosphorylation by the persisting kinases (Queralt *et al*, 2006). The implications of this became clear from collaborating with Bela Novak, who developed a quantitative model for mitotic exit. PP2A<sup>Cdc55</sup> downregulation by separase initiates Net1 phosphorylation, but as kinase activity declines during mitotic exit this mechanism can only lead to a transient Cdc14 release. Such transient Cdc14 activation is indeed observed in cells lacking the MEN (Stegmeier *et al*, 2002). This signalling cascade is therefore required to maintain Cdc14 activity at later stages of mitotic exit. Activation of the MEN in turn depends on initial Cdc14 activation and kinase downregulation (Jaspersen and Morgan, 2000; Stegmeier *et al*, 2002), explaining why both, separase and MEN, are essential for mitotic exit (Figure 2).

Mitotic exit comprises a carefully ordered series of events, all under control of Cdc14. First, the elongating anaphase spindle is stabilised, but not long thereafter the spindle is disassembled again in preparation for cytokinesis. What ensures that one occurs before the other? Ordered dephosphorylation of individual Cdk targets, and ordered destruction of distinct APC substrates (Lindon and Pines, 2004; Rape *et al*, 2006), are probably involved. How such ordering is achieved will provide fertile ground for future quantitative studies. Whether separase controls Cdc14 activation and mitotic exit in organisms other than budding yeast is another open question. Budding yeast differs from many organisms in that in response to genotoxic damage the cell-cycle arrests not in G<sub>2</sub>, but in mitosis. Exit from mitosis might therefore be particularly tightly controlled in budding yeast. So far, analyses of metazoan mitotic exit have yielded mixed results as to a role of separase. An intriguing link has been made between separase and centriole disengagement during vertebrate mitotic exit, and an important role has been uncovered in Cdk downregulation during exit from meiosis I (Pandey *et al*, 2005; Gorr *et al*, 2006; Kudo *et al*, 2006; Tsou and Stearns, 2006).

### Fine-tuning chromosome segregation timing

During Matt's analysis of Cdc14 nucleolar release in anaphase, he made an unexpected observation. In response to TEV protease expression sister chromatids efficiently separated, when looking at fluorescently tagged chromosomal loci or the DAPI-stained DNA mass, but the nucleolus did not separate and stubbornly remained as a single organelle between the dividing nuclei. Cohesin cleavage was sufficient to separate much of the genome, but not the sister rDNA sequences on the long arm of chromosome XII on which the nucleolus assembles (D'Amours *et al*, 2004; Sullivan *et al*, 2004a). A candidate for such cohesin-independent sister-chromatid cohesion had long been named as DNA catenation (Murray and Szostak, 1985). After termination of DNA replication the two sister chromatids are left catenated, but whether, where, and for how long catenation persists be-

tween sister chromatids is still largely unknown. When Matt inactivated topoisomerase II at anaphase onset, the enzyme required to resolve DNA catenation, it likewise blocked rDNA segregation.

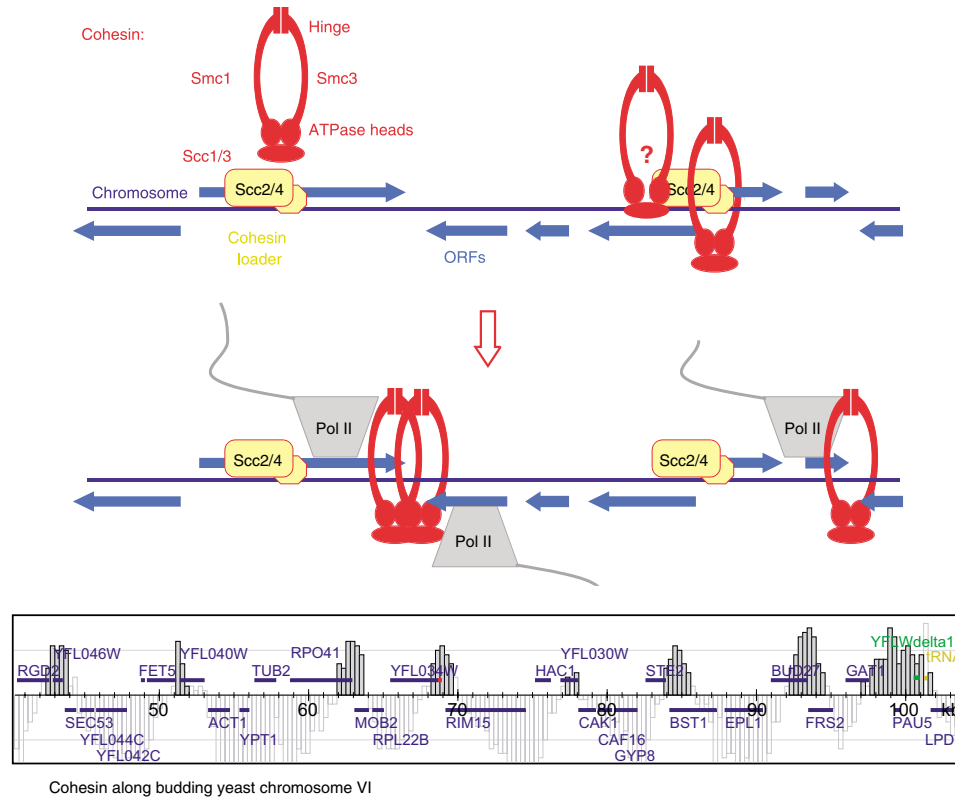
Problems with nucleolar segregation had been noted in *cdc14*-mutant cells before (Granot and Snyder, 1991), and again separase's role in activating this phosphatase was key to rDNA resolution (Figure 2). We do not know the Cdc14 substrate that regulates decatenation, but we and others realised that Cdc14 promotes recruitment and activation of the condensin complex at the rDNA, which in turn may stimulate decatenation of the locus by interaction with topoisomerase II (Bhat *et al*, 1996; Wang *et al*, 2004; C D'Ambrosio, unpublished observations).

Discovering the solution to an existing problem is a major driving force of scientific progress, but there is a problem with an unexpected discovery. Striking as it may look like, *a priori* it does not contain information as to its importance, in this case for chromosome segregation. We can only speculate about the reason for catenation-based rDNA cohesion and the Cdc14-dependence of its resolution. Might it be a relict from an evolutionarily ancient mechanism of sister-chromatid cohesion? DNA catenation as a form of cohesion has the danger that incomplete resolution during anaphase could lead to DNA breakage (Holm *et al*, 1989). Cohesin-based cohesion may therefore be a more gentle way of linking sister chromatids, and if forced, cohesin proteins can let go without the DNA being broken (Tanaka *et al*, 1999; Ocampo-Hafalla *et al*, 2007). In a repetitive locus, like the rDNA, catenation-based cohesion may be tolerable as a DNA break would at worst lead to loss of redundant information. The Cdc14 dependence of rDNA resolution in turn might regulate the segregation timing of the locus. Even though anaphase is a fast event in the cell cycle, individual human chromosomes have been shown to segregate with distinct, reproducible timing (Vig, 1981). In budding yeast, the rDNA is always the last part of the genome to segregate, opening the possibility that catenation-based cohesion is used at least at this locus to modulate its segregation timing during anaphase.

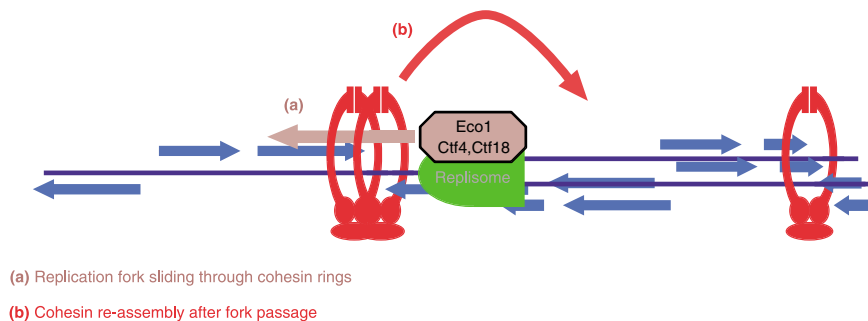
### A genome-wide view of sister-chromatid cohesion

The molecular mechanism by which the cohesin complex establishes and maintains cohesion between sister chromatids remains a topic of great interest. Studies at Kim's laboratory, together with electron micrographs from Anderson and Hirano, showed that the cohesin subunits assemble into an enormous protein ring (Anderson *et al*, 2002; Haering *et al*, 2002; Figure 3). The overall ring shape of the complex was not too surprising, as bacterial Smc proteins have been seen to form similar rings (Melby *et al*, 1998). Truly momentous was Kim's proposal that these cohesin rings might bind chromosomes by encircling the DNA. Cohesin's inner diameter of 35 nm is large enough even to enclose both sister chromatids after DNA replication. While this proposal remains the subject of animated discussion, Kim's laboratory has meanwhile provided compelling evidence that sister-chromatid cohesion indeed involves cohesin's topological embrace (Ivanov and Nasmyth, 2007). Such a simple mechanism for sister-chromatid cohesion of course does not exclude additional protein interactions between

**A** Cohesin loading onto chromosomes



**B** Cohesion establishment during DNA replication



**Figure 3** Cohesin rings in sister-chromatid cohesion. **(A)** Two structural maintenance of chromosomes subunits Smc1 and Smc3, with their extended coiled coil, form much of the circumference of the ring, kept closed by an interaction between the Smc subunits both at a dimerisation interface known as ‘hinge’ and by interaction of their ATPase subunits. Two additional subunits Scc1 and Scc3 associate with the Smc heads. Cohesin is loaded onto chromosomes by a loading factor, the Scc2/4 complex. This loading requires hydrolysis of ATP bound to the Smc head domains within the cohesin complex. Once loaded, cohesin translocates away from Scc2/4, in a manner consistent with it sliding along chromatin, and accumulates in regions of convergent transcriptional termination. **(B)** Establishment of sister-chromatid cohesion during DNA replication. Replication fork components are important for the establishment of sister-chromatid cohesion, in particular the proteins Eco1, Ctf4 and the RFC<sup>Ctf18</sup> complex. They might (a) help the replisome slide through cohesin rings, or (b) be involved in a reaction to re-assemble or stabilise cohesin around pairs of replication products in the wake of the fork.

cohesin and chromosomal proteins that contribute to sister-chromatid cohesion, for example, at heterochromatic or repetitive loci (Nonaka *et al*, 2002; Kobayashi *et al*, 2004; Chang *et al*, 2006). How cohesin rings are opened to be topologically loaded onto DNA is a fascinating question. An intact ATPase is required for the loading reaction, but what conformational changes ATP binding and hydrolysis introduce into the complex to open the ring still remains to be worked out (Arumugam *et al*, 2003; Weitzer *et al*, 2003; Gruber *et al*, 2006; Mc Intyre *et al*, 2007).

An approach that we have taken to study cohesin function was to ask where along chromosomes the cohesin complex binds, and what we could learn from the association pattern. It started when Etienne Schwab told us about Katsuhiko Shirahige’s chromatin immunoprecipitation experiments, hybridised to high-resolution oligonucleotide microarrays. Using this technique, Katsu had obtained exciting results about the role of DNA replication checkpoint proteins at replication forks (Katou *et al*, 2003). They had also mapped the localisation of the cohesin complex along chromosome

VI, and the clarity of the map was astounding. Almost all the cohesin peaks coincided with transcriptional termination sites between convergently transcribed genes. Armelle Lengronne's experimental finesse led us to a model how cohesin gets to these sites (Figure 3A). Cohesin is loaded onto chromosomes by a loading factor, the Scc2/4 complex (Ciosk *et al*, 2000), but instead of convergent termination sites, Scc2/4 locates to sites of strong transcriptional activity. Only after loading at these sites, cohesin translocates away towards convergent intergenes. Transcriptional activity of a gene redistributes cohesin downstream, as if the process of transcription pushes cohesin towards the termination sites (Glynn *et al*, 2004; Lengronne *et al*, 2004). Whether cohesin rings simply slide along chromosomes during translocation remains to be seen. Independent of the mechanism, movement away from the loading site might change cohesin from an ATPase mode engaged in a ring-opening reaction, to a structural mode that maintains stable DNA entrapment. After that, cohesin still responds to changes in the transcriptional programme, and maybe other requirements of chromosomal metabolism, by lateral movement without losing stable topological contact to the DNA.

Cohesin is loaded onto chromosomes in G1, but a key moment for the complex comes as the replisome travels along the DNA during S-phase. Then, two sister chromatids are produced and cohesin establishes linkages between them. Does cohesin transiently dissociate from DNA, like most DNA-bound proteins, as the helicase unwinds the double helix and polymerases replicate the single strands? Cohesin's topological embrace offers an alternative solution. Armelle found that the same cohesin molecules remain bound to chromosomes, without exchange with soluble complexes, during replication fork passage (Lengronne *et al*, 2006). Might cohesin rings simply let the replication fork slide through it to leave replication products trapped inside (Figure 3B)? This would not only provide a secure mechanism to connect two replication products, but would also ensure that linkages are only established between replication products, and not any other two DNA strands that may come close to each other during S-phase. Even if true in principle, cohesion establishment during S-phase involves additional players (Figure 3B). These include the establishment of cohesion protein Eco1, an acetyl transferase (Skibbens *et al*, 1999; Tóth *et al*, 1999; Ivanov *et al*, 2002). As the name suggests, the protein is required for cohesion establishment during S-phase, but not for loading cohesin

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onto DNA before, or maintenance of cohesion after replication. Eco1 is recruited to the replication fork, probably by interaction with PCNA (Lengronne *et al*, 2006; Moldovan *et al*, 2006). What Eco1, together with a number of other replication fork associated proteins, does to promote sister-chromatid cohesion during S-phase, and what really happens to the cohesin complex while the replication fork passes, are exciting questions for further study.

## Outlook

The cohesin complex is only one of three essential Smc subunit containing protein complexes in all eukaryotes. These complexes are involved in many aspects of chromosome biology, and are evolutionarily older than, for example, histones. By studying these complexes we hope to learn fundamental aspects about how cells shape and segregate their genetic material. The question that I have to consider working at a cancer institute is for how long we will continue to perform our experiments with a simple unicellular yeast. Ultimately, we aim to understand how humans came to life, and live healthy without developing disease. At the same time, all biological processes, irrespective of which organism they occur in, are part of nature just as much as we are. In a time of research 'strategies', it seems prudent to remind ourselves that discoveries are what advances our knowledge, and that there is no strategy to make discoveries other than exploring any suitable model organism in a curiosity-driven way. Curiosity to learn more about how cells work with their centimetre-long DNA molecules in micrometre-sized nuclei will keep me motivated to continue our quest with test tubes, molecules and microscopes.

## Acknowledgements

This review describes research and results on chromosome duplication and segregation, selected exclusively from a personal history rather than from the conceptual context. Many scientists provided seminal contributions to the field that I have not mentioned, but on which this research depended both at its outset and in the interpretation of the results. My gratitude goes to all of them for making it such a fascinating field to work in. I would especially like to thank my great mentors and co-workers who taught me, Hans Probst, Jerry Hurwitz, Kim Nasmyth, and many others. Much of our current work has its roots in what I was lucky to study in Kim's laboratory. Many thanks to all the past and present members of my laboratory who have done much of what is described here. They, together with wonderful co-worker in many countries, make it all so enjoyable.

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