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RESEARCH ARTICLE

CK1δ and CK1ε are components of human 40S subunit precursors required for cytoplasmic 40S maturation

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ABSTRACT

Biogenesis of 40S pre-ribosomal subunits requires many trans-acting factors, among them several protein kinases. In this study, we show that the human casein kinase 1 (CK1) isoforms δ and ε are required for cytoplasmic maturation steps of 40S subunit precursors. We show that both CK1δ and CK1ε isoforms are components of pre-40S subunits, on which they phosphorylate the ribosome biogenesis factors ENP1/BYSL and LTV1. Inhibition or co-depletion of CK1δ and CK1ε results in failure to recycle a series of trans-acting factors including ENP1/BYSL, LTV1, RRP12, DIM2/PNO1, RIO2 and NOB1 from pre-40S particles after nuclear export. Furthermore, co-depletion of CK1δ and CK1ε leads to defects in 18S-E pre-rRNA processing. Together, these data demonstrate that CK1δ and CK1ε play a decisive role in triggering late steps of pre-40S maturation that are required for acquisition of functionality of 40S ribosomal subunits in protein translation.

KEY WORDS: Ribosome biogenesis, Pre-40S maturation, Protein kinase, CK1δ, CK1ε

INTRODUCTION

One of the most energy-consuming processes in a cell is the biogenesis of ribosomes, which provides cells with the machinery to drive protein synthesis (Warner, 1999). Ribosome biogenesis starts in the nucleolus by RNA Pol I-mediated transcription of a long ribosomal RNA (rRNA) precursor on which ribosomal and non-ribosomal proteins, so called trans-acting factors, assemble to form an early 90S particle. Cleavage of the precursor rRNA (pre-rRNA) yields a pre-40S and a pre-60S particle, both of which undergo independent maturation steps in the nucleoplasm and are exported to the cytoplasm, where they become translation-competent 40S and 60S ribosomal subunits, respectively. This process involves more than 200 trans-acting factors, which join and are released from pre-ribosomal particles at different time-points along the maturation pathway (reviewed in Fromont-Racine et al., 2003; Zemp and Kutay, 2007; Henras et al., 2008; Kressler et al., 2010; Panse and Johnson, 2010). Trans-acting factors belong to numerous protein classes and provide different enzymatic activities, such as GTPases, exo- and endonucleases and protein kinases. In the budding yeast *Saccharomyces cerevisiae*, the composition of different pre-ribosomal particles has been thoroughly studied (reviewed in Fromont-Racine et al., 2003; Tschochner and Hurt, 2003), and, for an increasing number of trans-acting factors, the mechanism through which they regulate ribosome production is being elucidated. Less is known about trans-acting factors and their mode of action in mammalian ribosome biogenesis. However, many yeast trans-acting factors are conserved in humans. Recent RNA interference (RNAi) screening approaches (Wild et al., 2010), as well as analysis of tandem affinity purification (TAP) particles (Wyler et al., 2011), led to the identification of numerous human ribosome biogenesis factors, including some that are specific to vertebrate cells. Although the general pathway is similar between yeast and human, it has been shown that several steps, such as pre-rRNA processing (Rouquette et al., 2005) and ribosome export (Wild et al., 2010), differ and that certain trans-acting factors play different roles in these organisms (Carron et al., 2011; Sloan et al., 2013).

Among the enzymes that support ribosomal subunit maturation are protein kinases. Recent data demonstrate that the human protein kinases RIO1, RIO2 and RIO3 are components of pre-40S particles and are required for their cytoplasmic maturation (Rouquette et al., 2005; Zemp et al., 2009; Baumas et al., 2012; Widmann et al., 2012). Although phosphorylation substrates for RIO kinases have not been found, recycling of certain 40S trans-acting factors and pre-rRNA processing are dependent on the kinase/ATPase activity of the RIO kinases (Zemp et al., 2009; Widmann et al., 2012). Recent structural and biochemical analysis of RIO2 from the lower eukaryote *Chaetomium thermophilum* confirmed this conclusion (Ferreira-Cerca et al., 2012). It has been speculated that the ATPase activity of RIO2 could drive structural changes in pre-40S particles to promote their cytoplasmic maturation (Zemp et al., 2009; Ferreira-Cerca et al., 2012).

In budding yeast, an additional kinase is required for 40S, as well as 60S, maturation: the casein kinase I (CK1) homolog Hrr25. This serine/threonine kinase phosphorylates Tif6, a 60S trans-acting factor involved in processing of 35S pre-rRNA (Basu et al., 2001). Furthermore, it has been shown that Hrr25 also participates in 40S subunit maturation by phosphorylating a pre-ribosomal subcomplex consisting of Rps3 and the trans-acting factors Enp1 and Ltv1 (Schäfer et al., 2006). Phosphorylation of these components is part of a mechanism that leads to dissociation of the subcomplex from maturing pre-40S particles.

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Received 18 July 2013; Accepted 14 December 2013

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Subsequent dephosphorylation of Rps3 has been suggested to allow stable incorporation of Rps3 into the pre-40S particle, which then induces the formation of the protruding 40S 'beak' structure. When Hrr25 is depleted, Rps3 and Enp1 cannot be released from pre-ribosomes, while Ltv1 is still partly phosphorylated and able to dissociate from pre-40S (Schäfer et al., 2006). Interestingly, a cryo-EM structure of pre-40S particles revealed that the Enp1–Ltv1–Rps3 complex bound to pre-40S seems to hinder the opening of the mRNA channel, which indicates that Enp1 and Ltv1 need to dissociate in order for 40S subunits to become translation competent (Strunk et al., 2011).

In mammalian cells, there are seven different isoforms of CK1: α, β, γ1, γ2, γ3, δ and ε, which are involved in numerous processes, such as establishment of circadian rhythm (Lee et al., 2001), cell proliferation (Peters et al., 1999) and development of cancer (Knippschild et al., 1997). Notably, CK1 has also been linked to 60S biogenesis because it has been shown that mammalian eIF6, the ortholog of yeast Tif6, is phosphorylated by CK1 (Basu et al., 2001), which leads to changes in the subcellular distribution of eIF6 (Basu et al., 2001; Biswas et al., 2011). However, it remains unclear how this phosphorylation affects nuclear export or cytoplasmic maturation of 60S precursors.

Here, we show that human CK1 is required for the biogenesis of small ribosomal subunits. Chemical inhibition of CK1 causes defects in late 40S maturation, in particular in recycling of 40S trans-acting factors in the cytoplasm, indicating that CK1 kinase activity is required for this process. We identified the CK1 isoforms δ and ε as novel components of pre-40S particles. Like chemical inhibition, co-depletion of CK1δ and CK1ε leads to defects in recycling of 40S trans-acting factors, as well as to defective processing of 18S-E pre-rRNA. In addition, we observed CK1-dependent phosphorylation of ENP1 and LTV1 on 40S precursors, indicating that this process is involved in release of trans-acting factors from cytoplasmic pre-40S particles.

RESULTS

Cytoplasmic recycling of 40S trans-acting factors is blocked upon CK1 inhibition

To investigate whether other kinases, besides the RIO kinases, might be required for maturation of the human 40S subunit, we treated cells with the cell-permeable CK1 inhibitor D4476 (Rena et al., 2004; Bain et al., 2007; Bryja et al., 2007). We followed the effects of CK1 inhibition by analyzing the localization of a series of trans-acting factors by immunostaining. Mislocalization or altered shuttling behavior of human 40S biogenesis factors can indicate at which steps of 40S maturation defects occur (Zemp et al., 2009; Wild et al., 2010; Wyler et al., 2011). Compared to control cells, ENP1/BYSL, DIM2/PNO1 and RRP12 displayed partial relocalization from the nucleolus to the cytoplasm upon treatment of cells with D4476 (Fig. 1A). NOC4 (also known as NOC4L, the homolog of yeast Noc4), an early 40S maturation factor, did not show altered localization upon CK1 inhibition.

LTV1, NOB1 and RIO2 are cytoplasmic proteins at steady state but shuttle between the nucleus and the cytoplasm to support 40S maturation (Zemp et al., 2009). Cytoplasmic localization of all three factors is known to be dependent on the activity of the pre-40S exportin CRM1 (also known as exportin 1). Inhibition of CK1 by D4476 did not change the cytoplasmic localization of LTV1, NOB1 or RIO2. However, when combining D4476 treatment with inhibition of CRM1 by leptomycin B (LMB), LTV1, NOB1 and RIO2 remained cytoplasmic and failed to accumulate in the nucleus, whereas accumulation of these factors was observed in control cells (Fig. 1A). Thus, several 40S maturation factors appear to be confined to the cytoplasm upon D4476 treatment, indicating defects in their release from cytoplasmic pre-40S during their maturation. Furthermore, when analyzing extracts prepared from solvent- or D4476-treated cells, the nucleolar factors RRP12, DIM2 and, to a lesser extent, ENP1 are more readily extracted (Fig. 1B), supporting the idea that these factors are trapped in the cytoplasm. To determine whether these proteins accumulate in the cytoplasm as free proteins or as part of 40S precursors, we analyzed these extracts by sucrose gradient centrifugation. The major pool of all six trans-acting factors co-sedimented with 40S subunits after D4476 treatment, and for several factors, such as RIO2, ENP1 and NOB1, the ratio between 40S-associated and free protein was increased (Fig. 1C). These data indicate that these proteins are trapped in the cytoplasm as part of 40S precursors upon CK1 inhibition.

Isoforms CK1δ and CK1ε are required for cytoplasmic recycling of 40S trans-acting factors

To investigate further the observed 40S maturation defects upon CK1 inhibition, we wanted to determine which of the CK1 isoforms are required in 40S maturation. We tested their role in 40S biogenesis by targeting the individual isoforms by small interfering RNA (siRNA)-mediated knockdown. Notably, CK1 depletion can have effects distinct from those of CK1 inhibition as the former method leads to absence of the protein, whereas the latter does not alter protein levels but inhibits the kinase activity. Depletion of the individual CK1 isoforms did not change the localization of the 40S trans-acting factors (Fig. 2A and data not shown). As the CK1 isoforms might act in a redundant manner, we next performed co-depletion experiments targeting all possible combinations of two CK1 isoforms. With one exception, these experiments, again, did not result in changes in the localization of 40S trans-acting factors (Fig. 2A and data not shown). Only the co-depletion of CK1δ and CK1ε resulted in increased cytoplasmic localization of DIM2, ENP1 and RRP12 (Fig. 2A,B). Correspondingly, DIM2, RRP12 and, to a lesser extent, ENP1 are more efficiently extracted from CK1δ and CK1ε co-depleted cells (Fig. 2C). Moreover, RIO2, LTV1 and NOB1 failed to accumulate in the nucleus upon LMB treatment in the absence of CK1δ and CK1ε (Fig. 2A). Thus, the effects of double depletion of CK1δ and CK1ε led to mislocalization of trans-acting factors that closely resembles the effects of CK1 inhibition by D4476. Sucrose gradient centrifugation of extract from CK1δ and CK1ε co-depleted cells revealed that the six trans-acting factors trapped in the cytoplasm all co-sedimented with 40S precursors (Fig. 2D). Thus, in the absence of CK1δ and CK1ε, pre-40S particles are exported to the cytoplasm but fail to mature fully because several trans-acting factors are not released from the precursors. CK1δ and CK1ε therefore play redundant roles in cytoplasmic 40S maturation.

To verify the role of CK1 kinase activity in cytoplasmic 40S maturation, we performed rescue experiments by depleting CK1δ as above, and CK1ε with an siRNA oligo targeting the 3′UTR. Expression of wild-type CK1ε, but not of kinase-dead CK1ε(D149A), partially restored nucleolar ENP1 localization,

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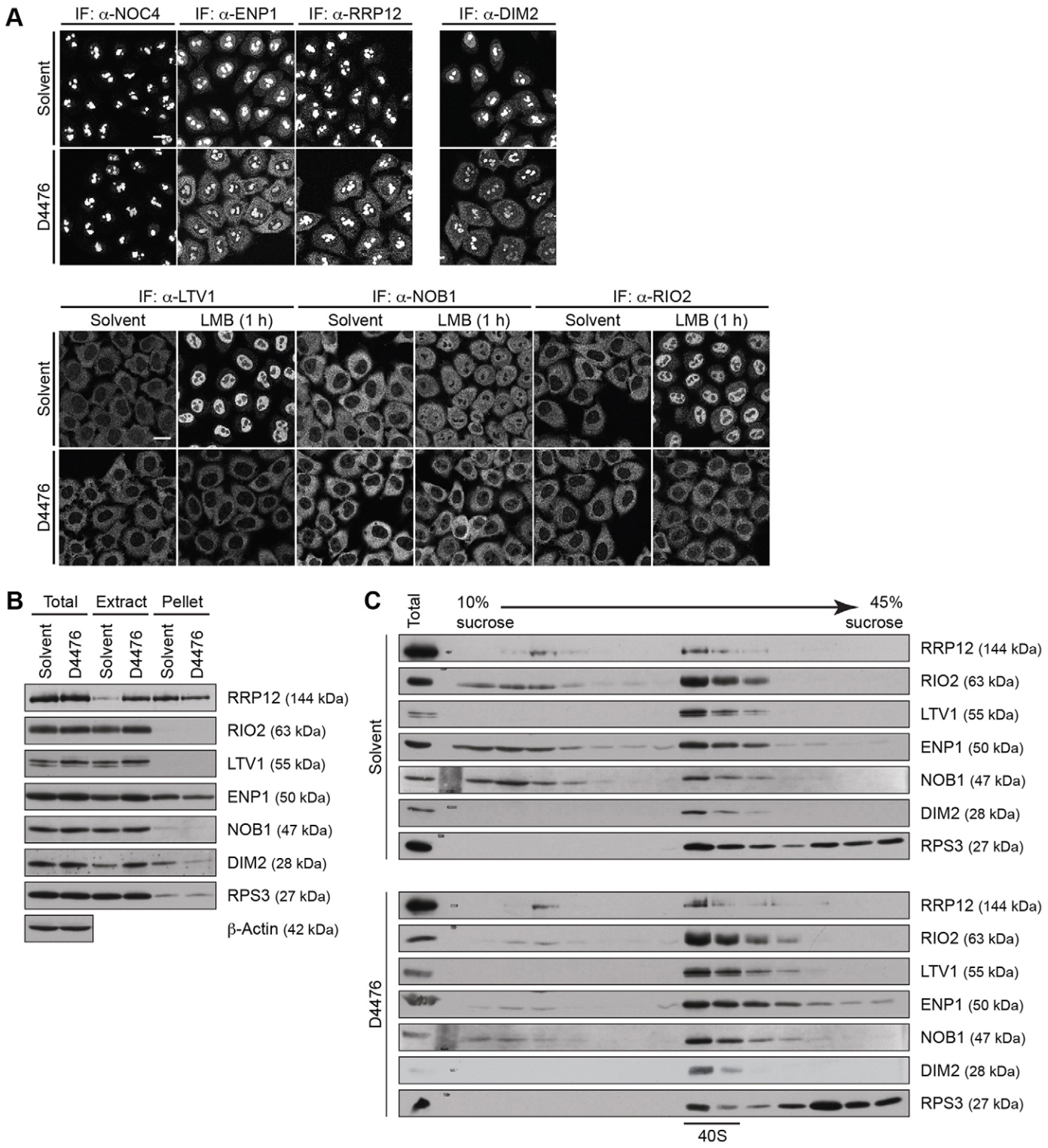

Fig. 1. CK1 inhibition prevents release of trans-acting factors from cytoplasmic 40S precursors. (A) Immunofluorescence analyses for 40S trans-acting factors after CK1 inhibition. HeLaK cells were treated with 100 μM D4476 or solvent (DMSO) for 8 h, followed by immunofluorescence analysis for the indicated factors. For LTV1, NOB1 and RIO2, cells were in addition treated with 20 nM LMB or solvent (ethanol) for 1 h prior to fixation. Scale bars: 20 μm. Upon CK1 inhibition, ENP1, DIM2 (analyzed in an independent experiment) and RRP12 are partially mislocalized to the cytoplasm, and LTV1, NOB1 and RIO2 fail to shuttle to the nucleus. (B) Western blot analysis of extracts from solvent- or D4476-treated cells. HeLaK cells were treated with 100 μM D4476 or solvent. After 8 h, extracts were prepared and then analyzed using antibodies against the indicated factors. The nucleolar proteins RRP12, DIM2 and, to a lesser extent, ENP1 are more readily extracted upon CK1 inhibition. (C) Sucrose gradient analysis of extracts from D4476-treated cells. Extracts prepared in B were centrifuged on a 10–45% sucrose gradient. Samples of total cells and of gradient fractions were analyzed by western blotting against the indicated factors. Fractions containing pre-40S particles are indicated (40S). The trans-acting factors trapped in the cytoplasm upon CK1 inhibition are part of 40S subunit precursors.

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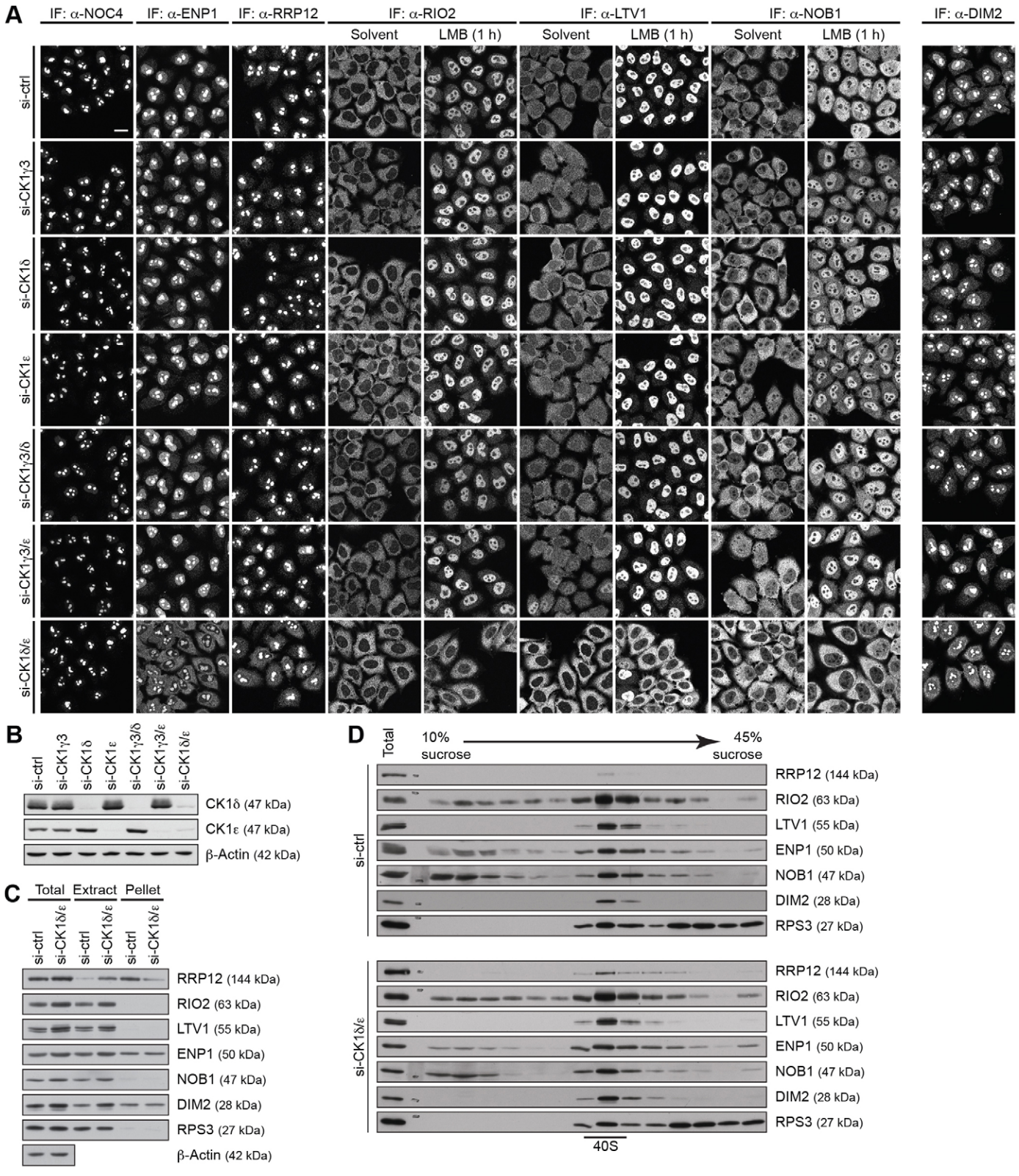

Fig. 2. See next page for legend.

confirming that CK1ε kinase activity was required for ENP1 recycling (Fig. 3). However, the rescue was only partial, probably as a result of dominant-negative effects of transient overexpression of CK1 constructs on the localization of ENP1.

The conclusions derived from the RNAi experiments were further supported by tests of different small-molecule inhibitors of CK1. LH846 and PF4800567 are two CK1 inhibitors that preferentially target the δ and ε isoforms, respectively (Walton

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Fig. 2. Co-depletion of CK1δ and CK1ε leads to defects in recycling of 40S trans-acting factors. (A) Immunofluorescence analyses for 40S trans-acting factors after RNAi-mediated depletion of CK1 isoforms. HeLaK cells were transfected with the siRNAs indicated. 48 h after transfection, cells were fixed directly or after treatment with solvent (ethanol) or 20 nM LMB for 1 h, followed by immunofluorescence analysis for the indicated factors. Scale bar: 20 μm. Upon co-depletion of CK1δ and CK1ε, DIM2 (analyzed in an independent experiment), ENP1 and RRP12 are partially mislocalized to the cytoplasm, and RIO2, LTV1 and NOB1 fail to shuttle to the nucleus. (B) Western blot analysis of experiment in A, using the indicated antibodies, to reveal efficient depletion of CK1δ and CK1ε by RNAi. CK1γ3 mRNA levels were depleted by more than 95%, as verified by RT-PCR (data not shown). (C) Western blot analysis of extracts from control or CK1δ- and CK1ε-depleted cells. HeLaK cells were transfected with the respective siRNAs, extracts were prepared 48 h after transfection and were then analyzed using antibodies against the indicated factors. The nucleolar proteins RRP12, DIM2 and, to a lesser extent, ENP1 are more readily extracted upon co-depletion of CK1δ and CK1ε. (D) Sucrose gradient analysis of extracts from CK1δ- and CK1ε-depleted cells. Extracts prepared in C were centrifuged on a 10–45% sucrose gradient. Samples of total cells and of gradient fractions were analyzed by western blotting against the indicated factors. Fractions containing pre-40S particles are indicated (40S). The trans-acting factors trapped in the cytoplasm upon CK1 inhibition are part of 40S subunit precursors.

et al., 2009; Lee et al., 2011), whereas PF670462 potently inhibits both CK1δ and CK1ε (Badura et al., 2007). Treatment of cells with the CK1δ and CK1ε inhibitor PF670462 caused accumulation of ENP1 in the cytoplasm similar to treatment with D4476, whereas individual application of LH846 and PF4800567 at the chosen concentration had no effect (Fig. 4). Only combined application of LH846 and PF4800567 led to cytoplasmic accumulation of ENP1, providing additional evidence for the redundant role of CK1δ and CK1ε in the cytoplasmic steps of 40S subunit maturation.

CK1δ and CK1ε are not required for pre-40S export

Studies in yeast have shown that repression of the CK1 homolog Hrr25 leads to nuclear accumulation of an Rps2–GFP reporter (Schäfer et al., 2006), which would indicate a pre-40S maturation defect prior to nuclear export. This is in contrast to our findings where CK1δ and CK1ε affect late cytoplasmic 40S biogenesis steps. To investigate further whether knockdown of CK1δ and CK1ε leads to pre-40S export defects, we used a HeLa cell line expressing RPS2–YFP in a tetracycline-inducible manner to visualize newly synthesized (pre-)40S subunits (previously described in Zemp et al., 2009). As expected, depletion of the 40S nuclear export receptor CRM1 led to nucleoplasmic accumulation of RPS2–YFP and also ENP1 (Fig. 5A). Neither single depletion nor co-depletion of CK1δ and CK1ε led to nuclear accumulation of RPS2–YFP or ENP1, whereas CK1δ and CDK1ε co-depletion, again, caused partial relocalization of ENP1 to the cytoplasm. Thus, compared with their yeast homolog Hrr25, CK1δ and CK1ε appear to be required at a later stage of pre-40S maturation.

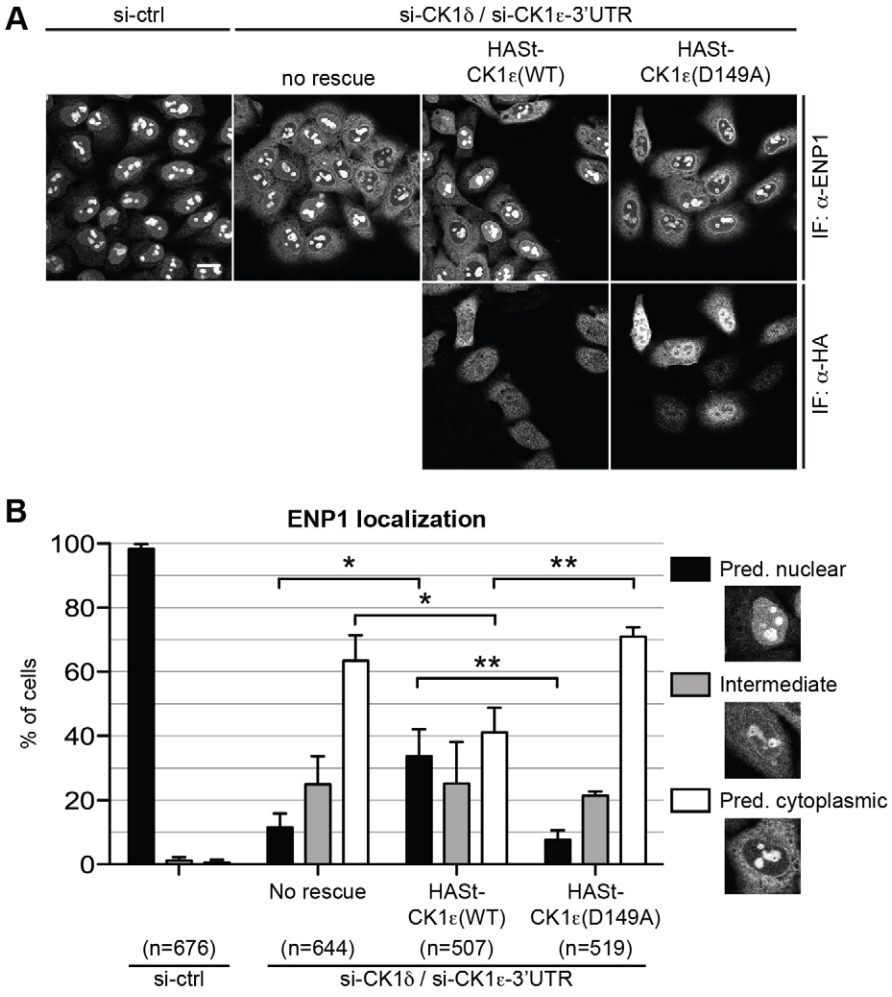

Fig. 3. Wild-type but not kinase-dead CK1ε partially rescues ENP1 mislocalization after CK1δ and CK1ε co-depletion. (A) HeLaK cells were treated with the indicated siRNAs. 24 h after transfection, cells were either left untreated or transiently transfected with a plasmid expressing either HASt–CK1ε wild-type (WT) or kinase-dead HASt–CK1ε(D149A). 48 h after siRNA transfection, cells were fixed, followed by co-immunostaining for HA (to detect the HASt constructs) and ENP1. Scale bar: 20 μm. CK1δ/ε co-depletion partially relocalizes ENP1 to the cytoplasm, as in Fig. 2. This phenotype is partially rescued in cells expressing CK1ε(WT) but not in cells expressing CK1ε(D149A).
(B) Quantification of three independent experiments performed as in A. Cells were scored for predominantly nucleolar (pred. nucleolar), intermediate or partially cytoplasmic (part. cytoplasmic) localization of ENP1 (see example cells). For rescues, only cells displaying detectable HASt–CK1ε expression were counted. For each experiment, between 80 and 330 cells were counted per condition. A *t*-test was performed and statistically significant differences are indicated (* *P* value <0.05; ** *P* value <0.01). HASt: N-terminal tandem HA/streptavidin-binding peptide tag.

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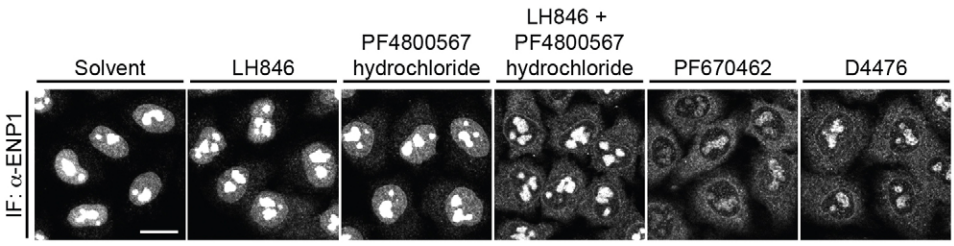

Fig. 4. Combined inhibition of CK1δ and CK1ε leads to ENP1 mislocalization. HeLaK cells were treated with the indicated inhibitors of CK1 (LH846, 7.5 μM; PF4800567, 1.5 μM; PF670462, 1 μM; D4476, 100 μM) for 6 h, followed by immunostaining for ENP1. LH846 and PF4800567 potently inhibit CK1δ and CK1ε, respectively, whereas PF670462 is an inhibitor of both isoforms. Combination of LH846 and PF4800567 leads to partial relocalization of ENP1 to the cytoplasm, comparable to PF670462 or D4476 treatment. Scale bar: 20 μm.

18S-E pre-rRNA processing requires CK1δ and CK1ε

Besides recycling of the 40S-associated trans-acting factors, cytoplasmic 40S maturation includes a pre-rRNA processing step, which produces mature 18S rRNA from 18S-E pre-rRNA (Rouquette et al., 2005). As CK1δ and CK1ε co-depletion blocks the release of trans-acting factors from cytoplasmic 40S precursors, we wanted to test whether 18S-E pre-rRNA processing was also affected. Therefore, we performed fluorescence *in situ* hydridization (FISH) analysis, which enables the detection of defects in 18S-E pre-rRNA processing by visualizing an increase in cytoplasmic 5′ internal transcribed spacer region 1 (ITS1) signal (Rouquette et al., 2005). As previously described (Rouquette et al., 2005; Zemp et al., 2009), depletion of RIO2 blocked 18S-E pre-rRNA processing (Fig. 5B). Co-depletion of CK1δ and CK1ε by RNAi led to a strong increase in cytoplasmic 5′ITS1 signal, similar to the effect observed after depletion of RIO2, indicating that CK1δ and CK1ε are required for 18S-E pre-rRNA processing.

CK1δ and CK1ε are components of human 40S subunit precursors

Inhibition of CK1 kinase activity and depletion of the CK1δ and CK1ε isoforms leads to defects in cytoplasmic 40S maturation. CK1 is a pleiotropic protein kinase involved in many different cellular processes, raising the question whether the defects we observed were a direct or indirect consequence of CK1 inhibition and depletion. We therefore tested whether CK1δ and CK1ε are associated with 40S precursors. Pre-40S particles were purified using immunoprecipitation of RIO2 (Zemp et al., 2009), a 40S trans-acting factor that is cytoplasmic at steady state. The isolated particles contained ribosomal proteins of the small subunit and several 40S trans-acting factors, as expected (Fig. 6A). By contrast, the 60S-specific trans-acting factor NMD3 was only detected in the NMD3 immunoprecipitate, which we used as a negative control, but not in the RIO2-associated particle. The nucleolar factor NOC4 was detected in neither immunoprecipitate. Analysis for the CK1 isoforms δ and ε revealed that both proteins are specifically present in the RIO2 immunoprecipitate (Fig. 6A), indicating that they are components of 40S subunit precursors and suggesting that the 40S maturation defects observed after CK1 inhibition and depletion are direct.

To further support this notion, we analyzed the sucrose gradient sedimentation behavior of CK1δ and CK1ε. The majority of both proteins was detected at, or near, the top of the gradient, suggesting that they are present as free proteins or in small complexes (Fig. 6B). However, an additional, smaller pool of CK1δ and CK1ε reproducibly co-sedimented with 40S subunits. To strengthen this conclusion, we repeated the analysis with extract from cells treated with Actinomycin D (ActD). ActD inhibits pre-rRNA transcription, and treatment of cells with ActD for 2 h reduces the levels of 40S precursors, as

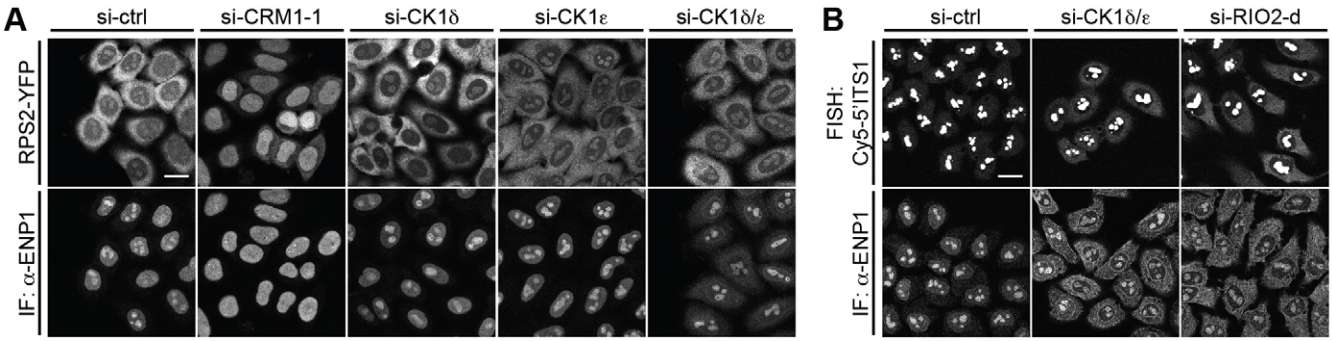

Fig. 5. CK1δ and CK1ε are not required for pre-40S export, but for efficient 18S-E pre-rRNA processing. (A) Analysis of RPS2–YFP and ENP1 localization after RNAi-mediated depletion of CK1δ and CK1ε. RPS2–YFP reporter cells were transfected with siRNAs as indicated. Expression of RPS2–YFP was induced by the addition of tetracycline 52 h after transfection. Cells were chased for 4 h in tetracycline-free medium after 16 h of induction, followed by fixation and immunostaining for ENP1. Depletion of the 40S export receptor CRM1 leads to nuclear accumulation of RPS2–YFP and ENP1, but neither single nor co-depletion of CK1δ and CK1ε causes a pre-40S export defect. Scale bar: 20 μm. (B) Fluorescence *in situ* hybridization (FISH) detecting 18S rRNA precursors after CK1δ and CK1ε co-depletion. Cells were depleted for CK1δ and CK1ε as in Fig. 2, followed by FISH analysis using a 5′ITS1 probe and immunofluorescence detection of ENP1. Scale bar: 20 μm. FISH pictures were processed in parallel by enhancing levels, followed by setting γ correction to 0.75. Depletion of RIO2 was performed as a positive control, leading to cytoplasmic ENP1 and FISH signals owing to defects in ENP1 recycling and 18S-E pre-rRNA processing, as described previously (Zemp et al., 2009). Similarly, CK1δ and CK1ε co-depletion also resulted in cytoplasmic 5′ITS1 signal, indicating defects in 18S-E pre-rRNA processing.

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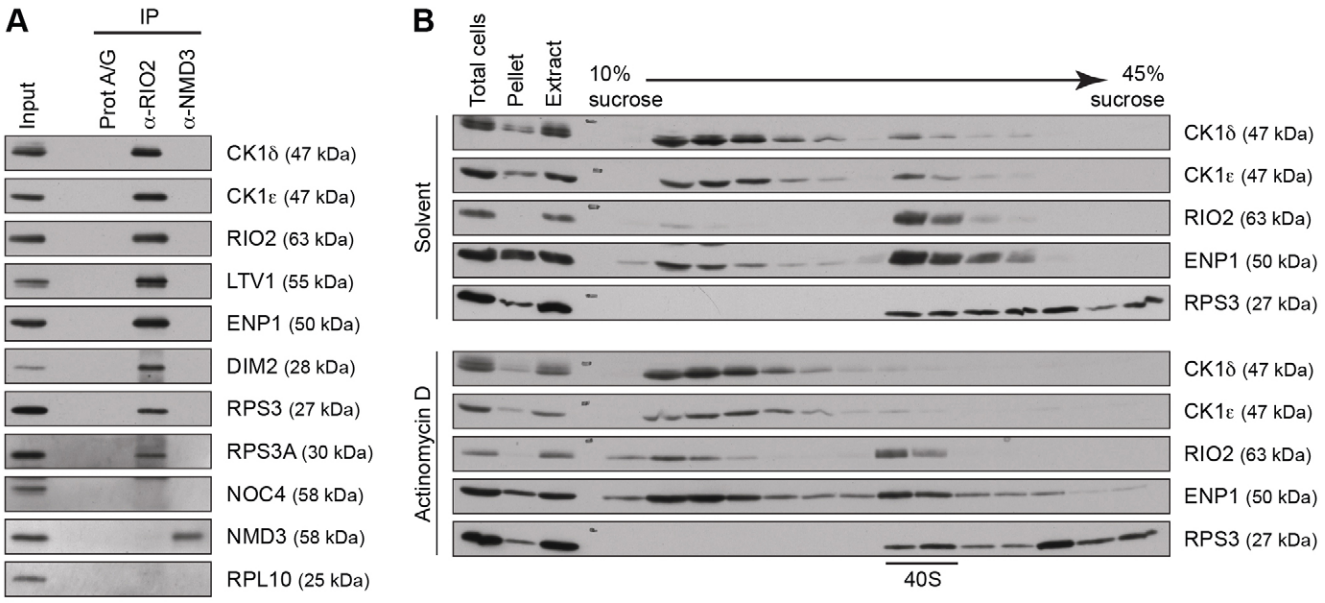

Fig. 6. CK1δ and CK1ε are components of pre-40S particles. (A) Western blot analysis of a RIO2-associated 40S precursor. A RIO2-associated 40S precursor was immunoprecipitated and analyzed by western blotting for the factors indicated. Immunoprecipitation of NMD3 served as a control. Load corresponds to 0.25% of the input and 15% of the eluates. Both CK1δ and CK1ε are associated with the pre-40S particle. (B) Sucrose gradient analysis of sedimentation behavior of CK1δ and CK1ε. Extracts were prepared from solvent- (ethanol) or Actinomycin D (ActD)-treated (100 nM, 2 h) cells and centrifuged on a 10–45% sucrose gradient. Samples of total cells, pellet, extract and gradient fractions were analyzed by western blotting against the indicated factors. Fractions containing pre-40S particles are indicated (40S). CK1δ and CK1ε co-sediment with 40S precursors in control but not ActD-treated cells.

indicated by the increased ratios of free versus 40S-associated RIO2 and ENP1 in extracts from ActD-treated compared to control cells (Fig. 6B). The small pool of CK1δ and CK1ε co-sedimenting with 40S subunits was strongly reduced by ActD treatment, indicating that their co-sedimentation with 40S-containing fractions is not due to an association with mature 40S subunits but with pre-40S particles. Therefore, these data support our conclusion that both CK1δ and CK1ε are components of 40S subunit precursors associated with the cytoplasmic trans-acting factor RIO2.

CK1δ and CK1ε localize to both the cytoplasm and nucleus as well as to specific cellular structures, such as the centromere (reviewed in Gross and Anderson, 1998; Knippschild et al., 2005; Cheong and Virshup, 2011). Moreover, CK1δ accumulates in the nucleus upon treatment with LMB, indicating a shuttling behavior of this protein (Milne et al., 2001). Thus, it is possible that CK1 is loaded onto pre-40S subunits already in the nucleus. In order to investigate whether CK1δ and CK1ε are also components of nuclear pre-40S subunits, we generated cell lines expressing a HASt-tagged version of the 40S trans-acting factor C21orf70. This factor has been shown to associate with early pre-40S subunits, and depletion of C21orf70 leads to nuclear accumulation of 40S precursors (Wyler et al., 2011). In HeLa cells, HASt–C21orf70 localizes to the nucleus and is enriched in nucleoli (Fig. 7A), as observed with endogenous C21orf70 (data not shown). Unlike ENP1, a nucleolar factor that shuttles together with pre-40S subunits to the cytoplasm, C21orf70 remains nuclear upon co-depletion of CK1δ and CK1ε, NOB1, RIO2 or RPS3 (Fig. 7A), indicating that C21orf70 is not associated with late cytoplasmic pre-40S subunits. To investigate whether CK1δ and CK1ε are part of C21orf70-associated pre-40S subunits, we used C21orf70 as bait in a TAP experiment. We generated a stable HEK 293 cell line expressing HASt–C21orf70 under the control of a tetracycline-inducible promoter, as described previously (Wyler et al., 2011). TAP of HASt–C21orf70 followed by mass spectrometry (MS) (Fig. 7B), as well as western blot analysis (Fig. 7C), revealed that C21orf70 co-purifies nuclear 40S precursors, indicated by the presence of the early pre-40S trans-acting factor NOC4 (Fig. 7B,C) and the absence of late-joining ribosomal proteins RPS10 and RPS26 (Fig. 7C). Strikingly, both CK1δ and CK1ε efficiently co-purified with HASt–C21orf70 (Fig. 7B,C), suggesting that they can join pre-40S subunits in the nucleus.

CK1 phosphorylates ENP1 and LTV1 in pre-40S particles

CK1 inhibition and co-depletion of CK1δ and CK1ε prevent the normal release of 40S trans-acting factors from cytoplasmic 40S precursors. In an attempt to determine which targets are phosphorylated by CK1 in this process, we purified cytoplasmic pre-40S particles by TAP using tagged LTV1 and ENP1 as bait proteins, as described previously (Wyler et al., 2011). We then incubated the isolated 40S precursors in the presence of $\gamma[^{32}P]$ATP to radiolabel proteins that undergo phosphorylation and analyzed these reactions by autoradiography and silver staining (Fig. 8A). Because several protein kinases are present in late 40S precursors, we performed these reactions in the presence or absence of CK1 inhibition. Control reactions revealed that the presence of the CK1 inhibitor IC261, which preferentially inhibits CK1δ and CK1ε (Mashhoon et al., 2000), prevented CK1δ-mediated phosphorylation of casein, as expected, but did not interfere with RIO2 autophosphorylation. Phosphorylation analysis of the LTV1-TAP particle revealed two strongly phosphorylated bands. For both of these, phosphorylation intensity was strongly decreased in the presence of the inhibitor. Our MS analyses identified these bands as LTV1 and ENP1. In addition, one high-molecular-mass protein appears to be phosphorylated by CK1, but we have not been able to identify this

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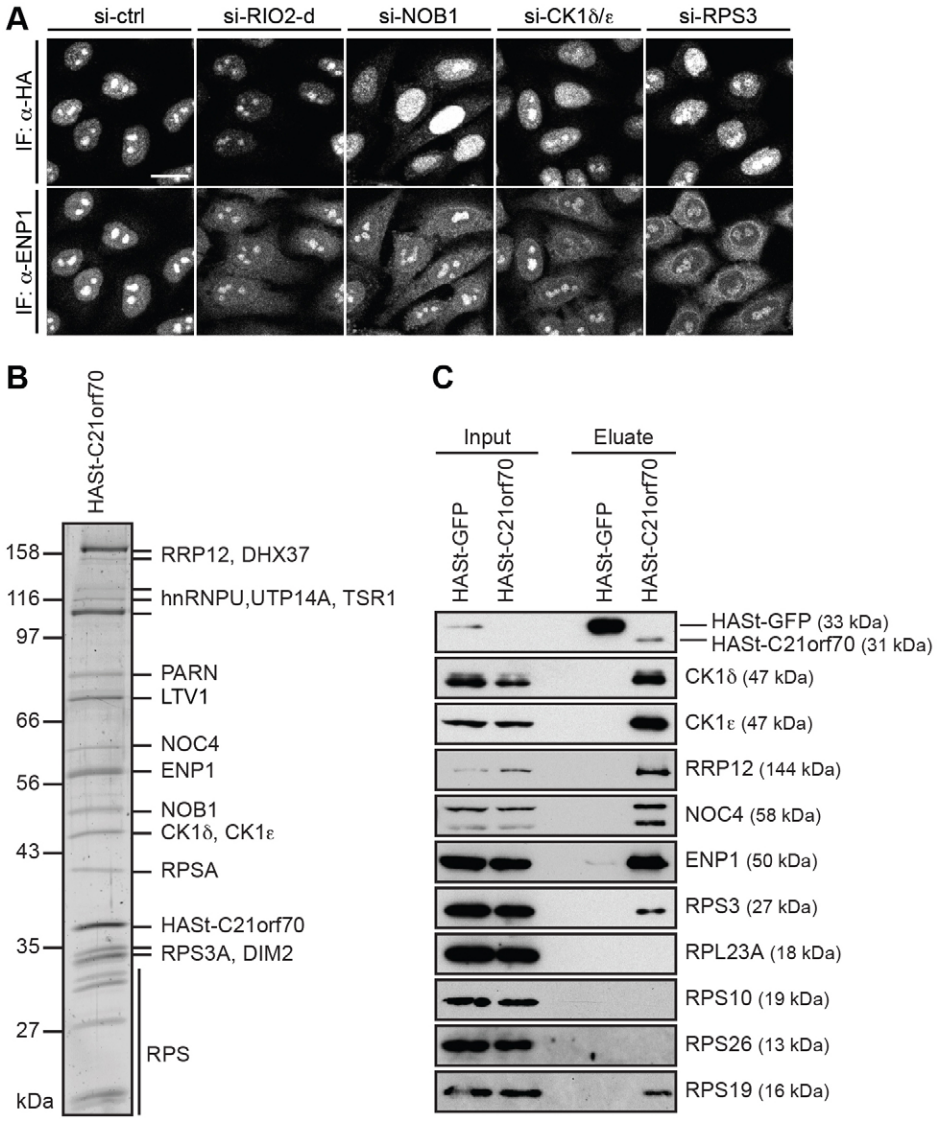

Fig. 7. CK1δ and CK1ε are associated with early 40S precursors. (A) Analysis of HASt-tagged C21orf70 localization by immunofluorescence. HeLa cells expressing HASt–C21orf70 were treated with the indicated siRNAs for 72 h. HASt–C21orf70 expression was induced by the addition of tetracycline 24 h prior to fixation, followed by co-immunofluorescence analysis for HASt–C21orf70 (α-HA) and ENP1. Upon depletion of several 40S trans-acting factors, ENP1 partially relocalizes to the cytoplasm whereas C21orf70 remains nuclear. Scale bar: 20 μm. (B) Mass spectrometric and (C) western blot analysis of proteins binding to tandem affinity purified HASt–C21orf70. Expression of HASt–C21orf70 was induced in a stable HEK 293 cell line by the addition of tetracycline 24 h before harvesting. Proteins eluted from tandem affinity purification were analyzed by SDS-PAGE. Tandem affinity purification of HASt–GFP served as a negative control. Note that HASt–GFP and HASt–C21orf70 were detected with an antibody against HA. Load corresponds to 0.06% of the input and to 20% of the eluates. C21orf70 co-purifies early 40S precursors along with CK1δ and CK1ε. HASt: N-terminal tandem HA/streptavidin-binding peptide tag.

protein. Although RPS3 was identified as a major component of particles isolated by both ENP1-TAP and LTV1-TAP by MS analysis, there was only a faint phosphorylated band migrating at the corresponding region of the gel, suggesting that RPS3 is not a predominant phosphorylation target on these particles. In the ENP1-TAP particle, CK1-dependent phosphorylation of LTV1 and ENP1 was, again, observed. The differing migration behavior of the bands in the two respective complexes is due to the presence of either endogenous or HASt-tagged LTV1 and ENP1 and confirms the conclusion of MS analyses. These data suggest that CK1δ and CK1ε phosphorylate LTV1 and ENP1 in late pre-40S particles.

To confirm this conclusion, we isolated pre-40S particles by LTV1-TAP. Immunoblot analysis indeed revealed that CK1δ and CK1ε were isolated together with ribosomal proteins and other pre-40S trans-acting factors by TAP of HASt–LTV1, whereas CK1α was absent (Fig. 8B). In addition, phosphorylation analysis showed that the CK1δ/ε-specific inhibitor PF670462 inhibited phosphorylation of LTV1 and ENP1, consistent with its ability to inhibit CK1δ and CK1ε potently *in vitro* (Fig. 8C). Thus, phosphorylation of LTV1 and ENP1 is mediated by the pre-40S components CK1δ and CK1ε, which could contribute to their release from 40S precursors.

DISCUSSION

Along their maturation pathway from nucleoli to the cytoplasm, ribosomal subunit precursors undergo large changes in structure and composition. Importantly, to become translation competent, pre-60S and pre-40S particles require cytoplasmic maturation steps to remove remaining trans-acting factors because these block functionally important positions of the subunits (reviewed in Zemp and Kutay, 2007; Panse and Johnson, 2010; see also Strunk et al., 2011; Strunk et al., 2012). In this study, we have established a novel role of the CK1 isoforms δ and ε in cytoplasmic maturation of 40S ribosomal particles. The CK1 isoforms δ and ε are required for recycling of the 40S trans-acting factors ENP1, LTV1, RRP12, DIM2, RIO2 and NOB1, as well as for processing of 18S-E pre-rRNA. The two CK1 isoforms δ and ε are 98% identical in their kinase domain and 53% identical in their C-terminal regulatory domain (Fish et al., 1995). Although redundancy with respect to substrate phosphorylation has been

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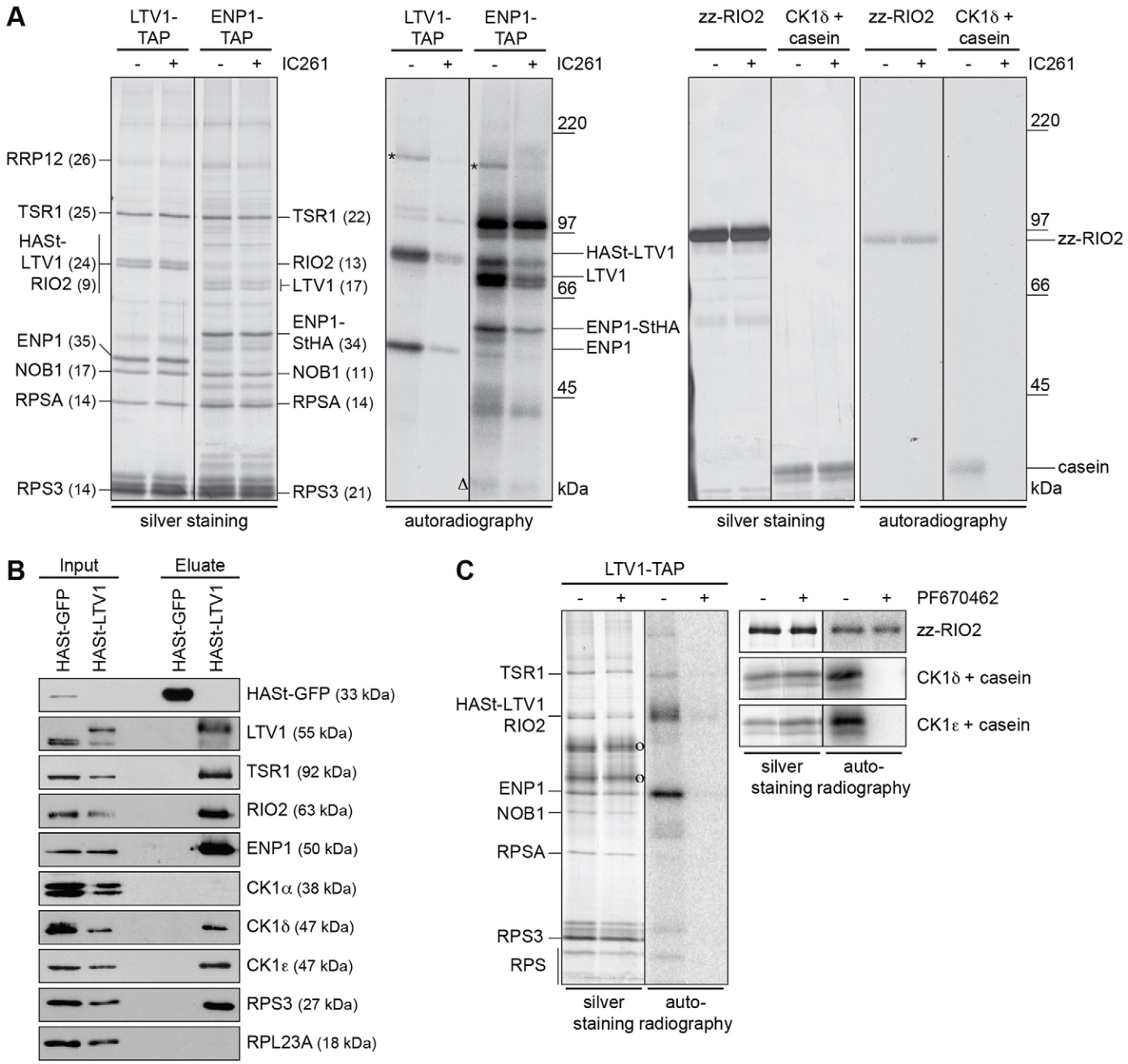

Fig. 8. LTV1 and ENP1 are phosphorylated in a CK1-dependent manner in pre-40S particles. (A) Autoradiography analysis and silver staining of kinase assay on 40S precursors. HASt–LTV1 and ENP1–StHA-associated 40S precursors were isolated by tandem affinity purification. γ[^{32}P]ATP was added to the isolated particles in the absence or presence of 4 μM CK1 inhibitor IC261 (Behrend et al., 2000; Mashhoon et al., 2000). Proteins were separated by SDS-PAGE on an 8% gel, followed by silver staining (left) and autoradiography (middle). Note that IC261 was used instead of D4476 because of better solubility. The identity of proteins was determined by mass spectrometry. The numbers of identified unique peptides are given in brackets. Control kinase reactions using recombinant, purified zz-RIO2 or recombinant CK1δ and casein were performed (right). Vertical lines separate different parts of the same gel. Phosphorylation of LTV1 and ENP1 is observed in the absence of inhibitor, and phosphorylation is reduced in the presence of the CK1 inhibitor IC261. Asterisk denotes unidentified high-molecular-mass protein whose phosphorylation is reduced in the presence of CK1 inhibitor. One protein isolated by ENP1-TAP and migrating at 97 kDa, the same apparent molecular mass as TSR1, is strongly phosphorylated but insensitive to CK1 inhibition. The triangle indicates the faint phosphorylation signal observed in the region where RPS3 migrates. HASt and StHA: N- and C-terminal tandem streptavidin-binding peptide/HA tag, respectively. (B) CK1δ and CK1ε are associated with the pre-40S isolated by HASt–LTV1. HASt–LTV1-associated 40S precursors were isolated by TAP and analyzed by immunoblotting for the indicated proteins. TAP of HASt–GFP served as negative control. Load corresponds to 0.04% of the input and to 20% of the eluates. HASt–GFP was detected with anti-GFP antibody. Note that both CK1δ and CK1ε are associated with the LTV1-associated pre-40S particle, whereas two isoforms of CK1α recognized by an antibody against CK1α are absent. (C) The CK1δ/ε-specific inhibitor PF670462 inhibits phosphorylation of LTV1 and ENP1. The experiment was performed as described in A. Proteins were separated by SDS-PAGE on a 10% gel. Note that PF670462 does not inhibit autophosphorylation activity of zz-RIO2, whereas phosphorylation of casein by recombinant CK1δ and CK1ε is strongly inhibited. Open circles mark nonspecific bands.

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suggested based on their high similarity, there are also examples of distinct roles for these two proteins, such as CK1δ, but not CK1ε, acting in maintenance of circadian cycle length (Etchegaray et al., 2009) or in Wnt-mediated neurite outgrowth (Greer and Rubin, 2011). In our analysis, single depletion of either CK1 isoform did not result in detectable defects in 40S maturation, indicating that CK1δ and CK1ε play at least partially redundant roles in this process.

Previously, a function of the *S. cerevisiae* CK1 homolog Hrr25 in 40S biogenesis has been described (Schäfer et al., 2006). Hrr25 phosphorylates a subcomplex consisting of Rps3, Enp1 and Ltv1, leading to their dissociation from 40S precursors. Dephosphorylation of Rps3 then leads to its stable incorporation into pre-40S particles and formation of the 40S 'beak' structure. We show that CK1δ and CK1ε play a similar role in mammalian 40S biogenesis with respect to their ability to phosphorylate ENP1 and LTV1 on pre-40S particles and their requirement for release of these factors from 40S precursors. However, in contrast to Hrr25-depleted cells, which display nuclear accumulation of Rps2-GFP (Schäfer et al., 2006), we did not observe export defects of pre-40S particles upon co-depletion of CK1δ and CK1ε. Instead, CK1δ and CK1ε appear to affect 40S maturation steps only after export, being required for cytoplasmic release of 40S trans-acting factors and 18S-E rRNA processing. Together with our previous observation, that recycling of ENP1 and LTV1 can be uncoupled using a kinase-dead RIO2 mutant (Zemp et al., 2009), these data indicate differences between yeast and human cells in the release of these factors from 40S precursors.

Using immunoprecipitation of RIO2, and TAP of C21orf70 and LTV1, we show that both CK1δ and CK1ε are components of pre-40S particles. Association of both kinases with nuclear pre-40S subunits isolated by C21orf70-TAP suggests that CK1δ and CK1ε are, likely, loaded on 40S precursors in the nucleus. They remain on the particle until they reach the cytoplasm, indicated by their presence in cytoplasmic pre-40S precursors isolated by RIO2 immunoprecipitation. Together, these data support the idea that CK1δ and CK1ε associate with pre-40S subunits before nuclear export and accompany them to the cytoplasm. Although it is possible that CK1δ and CK1ε already phosphorylate their targets in the nucleus, our data demonstrate that kinase activity is only required for further maturation of 40S pre-ribosomes in the cytoplasm. Accordingly, the simplest model reconciling our findings is that the activity of CK1 is inhibited until pre-40S particles reach the cytoplasm to avoid premature phosphorylation and dissociation of 40S trans-acting factors. Possibly, an inhibitory component with a binding site close to the LTV1–ENP1–RPS3 complex is removed from 40S precursors in the cytoplasm, in turn allowing CK1 to phosphorylate its targets. Alternatively, a conformational change of pre-40S particles in the cytoplasm might take place to provide accessibility of the substrates to the active site of CK1. Similar structural rearrangements have been proposed to allow NOB1 to access and cleave the 18S-E pre-rRNA (Lamanna and Karbstein, 2011; Lebaron et al., 2012).

Several protein kinases associate with, and contribute to, the maturation of late 40S precursors (Rouquette et al., 2005; Zemp et al., 2009; Baumas et al., 2012; Widmann et al., 2012; this study), indicating a prominent role for phosphorylation in this process. The substrates of RIO family members have not been identified to date, and for RIO2 it has even been proposed that its ATPase activity mediates a conformational switch, rather than phosphorylation (Ferreira-Cerca et al., 2012). By contrast, in both yeast and human cells, CK1 is able to phosphorylate LTV1 and ENP1 on pre-40S subunits, and its kinase activity is needed for release of several 40S trans-acting factors in the cytoplasm. A comparison of the different requirements of RIO1, RIO2 and CK1 and their kinase activities for recycling of the 40S trans-acting factors does not result in a clear, linearly ordered model for the release of these proteins from 40S precursors. Most likely, release events can occur to some extent in a parallel manner on different surfaces of the pre-40S particle. However, these processes still influence each other, and loss of any of these kinases, or of their activity, is sufficient to prevent final maturation of the subunit. Because the kinase activity of CK1 is needed for cytoplasmic release of all investigated trans-acting factors, CK1 probably acts early in cytoplasmic maturation, triggering a series of steps that allow 40S subunits to become translation competent. In cryo-electron microscopic studies, the CK1 phosphorylation targets ENP1 and LTV1 were placed on a surface of 40S precursors that was distant from the binding site of RRP12, DIM2, RIO2 and NOB1 (Strunk et al., 2011), although these factors also depend on CK1 for their recycling. How the CK1 signal is transmitted from ENP1 and/or LTV1 to the other trans-acting factors remains unclear, but it is likely to involve structural rearrangements of the 40S precursor.

It has recently been shown that ribosome biogenesis is coordinated by the circadian clock (Jouffe et al., 2013). While the circadian clock exerts effects on ribosome synthesis by means of transcriptional control, the fact that CK1δ and CK1ε are, on the one hand, essential for establishing the circadian rhythm (Lee et al., 2001) and, on the other hand, directly involved in late 40S maturation provides a potentially interesting additional link between these two processes.

MATERIALS AND METHODS

Cell lines, inhibitors and antibodies

HeLaK cells inducibly expressing RPS2–YFP have been described previously (Zemp et al., 2009). ENP1–StHA- and HASt–LTV1-expressing HEK 239 FlpIn TRex cells have been previously described (Wyler et al., 2011). Polyclonal HASt–C21orf70-expressing HEK 239 FlpIn TRex cells were generated as described previously (Wyler et al., 2011). Monoclonal HASt–C21orf70-expressing HeLa FlpIn cells were generated as described elsewhere (Wyler et al., 2011), except that cells were selected in standard DME medium containing 400 µg/ml hygromycin. Single colonies were picked and screened for HASt–C21orf70 expression by immunofluorescence analysis.

The CK1 inhibitor IC261 was purchased from Santa Cruz Biotechnologies (Heidelberg, Germany; sc-3561), D4476, LH846, PF4800567 hydrochloride and PF670462 from Tocris Bioscience (Abingdon, UK), LMB from LC laboratories (Woburn, MA; L-6100) and Actinomycin D from Sigma-Aldrich (Buchs, Switzerland).

Antibodies against trans-acting factors used in immunofluorescence and western blot analysis have been described previously (Zemp et al., 2009; Wyler et al., 2011). Anti-TSR1 was raised against a purified, recombinant N-terminal fragment of TSR1 (amino acids 1–245) and affinity purified with this antigen coupled to SulfoLink beads. Anti-β-actin was purchased from Sigma-Aldrich (A1978), anti-CK1ε from BectonDickinson (Allschwil, Switzerland; 610445), anti-CK1α and anti-CK1δ from Abcam (Cambridge, UK; ab108296 and AF12G4, respectively), anti-hemagglutinin (HA) from Covance (Geneva, Switzerland; MMS-101P), anti-RPS10 and anti-RPS26 from Abcam (Cambridge, UK; ab151550 and ab104050, respectively) and secondary antibodies for immunofluorescence from Invitrogen (LuBioScience, Lucerne, Switzerland).

Inhibitor treatment

Treatment of cells with D4476, at a final concentration of 100 µM, was carried out as previously described (Rena et al., 2004). HeLaK cells were

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treated with LH846 (7.5 μM), PF4800567 hydrochloride (1.5 μM) and PF670462 (1 μM) in complete medium for 6 h.

Molecular cloning

The coding region for CK1ε was amplified from HeLaK cDNA and inserted into the pcDNA5-FRT-TO-nHASt-TAP vector (described in Wyler et al., 2011) using *Kpn*I/*Not*I. Based on sequence alignment with protein kinase A, Asp149 was identified as a conserved residue in the CK1ε kinase domain required for its catalytic activity. The inactivating D149A mutation was introduced using the QuikChange kit (Agilent Technologies, Basel, Switzerland). The C21orf70 coding region was amplified from cDNA and inserted into the pcDNA5-FRT-TO-nHASt-TAP vector (described in Wyler et al., 2011) using *Bam*HI/*Xho*I (*Sal*I for the insert).

RNAi and rescue experiments

siRNAs were transiently transfected using INTERFERin siRNA transfection reagent (Polyplus Transfection, Illkirch, France) according to the manufacturer's instructions. siRNAs were used at 5 nM concentration or 9 nM for RPS2–YFP cells. Forty-eight hours after transfection, cells were harvested for western blotting or extract preparation, or fixed with 4% PFA for immunofluorescence or FISH analysis. RPS2–YFP expressing cells were induced by the addition of tetracycline (125 ng/ml) 20 h before fixation and incubated in tetracycline-free medium 4 h before fixation. HASt–C21orf70-expressing cells were transiently transfected with siRNAs at 9 nM final concentration using the RNAiMAX transfection reagent (Invitrogen, LuBioScience, Lucerne, Switzerland) according to the manufacturer's instructions. At 24 h prior to fixation, expression of HASt–C21orf70 was induced with tetracycline (50 ng/ml). The following siRNA oligonucleotides, obtained from Microsynth (Balgach, Switzerland), were used (sense strand indicated): si-CK1γ3 (5′-GAATATGA-CTGGATTGGTA-3′), siCK1δ (5′-CCATCGAAGTGTTGTGTAA-3′), siCK1ε (5′-ACATCGAGAGCAAGTTCTA-3′), siCK1ε-3′UTR (5′-CC-CGTTCTCCTGTGTCTACTA-3′, used for rescue experiments), si-NOB1 (5′-GCCCAGAGATCATGCATTT-3′), si-RPS3 (5′-GCAAGAU-GGCAGUGCAAAU-3′). si-CRM1-1 and si-RIO2-d have been described previously (Zemp et al., 2009), and siRNA AllStars (QIAGEN, Hombrechtikon, Switzerland) was used as a negative control (si-ctrl).

For rescue experiments, cells were, in addition, transiently transfected with either HASt–CK1ε wild-type or HASt–CK1ε(D149A) using XtremeGene 9 (Roche, Rotkreuz, Switzerland) according to the manufacturer's protocol 24 h prior to fixation.

Immunofluorescence and FISH analysis

Immunofluorescence (IF) analysis and FISH were performed as described previously (Rouquette et al., 2005; Zemp et al., 2009). Images of fixed and stained cells were taken using a Leica SP1 or SP2 confocal scanning system. For FISH pictures, γ-correction was done using Photoshop software (Adobe).

Extract preparation

For preparation of extracts, cells were treated with 100 μg/ml cycloheximide (Sigma-Aldrich) for 10 min, washed briefly with PBS, detached with PBS containing 0.5 mM EDTA and 100 μg/ml cycloheximide, and centrifuged (2000 ***g***, 5 min, 4℃). The pellet was washed with 10 mM Tris/HCl pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 50 μg/ml cycloheximide, and the cells were pelleted again. Cells were resuspended in the same buffer supplemented with 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5% Triton X-100 and protease inhibitors, and incubated on ice for 20 min. After passing cells through a 27-gauge needle and centrifugation for 5 min at 2500 ***g***, supernatants were used as cell extracts. 400 μl lysis buffer was used per 6×10^6 cells.

Sucrose gradient analysis

Cell extracts corresponding to ~400 μg total protein were loaded onto linear 10–45% sucrose gradients in 50 mM HEPES/KOH pH 7.5, 100 mM potassium acetate, 3 mM $MgCl_2$ and centrifuged for 2 h at 55,000 rpm at 4℃ in a TLS55 rotor (Beckman Coulter, Nyon, Switzerland). Fractions of 160 μl were collected, precipitated with TCA, and analyzed by western blotting.

Immunoprecipitation

Cell extract corresponding to 250 μg total protein was supplemented with 2.5 M potassium acetate to a final concentration of 140 mM, diluted 10-fold in IP buffer (50 mM Tris/HCl pH 7.6, 150 mM potassium acetate, 3 mM $MgCl_2$) and centrifuged for 15 min at 15,000 ***g***, 4℃. The supernatant was added to RIO2- or NMD3-specific antibodies (Zemp et al., 2009) coupled to a 9:1 mixture of protein A:protein G Sepharose beads (GE Healthcare, Glattbrugg, Switzerland) and incubated on a rotating wheel for 2 h at 4℃. After three washes with IP buffer and one wash with 50 mM Tris/HCl pH 7.6, 150 mM NaCl, 3 mM $MgCl_2$, beads were eluted with SDS sample buffer without DTT. DTT was added after the elution, followed by SDS-PAGE and western blot analysis.

Tandem affinity purification and mass spectrometry

Mass spectrometry and tandem affinity purification were carried out as described previously (Wyler et al., 2011).

Kinase assay on pre-40S particles

Tandem affinity purification of ENP1- and LTV1-associated pre-40S particles was performed as previously described elsewhere (Wyler et al., 2011), except that purified complexes were not eluted from the HA agarose beads. 40S precursors were re-buffered on beads to 50 mM Tris/HCl pH 7.6, 50 mM NaCl and 5 mM $MgCl_2$, with 4 μM IC261, 5 μM PF670462 or solvent control. 1 μCi γ[^{32}P]ATP was added per reaction, followed by incubation at 30℃ for 20 min. 40S precursors were then eluted with SDS sample buffer without DTT. DTT was added after the elution, and samples were analyzed by SDS-PAGE. The gel was silver-stained, dried and analyzed by phosphoimaging. For control reactions, 0.9 μg recombinant zz-RIO2 purified as described elsewhere (Zemp et al., 2009) or 0.2 μg casein and 5 units (2.5 ng) rat CK1δ (New England Biolabs, Bioconcept, Allschwil, Switzerland; P6030S) or 10 ng CK1ε (LucernaChem, Lucerne, Switzerland; C66-10G) were incubated in 10 μl reactions containing 1 μCi γ[^{32}P]ATP in Tris/HCl pH 7.6, 50 mM NaCl and 5 mM $MgCl_2$. Reactions were stopped after 20 min incubation at 30℃ by addition of SDS sample buffer, followed by analysis as described above.

Acknowledgements

We thank the members of the Kutay lab for valuable discussion, Jens Pfannstiel and Berit Würtz (Serviceeinheit LSC, Universität Hohenheim, Germany) for mass spectrometry, and Marie-Françoise O'Donohue and Pierre-Emmanuel Gleizes for exploring a potential role of CK1 in exonucleolytic trimming of 18S-E pre-rRNA. Microscopy was performed on instruments of the ETH Zurich Light Microscopy and Screening Center (LMSC).

Competing interests

The authors declare no competing interests.

Author contributions

U.K. and I.Z. conceived the study, I.Z. designed and performed experiments for Figs 1, 2, 5B, 6, and 8A; F.W. for Figs 3, 4, 5A and 7; S.R. and F.W. for Fig. 8B,C; C.A. and C.M. generated cell lines and antibodies; E.W. contributed to Fig. 8A. I.Z., F.W., S.R. and U.K. analyzed and interpreted data. I.Z., F.W. and U.K. wrote the manuscript.

Funding

This work was supported by a grant of the Swiss National Science Foundation [grant number 31003A_144221 to U.K.]. Deposited in PMC for immediate release.

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