Supplementary information

Material and Methods

Yeast two hybrid screen

We used the C-terminal region (a. a 592-896) of Xenopus p150 fused to the lexA DNA binding domain (lexA-xp150) as a bait to screen a Xenopus oocyte (stages I to VI) GAL4-cDNA library (Iouzalen et al., 1998; Quivy et al., 2001). From 1.4 x 10^6 independent clones, 1000 clones were isolated by a first round of selective growth on histidine media. A second round of growth selection was used to reduce the number to 150. 83 of these clones were positive for the LacZ activation assay, and 65 were sequenced. One of them contained the ORF encoding *Xenopus* Dbf4 (xDbf4*)*.

Antibodies and Immunological procedures

Immunoprecipitation with anti-FLAG (M2, Sigma) and anti-GFP (BD Living Colors Full-Length A.v. Polyclonal Antibody, Clontech) were performed as in (Koundrioukoff et al., 2000). S100 extracts were immunodepleted from Cdc7 with mouse monoclonal antibody (ab10535, Abcam) and protein G coupled to Dynabeads (Dynal, Oslo). Anti beta-actin IgG1 (Sigma) were used for the mock-depletion. For Western-blot (Martini et al., 1998) unless specified we used the following antibodies at 1/1000 dilution: anti-xp150 (Quivy et al., 2001); anti-hp150 (Ab7655, Abcam); anti-PCNA (PC-10, DAKO); anti-p60 (pAb1, (Marheineke and Krude, 1998), anti-His-probe (sc-8036, TEBU) at 1/5000, anti-Cdc7 (ab10535, Abcam) at 1/500, anti-Dbf4 at 1/500 (see characterization of this antibody, fig. S3), anti xCdc7 (Walter, 2000) at 1/500 and horseradish peroxydase-conjugated secondary antibodies (Jackson) at 1/25000. Super Signal detection kit (Pierce) was used for visualization.

Protein expression and purification

Bacterially expressed GST, GST-PCNA, GST-xMcm2, His_6-p150 (human and *Xenopus*) and His_6-p150ΔCAF-1 (deletion of residues 642 to 678 of human p150) were prepared (Moggs et al., 2000; Quivy et al., 2001), immobilized on glutathione (Amersham) or Ni-NTA beads (Novagen) and purified by elution from the Ni-NTA beads according to manufacturer's instructions. The human Cdk2-Cyclin A and Cdc7-Dbf4 kinases were produced in *Sodoptera frugiperda* cells (SF9) and purified as in (Jiang et al., 1998; Jiang et al., 1999; Voitenleitner et al., 1997). XDbf4 cDNA was subcloned in pGEX-4T1 (Pharmacia), and xCdc7 cDNA amplified from a *Xenopus* cDNA library (gift from P. Lemaire) was inserted into pET-30a vector (Novagen). XCdc7-xDbf4 kinase was purified by GST affinity from *E. coli* BL21 (DE3) strain co-expressing His_6-xCdc7 and GST-xDbf4. Flag-tag was inserted in C-terminus of His_6-p150 between *DraIII* and *EcoRI* sites of pET-30a/hp150 (Quivy et al., 2001) (5'-GTGCATCCGATTATAAAGATGATGACGATAAATGAG-3', 5'-TCGACTCATTTATCGTCATCATCTTTATAATCGGATGCACCCA-3'). Recombinant His_6-p150-Flag was produced in *E.coli* and purified with an anti-FLAG M2 affinity gel (Sigma-Aldrich) according to the manufacturer's recommendation. To establish the epitope tagged Flag-p150 cell line, we inserted a Flag sequence with the p150 cDNA into the *Hind III* and *Xho I* sites of pcDNA5/FRT vector (Invitrogen) and used it for 293Flp-In (Invitrogen) cells transfection with Effectene (Qiagen).

Pull-down and kinase assays

Purified His_6-p150, His_6-p150-Flag and GST-Mcm2 (100ng) were phosphorylated (Koundrioukoff et al., 2000). S100 extract (70µg) prepared from human 293 cells (Stillman, 1986) was mixed with Ni-NTA bound p150 beads (70ng) and incubated for 30 min at 37°C in 8mM Hepes-KOH pH=7.6, 1mM $MgCl_2$, 0.1mM DTT, 0.8mM ATP, 4µM of each dGTP, dATP, dTTP, 1.6µM dCTP, 8mM phosphocreatine and 0.5µg creatine phosphokinase. After extensive washes in 150mM NaCl, or 500 mM, 20mM Hepes-KOH pH=7.6, 5mM $MgCl_2$, supplemented with 5mM imidazole and 0.025%

NP-40, bound proteins were recovered by boiling in SDS-PAGE loading buffer and analyzed by Western-blot. Where indicated, we treated S100 extracts with 800 units of lambda phosphatase (New England Biolabs) for 1h at 30°C, before the interaction assay. Staurosporine (Calbiochem) was added at 10μM during the binding reaction. GST pull-down were performed as (Koundrioukoff et al., 2000).

Supplementary references

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Supplementary figure legends

Figure S1: Purified p150 in monomeric and dimeric state *in vitro*.

In order to discriminate between wild type (wt) p150 and mutant p150Δ (deleted in the region critical for dimerization) in their respective oligomerization state, we developed an *in vitro* cross-link assay using purified recombinant proteins. Purified wt p150 (p150) and mutant p150 (p150Δ) are cross-linked with 0,25 or 1 mM EGS and subjected to SDS-PAGE to resolve monomeric and dimeric forms of p150. Western-blot shows migration position of monomer and dimer (white and black arrowhead). Asterisk (*) marks accumulation of non-specific products, and position of 150 and 250 kDa MW markers are indicated. In this assay, whereas the wt p150 protein gave rise to two bands, dimer and monomer (black and white arrowhead, respectively), the mutant p150Δ produced a single band (white arrowhead). These data are consistent with a dimer/monomer equilibrium in wt p150.

Figure S2: Cdc7-Dbf4 interacts with and phosphorylates p150 *in vitro*.

A) Xp150 and xDbf4 interaction in a yeast two-hybrid assay. The L40 yeast strain, expressing Gal4-xDbf4 and the indicated proteins was grown on non-selective (-His) or on a His-selective (+His) plate prior assessment of β-galactosidase activity (β-gal).

B) Purified xCdc7-xDbf4 specifically phosphorylates GST-xMcm2 in vitro. The Xenopus Mcm2 (xMcm2) cDNA was retrieved from a *Xenopus laevis* cDNA library (gift from P. Lemaire) and cloned into pGEX-4T1 to produce the GST-xMcm2 fusion protein. Xenopus His_6-xCdc7 and GST-xDbf4 were co-expressed in *E. coli* and the complex was purified by glutathione-sepharose 4B beads according to manufacturer's instructions (Amersham Bioscience). Purified GST (lane 1) or GST-xMcm2 (lane 2) were incubated with ATPγ32P and xCdc7-xDbf4 kinase. Following separation by PAGE, specific phosphorylation of GST-xMcm2 but not of GST is detected by autoradiography (right). Corresponding coomassie stained gel used as a loading control is shown

(left).

C) hCdc7-hDbf4 phosphorylates purified recombinant GST-xMcm2 and His_6-p150-Flag with the same efficiency *in vitro*. Left: silver staining and autoradiography of both proteins phosphorylated with ATPγ^{32}P by hCdc7-hDbf4. Right: quantification of the phosphorylation level, in arbitrary units (cpm normalized to the number of serine and threonine and the MW of the protein), is set at 100% for Mcm2.

Figure S3: Dbf4 antibody specifically recognizes endogenous Dbf4 in human cells.

The rabbit polyclonal anti-Dbf4 antibody (JDI74) was raised against a GST-tagged fragment of human Dbf4 spanning amino acids 374 to 523. Exponentially growing osteosarcoma U2OS cells were transfected with control siRNA (-), 5'-AACGUACGCGGAAUACUUCGA-3', or Dbf4 siRNA (+), 5'-AACAAGCCAUCUAGUAUGCAA-3'. Cells were harvested 24 hr later, and total cell extracts were prepared. Following Western-blot analysis with the polyclonal Dbf4 antibody (JDI74), a band migrating at the expecting molecular weight for Dbf4 (see arrow) is detected in the extract corresponding to cells transfected with the control siRNA, but not in the extract corresponding to cells transfected with the Dbf4 specific siRNA. The asterisks mark proteins non-specifically recognized by the antibody.

Figure S4: p150 directly interacts with and is phosphorylated by Cdk2-Cyclin A.

A) GST-pull down of p150 by GST-hCyclin A, GST-hCdk2 or GST beads. p150 and GST in the input (I, 50%), unbound (U) and bound (B) fractions are shown.

B) Purified p150 is phosphorylated with ATPγ^{32}P using increasing amounts of hCdk2-hCyclin A kinase (in fmol). Coomassie stained gel and corresponding autoradiography are shown.

Figure S5: Mutation of the xCdc7 D165 into N leads to a kinase dead xCdc7 mutant.

Change of D165 into N of the xCdc7 was achieved using the QuickChange site-directed

mutagenesis kit (Stratagene). Purified recombinant p150 was phosphorylated with ATPγ^{32}P and either wt xCdc7-xDbf4 (20 ng xCdc7) or increasing amounts of xCdc7(D165N)-xDbf4 (5, 10 and 15 ng xCdc7) and resolved by SDS-PAGE. Western blot analysis of p150, Dbf4, Cdc7 (top) and autoradiography of the gel are shown. In contrast to the wt Cdc7, the xCdc7(D165N) mutant failed to phosphorylate p150, Dbf4 and Cdc7.

Figure S6: Interaction between p150 and PCNA requires phosphorylation events but not the sole presence of Cdc7.

A) Scheme of the experimental procedure.

B) Control of phosphorylation inhibition. Left: since p60 migrates in SDS-PAGE as a doublet corresponding to both hypo- and phosphorylated forms of the protein (Keller and Krude, 2000; Marheineke and Krude, 1998; Martini et al., 1998) we followed these migration properties as a way to monitor the efficiency of the phosphorylation inhibition. A p60 Western-blot showing the efficiency of lambda phosphatase and staurosporine (λ-PPase+stauro) treatment is presented. Position of the phosphorylated (p60-P) and the hypo-phosphorylated (p60) p60 are indicated. Lambda phosphatase treatment in combination with staurosporine, led to a conversion into the hypophosphorylated form, indicative of an efficient dephosphorylation preserved till the end of the reactions. Right: efficiency of p150 phosphorylation inhibition. Autoradiography (^{32}P) and Western-blot (total) of purified His_6-p150-Flag (100ng) phosphorylated with ATPγ^{32}P by hCdc7-hDbf4 in the absence (-) or the presence (+) of staurosporine.

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phosphorylation events, PCNA interaction with p150 is severely reduced, yet Cdc7 was bound to p150 (compare lanes 6 and 12).

Figure S7: Cdc7 depletion decreases CAF-1 dependent nucleosome assembly coupled to DNA synthesis.

Under optimal conditions using as a control mock-depleted S100 extracts complemented with 100 ng of purified recombinant wt p150, Cdc7 depletion (to an extent that impaired p150 recruitment, fig. 5B), did not significantly impact on plasmid supercoiling (compare lane 7 with 9). This may appear contradictory with the results obtained in the PCNA/DNA loading assay (fig. 5D). Since the Cdc7 depletion is not complete (fig. 5B), a limited amount of p150 recruited onto DNA can be sufficient to propagate histone deposition and generate supercoiled molecules. A similar situation was observed when we analyzed the synergy of ASF1 with CAF-1. Such synergy could only be evidenced under limiting amount of CAF-1 (Mello et al., 2002). We thus decided to follow the same strategy and titrated the amount of p150 to 25 ng. Under these conditions, a modest but reproducible decrease in supercoiling can be revealed at early and late time of the reaction (see supercoiling ratio (Sc ratio) at the bottom of the magnified lanes 11, 13, 15 and 17). Furthermore, quantification of the distribution of the topoisomers indicates that bands of similar intensity (arrows) correspond to less supercoiled topoisomers in the depleted extract when compared to the mock-depleted extract (compare lanes 11 with 13 and 15 with 17).

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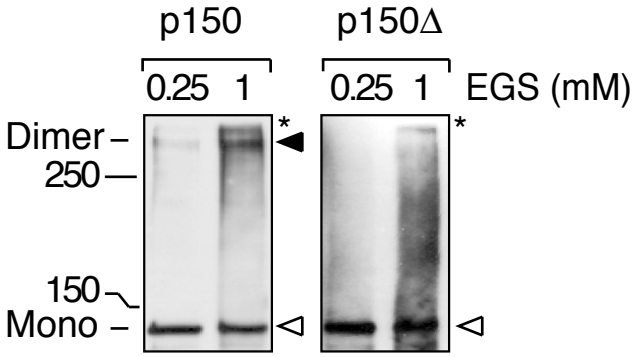

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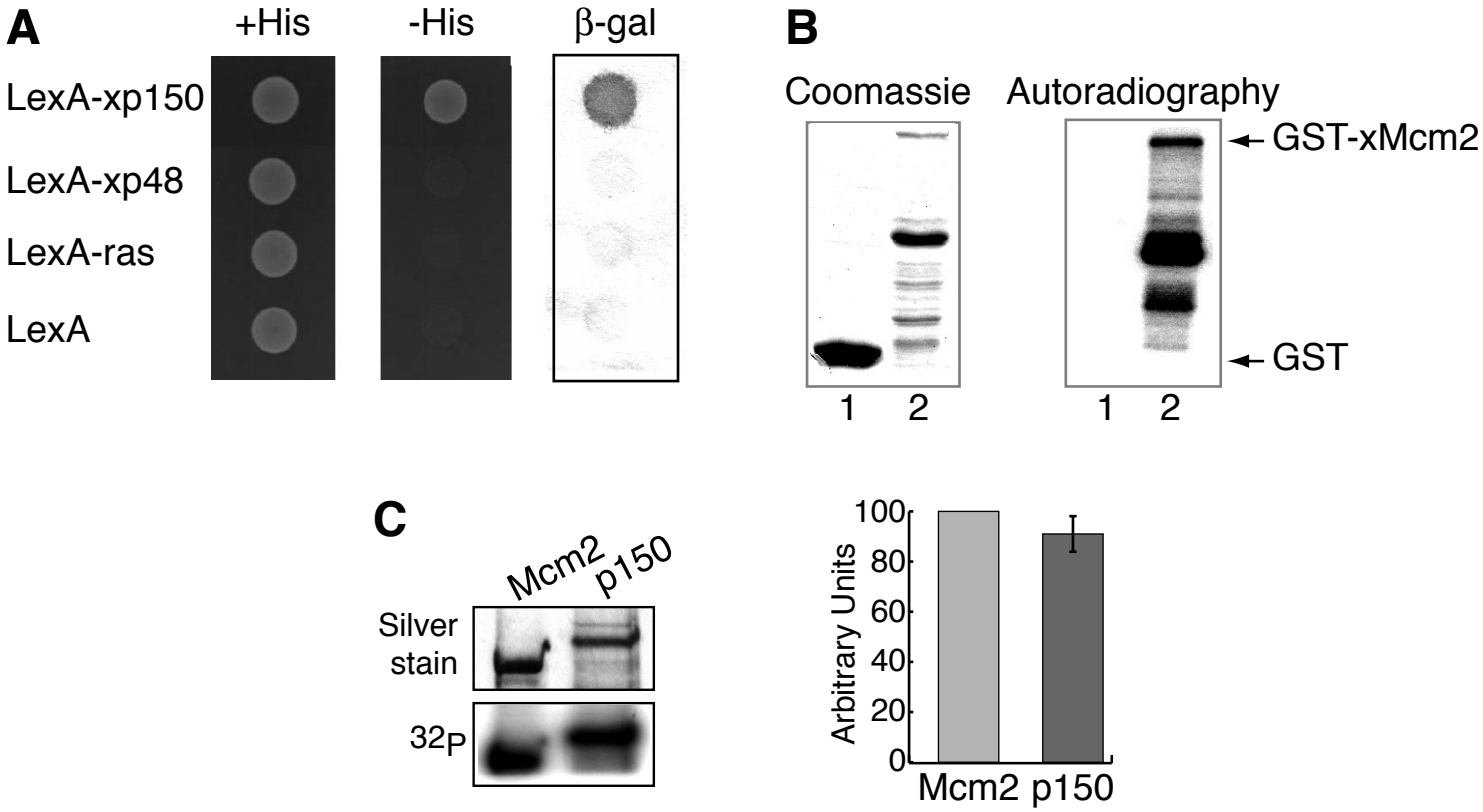

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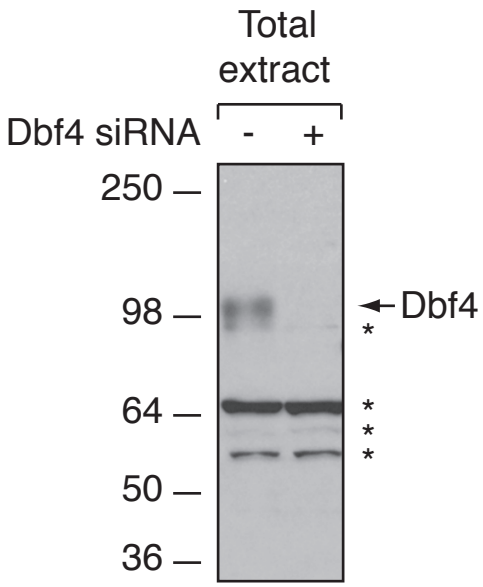

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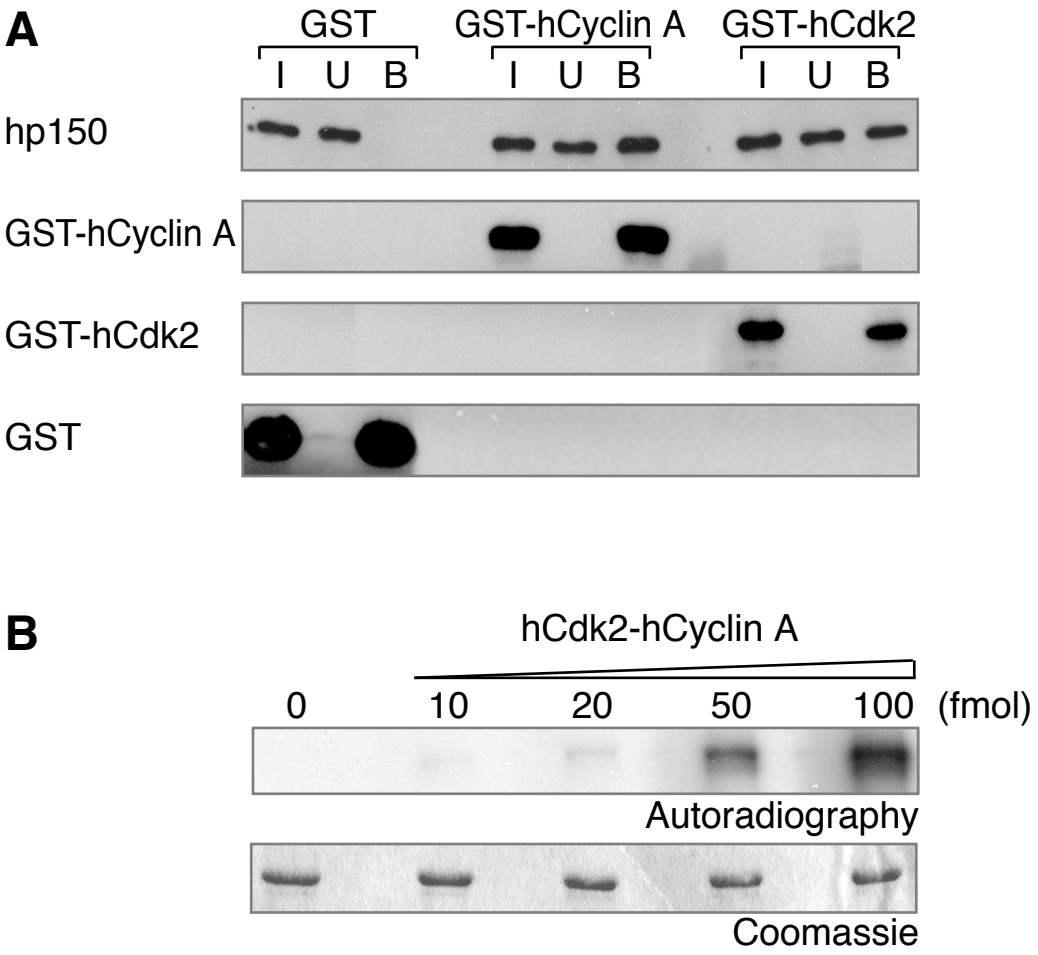

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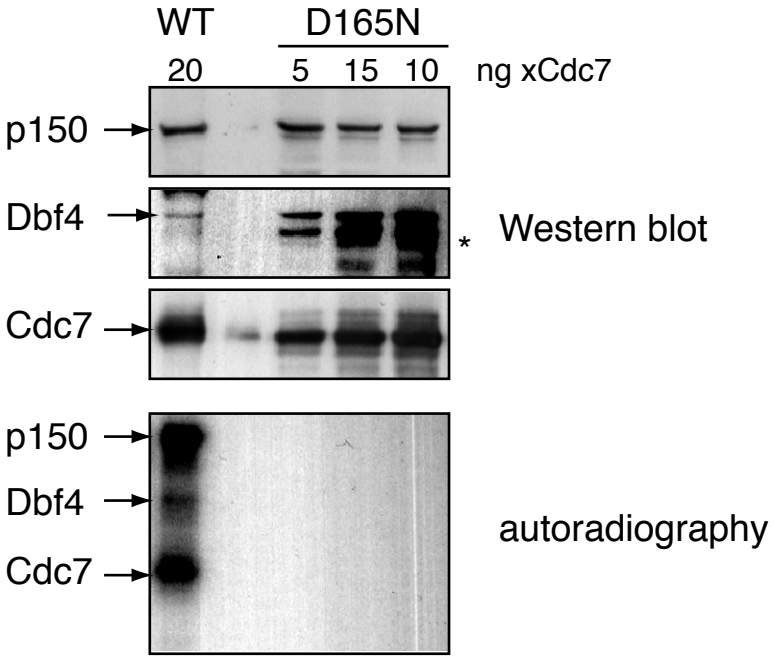

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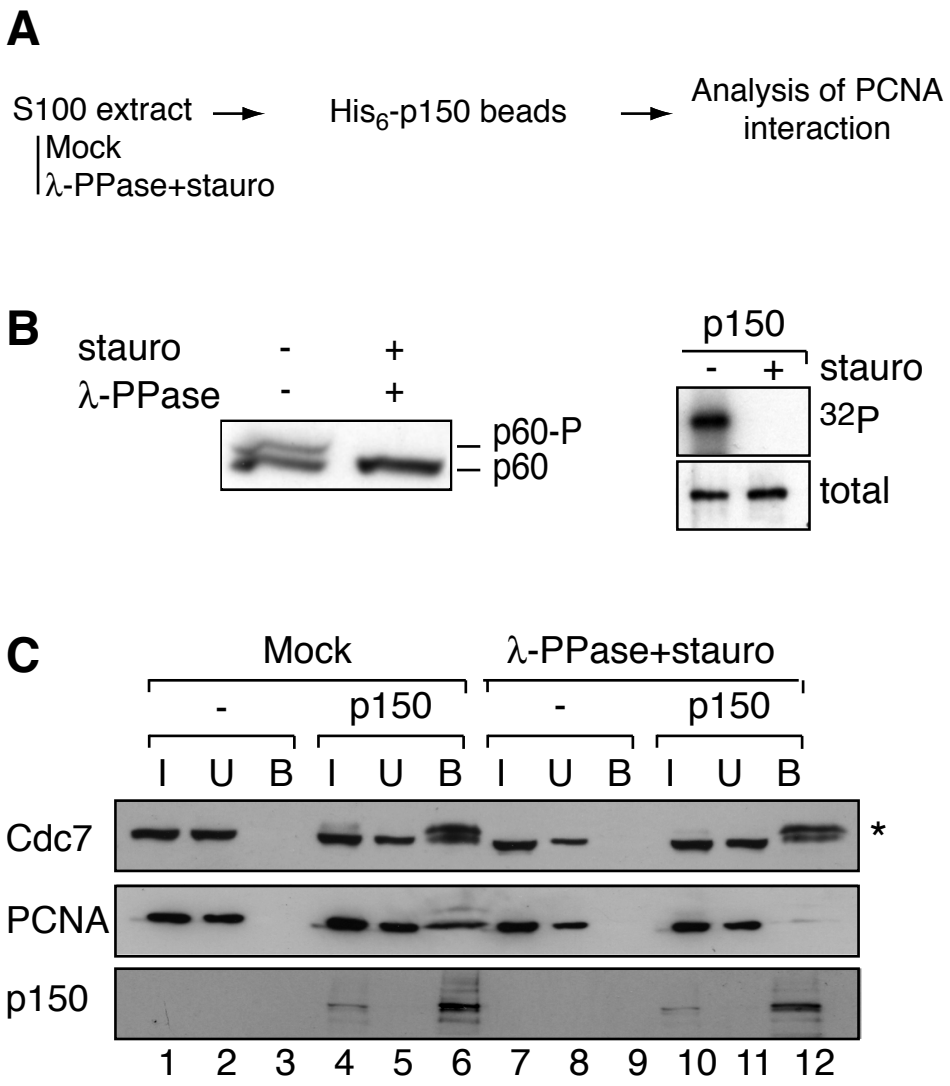

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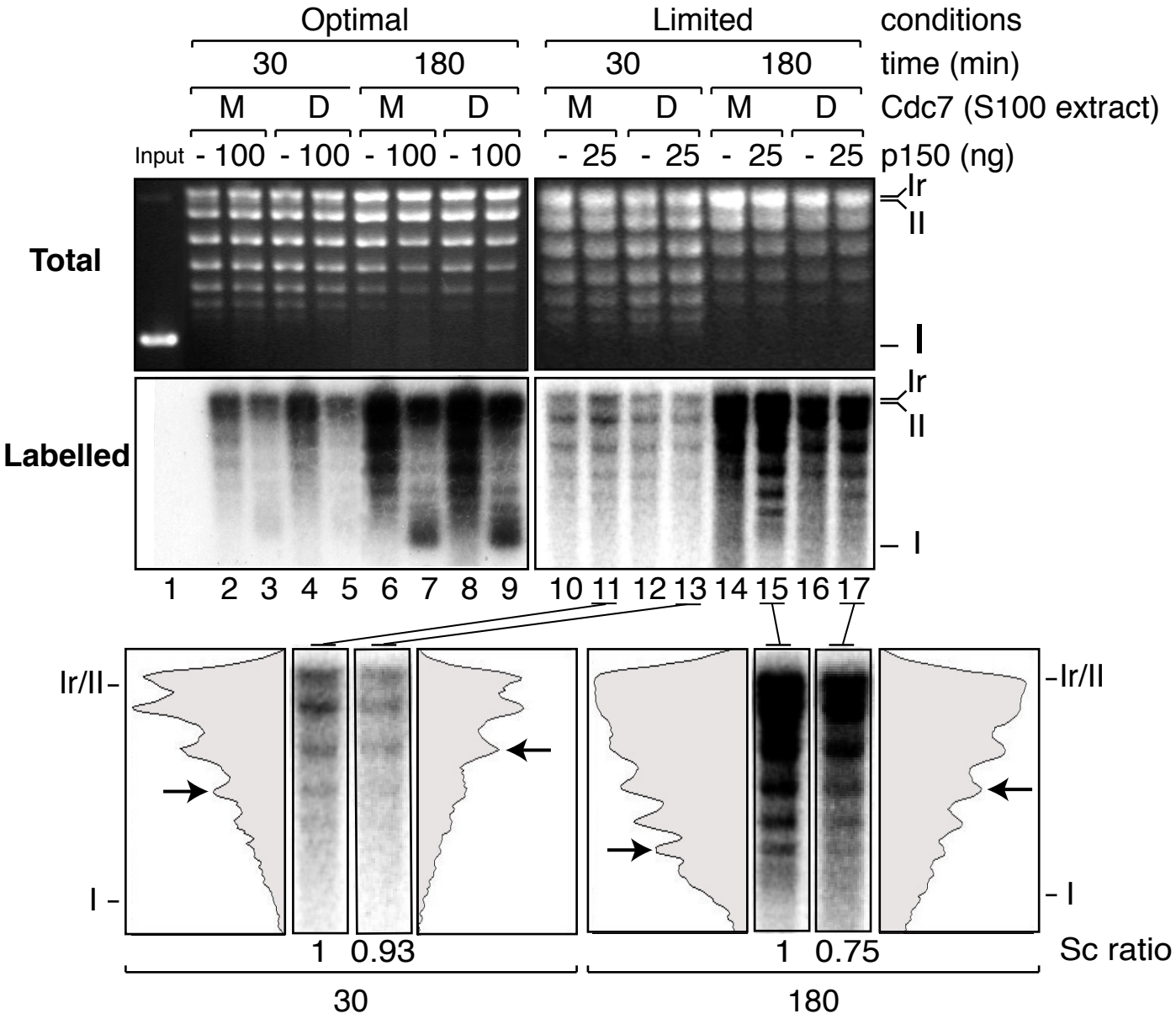

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