results here suggest that activated E2 associates only transiently with the E3 ligase, and that its dissociation may be a mandatory step in the ubiquitination reaction.

So how could release of Cdc34 be functionally important for ubiquitination? Dissociation of E2 loaded with ubiquitin may enable the sequential recruitment of several activated Cdc34 molecules into the SCF complex. In this case, the dissociation rate of Cdc34 should be similar to the rate of Sic1 polyubiquitination, which is exactly what the authors observed. One advantage of such a mechanism is that recruitment of loaded E2 and the ubiquitination of targets would be distinct steps: allowing recruitment of a new E2 while the 'released E2' still transfers ubiquitin to the bound substrate might make the system more efficient (Fig. 1). Deffenbaugh *et al.* observed that dissociation of Cdc34 was strongly increased when Cdc34 was first activated by thioester bond formation with ubiquitin. Consistent with this finding, a catalytically inactive version of Cdc34 dissociated poorly from the SCF^{Cdc4}. It has recently been shown that homodimerization of Cdc34 is required for the synthesis of polyubiquitin chains, and this interaction depends on the formation of Cdc34–ubiquitin thiol ester formation[6]. It is possible that the released E2 dimerizes to promote assembly of the polyubiquitin chain. Alternatively, dimerization may promote the recruitment of activated E2. Thus, the binding affinities combined with the dimerization properties may ensure a transient but efficient recruitment of activated Cdc34 into the SCF^{Cdc4} complex. The finding that Cdc34 must be released from its initial binding site on the E3 may also help explain how multiple lysine residues on the target protein can be ubiquitinated. This fact was hard to reconcile with a rigid model that fixes the position of the bound E2 and substrate. A released E2 may have more steric flexibility and may thus be able to target several lysine residues.

How is the recruitment and release of Cdc34 regulated? Deffenbaugh *et al.* observed that activated Cdc34 binds to the SCF complex with much lower affinity than uncharged Cdc34, raising the question of why uncharged E2 does not block SCF function. It is possible that Cdc34 *in vivo* exists predominantly in its activated state. It is unlikely that the E1 activates the unloaded E2 when it is bound to the SCF because the addition of the ubiquitin mixture containing the E1 did not stimulate dissociation of unloaded E2 that had been pre-bound to the SCF.

Although the observed dynamic properties of Cdc34 provide important new insight into the mechanism of ubiquitination by a RING-H2 E3-ligase, they also raise new questions. For example, how do proteins that are part of a multisubunit complex containing the target such as Cdc28–Clb5–Sic1, or SCF subunits themselves, escape ubiquitination? Several F-box proteins are targets of auto-ubiquitination, and it has been proposed that substrate binding may prevent their destruction[7,8]. Unless dimerization of F-box proteins is required for their ubiquitination, the released E2 must be able to access lysine residues that are quite distant form the substrate that is thought to fill part of the cleft. It is also unclear how released E2 can distinguish between different lysine residues of ubiquitin itself (Lys 48 versus Lys 63 or Lys 29). Future studies must now aim to elucidate the dynamic interaction between the F-box protein and its bound substrate during the ubiquitination reaction. Finally, it will be important to study the importance of neddylation/deneddylation of cullin for the dynamic interactions between Cdc34 and SCF^{Cdc4}. It was shown that the cullin subunit is modified by the ubiquitin-like protein Nedd8 (Rub1 in yeast), and both neddylation and deneddylation of cullins seem to be required for SCF function[9–12]. Although neddylation of Cdc53 is not essential in budding yeast, dynamic addition and removal of Nedd8 increases its activity *in vivo*[11]. Structural analysis revealed that the neddylated lysine residue of Cul1 is located close to the E2-binding site (less than 11 Å)[2], and it has been shown that the neddylated Cul1 has a higher affinity for the E2 than the non-neddylated form[13]. Available evidence is thus consistent with a model in which neddylation of cullins promotes recruitment of E2 into the SCF complex, whereas de-neddylation may ensure release of the charged E2, thereby perhaps regulating the 'hit-and-run' mechanism of Cdc34 in SCF^{Cdc4} (refs 9–12). □

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Of fertility, cystic fibrosis and the bicarbonate ion

Keith A. Sutton, Melissa K. Jungnickel and Harvey M. Florman

Mammalian sperm require activation within the female reproductive tract to fertilize eggs, and bicarbonate is essential for this process *in vitro*. A recent study implicates the cystic fibrosis transmembrane regulator (CFTR) as a possible regulator for bicarbonate release *in vivo*.

In 1951, M.C. Chang and C.R. Austin discovered that mammalian sperm must be activated, or capacitated, within the female reproductive tract to fertilize eggs. These observations led to the development of the *in vitro* techniques of capacitation and fertilization, which in turn provided the basis for new approaches to study development and to treat infertility. In contrast, the mechanisms of capacitation *in vivo* remain a mystery[1]. However, the recognition that clinical-assisted reproductive technology may be associated with certain developmental defects has redoubled interest in understanding the sperm–egg interaction as it occurs within the environment of the female reproductive tract[2]. On page 904 of this issue, a study by Wang *et al.* points to an unanticipated role of CFTR in the development of capacitation *in vitro* and provides a plausible link to events *in vivo*[3].

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Capacitation regulates at least two aspects of sperm behaviour. First, sperm develop a 'hyperactivated' flagellar motility with highly curved, asymmetric bends that generate arced or circular swimming paths *in vitro*[4]. This type of motility is characteristic of many sperm obtained from the ampulla region of the oviduct, where fertilization typically occurs, and, it is speculated, may facilitate contact with the egg. Second, sperm acquire the ability to undergo a receptor-activated acrosome reaction — an exocytic reaction in which sperm release a single secretory granule (acrosome) from the apical region of the head. The completion of the acrosome reaction is a key control step in the fertilization process. Although sperm bind to the egg extracellular matrix, or zona pellucida (zona), through the plasma membrane overlying the acrosome, they must complete the acrosome reaction before they can penetrate the zona or fuse with the egg plasma membrane. When sperm are first released into the female reproductive tract they are incapable of undergoing a zona-evoked acrosome reaction and acquire this ability only as a result of capacitation. The acrosome reaction may thus be viewed as a switch that may be thrown only in capacitated sperm[1].

Capacitation of mammalian sperm occurs within the complex environment of the female reproductive tract, with the final events occurring within the oviduct. In principle, active 'capacitating factors' might either be secreted by eggs or by the reproductive tract epithelium. These complications make the task of establishing the molecular events that drive this process daunting and have forced a reliance on *in vitro* analysis. So far, such studies have identified three key components: bicarbonate, calcium and albumin. The addition of these three components to base media drives high levels of sperm capacitation *in vitro* in some species, but represents a dramatic simplification of the capacitating environment *in vivo*.

Biochemical studies have indicated that bicarbonate, acting in concert with calcium in sperm, elevate intracellular cyclic adenosine-3,5-monophosphate (cAMP) levels[1,5]. Sperm express a 'soluble' adenylyl cyclase that is directly activated by bicarbonate and is proposed to function as a physiological bicarbonate sensor[6] (see Fig. 1). Elevated cAMP activates multiple pathways, including the opening of cyclic nucleotide-gated ion channels in the flagellum that may drive alterations in sperm motility and enhanced tyrosine phosphorylation of an array of sperm proteins. cAMP may possibly have effects on other elements of capacitation, such as membrane potential hyperpolarization and the alkalinization of intracellular pH.

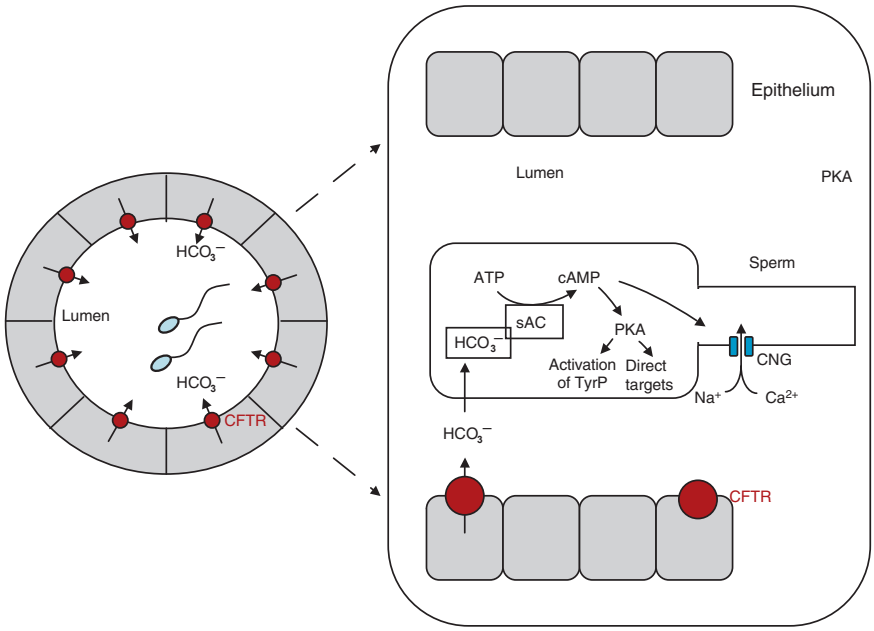

Figure 1 Proposed role of CFTR in sperm capacitation *in vivo*. Epithelial CFTR transports bicarbonate into the lumen of the uterus and oviduct (left). In the sperm head, bicarbonate activates the soluble adenylyl cyclase sAC (right). This triggers the production of cAMP, which activates proximal effectors, including the CNG cation channel and protein kinase A (PKA). PKA stimulation results in enhanced protein tyrosine phosphorylation (PYP) of an array of sperm proteins, as well as direct phosphorylation of other targets. These alterations are essential components of the sperm capacitation process.

Albumin, in contrast, accelerates cholesterol efflux from the sperm plasma membrane, thereby altering membrane composition and domain organization. Loss of cholesterol from the membrane is believed to shift the membrane to a more fusogenic state, which is required for successful completion of the acrosome reaction.

Do similar events control sperm activation *in vivo*? There have been hints that bicarbonate may be secreted at high concentrations from the female reproductive tract[7], but there was no obvious way to connect this to *in vitro* studies. The study by Wang and co-workers now points towards a possible link[3]. They reproduce earlier reports that sperm are capacitated *in vitro* in media conditioned by uterine epithelial cell cultures. They then suggest that the capacitating factor is bicarbonate, which is secreted by a process that depends on CFTR. This conclusion is supported by several findings: first, the uterine endometrium expresses CFTR; second, sperm were not capacitated efficiently in medium conditioned by endometrial cells in which CFTR expression had been suppressed using an antisense RNA strategy; third, sperm were also capacitated *in vitro* in medium conditioned by pancreatic duct cells expressing functional endogenous CFTR, but not by cells expressing the ΔF508 mutant CFTR, in which anion secretion is diminished through the failure of this channel protein to reach the plasma membrane[8]. Importantly, the failure of ΔF508 CFTR pancreatic duct cells to capacitate sperm was rescued by transfection with wild-type CFTR or by addition of bicarbonate to conditioned medium. These and other observations from the study support the notion that CFTR-dependent bicarbonate secretion can capacitate mouse sperm.

CFTR is a voltage-insensitive anion channel that is present in a wide range of epithelia. Chloride is the significant ion conductance of this channel and the reduction or ablation of chloride conductance was long believed to be the proximate cause of cystic fibrosis in *cftr* mutations[8]. Similarly, male and female infertility is associated with cystic fibrosis: in the case of the female, this is attributed to the properties of mucus secreted by the uterine cervix and can be accounted for by a primary defect in chloride secretion. It has been known for a decade that CFTR also transports bicarbonate, but this was not thought to be a significant factor in the aetiology of cystic fibrosis[9,10]. This issue is being re-assessed following the recent suggestion that CFTR may

transport bicarbonate and chloride by distinct mechanisms, with the severity of cystic fibrosis predicted more accurately by bicarbonate conductance[10–12]. The demonstration that uterine epithelial CFTR can drive sperm capacitation *in vitro* then suggests a new function for this channel as a bicarbonate transporter[3].

The study of Wang and co-workers strongly suggest that bicarbonate, a compound identified as a key regulator of sperm function and fertility *in vitro*, may also have a similar role *in vivo*[3]. A number of questions arise that must be resolved before the significance of this work can be fully appreciated. First, does uterine endometrial CFTR transport bicarbonate directly or is this caused by an indirect effect on another transporter? Second, the current study focuses on transport by uterine cells. In contrast, capacitation occurs within the oviducts, although some initial events may occur in the uterus. So is this function of CFTR relevant to oviduct cells? CFTR is also expressed in the oviductal epithelium, but whether it regulates capicitation here needs to be examined. Third, the work of Wang *et al.*[3] would predict that luminal bicarbonate in the uterus and oviducts should be lower in mice and humans with CFTR mutations that result in cystic fibrosis than when wild-type CFTR is present. Direct measurements of bicarbonate, although challenging, are required.

Wang and co-workers[3] suggest that the infertility of women with cystic fibrosis may not be caused exclusively by the failure of sperm to penetrate cervical mucus and instead may also follow from an inability of those sperm to capacitate within the uterus and oviduct. However, pregnancies may be produced in women with cystic fibrosis by intrauterine insemination[13], a surprising result if infertility were caused by a failure to capacitate. One explanation may lie in the fact that the nature of the *ctfr* mutation, particularly with regard to bicarbonate transport defects, was not examined in these women. In any case, the study of Wang *et al.*[3] provides a first step in which *in vitro* models of sperm capacitation can be tested *in vivo*. □

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Timing the cell cycle

Luca Cardone and Paolo Sassone-Corsi

Most cells contain two endogenous clocks, one devoted to the control of cell division and the other acting as circadian pacemaker. Although classically thought to be independent, recent findings challenge this view, as molecular components of the circadian clock directly regulate WEE1, a kinase that inhibits mitosis by inactivating Cdc2/cyclin B.

A fundamental feature of nearly all living organisms is the circadian rhythmicity of many of their physiological processes. The period of these oscillations is close to 24 h (thus the name circadian, from the latin *circa diem*), strongly implying that the circadian mechanism has been shaped by the day–night cycle during evolution[1]. Circadian rhythms are based on molecular clocks, self-sustaining pacemakers intrinsic to most cells[2]. These cell autonomous clocks dictate the timing of many physiological responses. Similarly, the cell cycle is also highly periodic. Consequently, researchers have wondered about possible intracellular links between the circadian pacemaker and the cell cycle. Findings by Matsuo and colleagues[3] published in a recent issue of *Science* shed some light on this fundamental question.

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Circadian clocks consist of a system of 'clock genes', some of which encode proteins able to feed back and inhibit their own expression[1]. Cells experience daily variations in the levels of clock proteins, and they interpret these changes to reflect different phases of the daily cycle. As a general hallmark, molecular pacemakers help to anticipate the needs of the organisms through the cyclic regulation of clock controlled genes. At the molecular level, the architecture of the circadian system is reminiscent of the cell cycle, in that both exhibit 'clock'-like properties. Both systems rely on sequential phases of transcription–translation, protein modification and degradation, constituting several interconnected autoregulatory loops. Besides, is it only fortuitous that most eukaryotic cells in culture undergo mitosis with a periodicity of about one day? As it is conceivable that most cells were sensitive to light–dark cycles in their evolutionary history, is what we study today as the 'cell cycle' a vestigial circadian clock? These questions make one wonder whether the two clocks operating within an individual cell are interlocked by sharing some critical elements.

Evidence exists that cell division of some unicellular organisms is controlled by a circadian mechanism. This is true for the green alga *Chlamydomonas reinhardtii*[4], of the flagellate *Euglena gracilis*[5] and of the dinoflagellate *Gonyaulax polyedra*[6], although uncoupling between the two cyclic processes is evident in cyanobacteria[7]. The situation is of course different in multicellular organisms, in which a link between the two intracellular clocks has been elusive. Indeed, adult neurons that constitute the suprachiasmatic nucleus (SCN), the centre of the mammalian clock, do not divide and yet display formidable circadian oscillations[1,2]. More importantly, circadian rhythmicity of gene expression persists in cultured fibroblasts even when cell division is blocked[8]. Thus, if a link exists, it seems that it can be unmasked only under particular circumstances. It could be that, if the circadian clock is to exert some type of influence on the cell cycle clock (or vice versa), the two mechanisms need to reach a certain degree of synchronization. The work by Matsuo and colleagues[3] reveals one setting in which the two clockworks become interlocked at the molecular level.