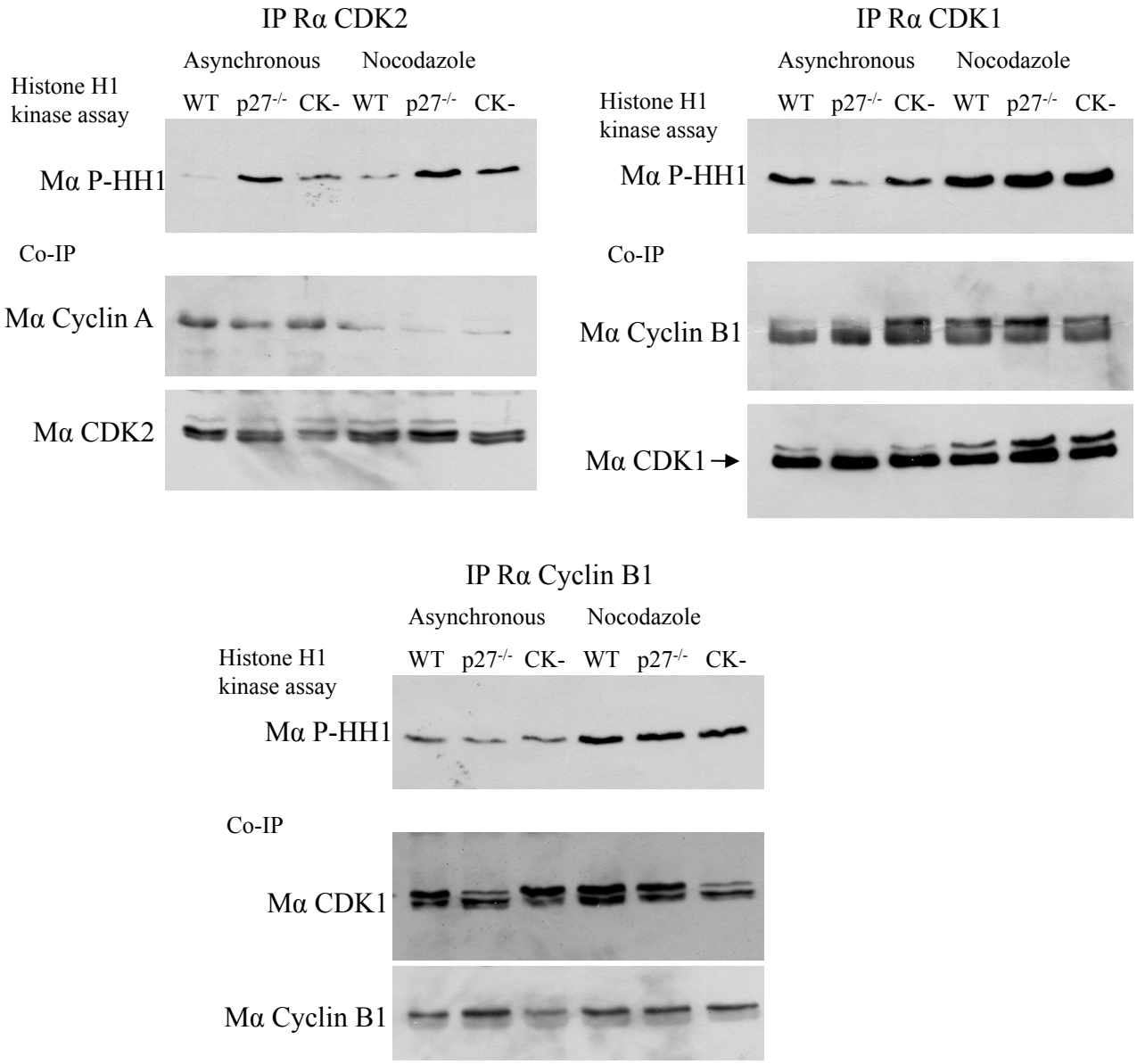

Figure S1: Cyclin-CDK complex formation and activity in asynchronously growing and nocodazole treated primary MEFs .

Passage 2-3 exponentially growing primary MEFs were either lysed (asynchronous) or treated for 5 h with 250 nM nocodazole before lysis. Five hundred µg of proteins were subjected to immunoprecipitation with the indicated polyclonal antibodies (CDK2 (M2); CDK1 (C19); Cyclin B1 (H433) (Santa Cruz Biotechnology) as described in the methods section. After washing of the beads, 20 µl of kinase assay buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM βglycerophosphate, 1 mM NaF, 2.5 mM EGTA, 0.1mM sodium orthovanadate) complemented with 1 µg of recombinant Histone H1 (Calbiochem) and 200 µM ATP were added to each tube. The kinase reaction was performed for 15 min at 30°C and stopped by adding 8 µl of 4X loading buffer. Immunoprecipitates were resolved on 12% SDS-PAGE and transferred on PVDF membranes. Western blotting was performed as described in the methods section by sequential probing using the indicated monoclonal antibodies (Phospho-Histone H1 (12D11, Millipore); and the Santa Cruz antibodies Cyclin A (E67.1), CDK2 (D12), Cyclin B1 (GNS1), CDK1 (17).

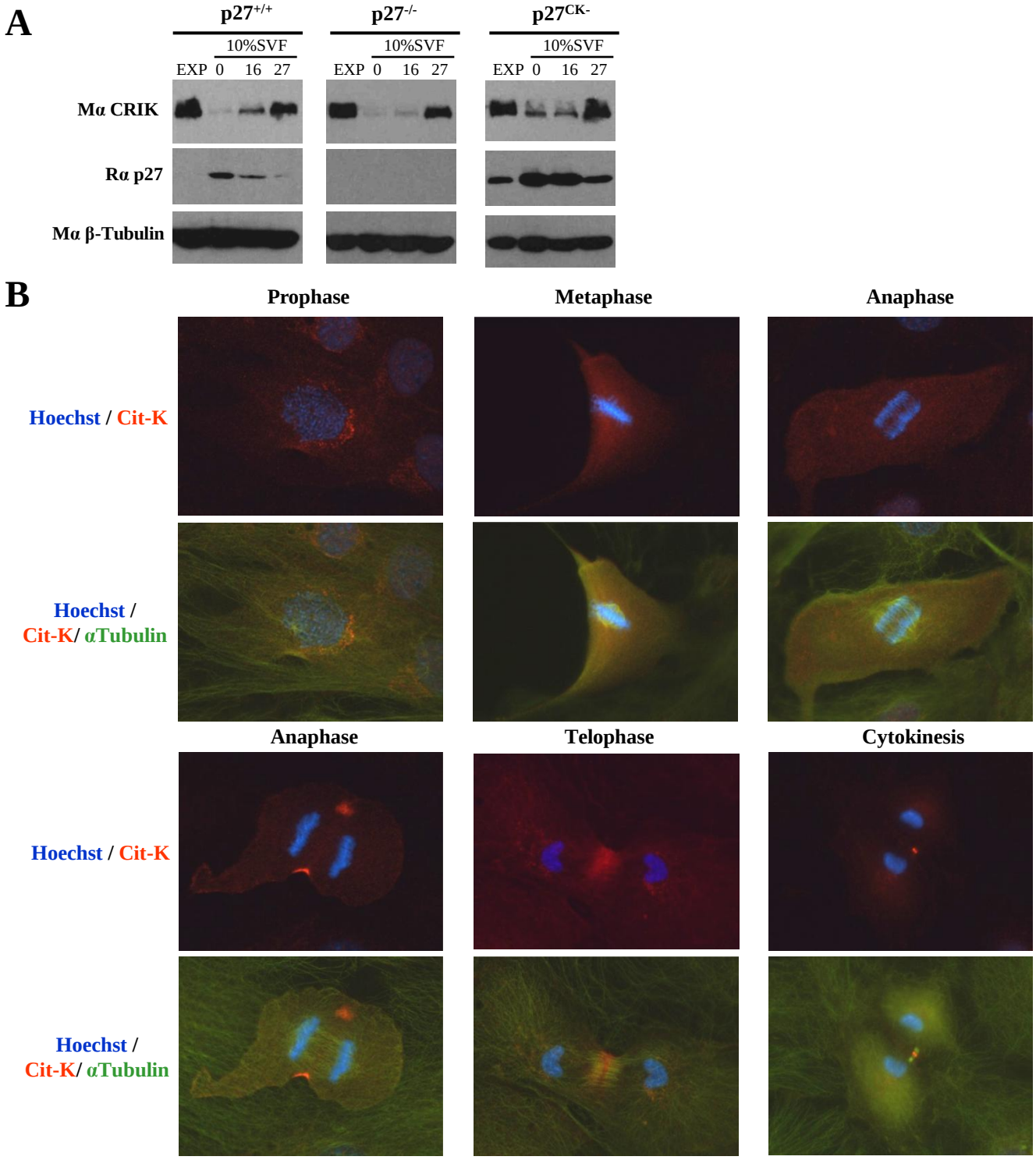

Figure S2: Citron-K expression and localization in MEFs.

(A) Expression levels of Citron-K and p27 in various culture conditions. Immunoblotting of immortalized MEF extracts either exponentially growing (EXP) or starved in 0.1% FCS DMEM for 48h (0) and stimulated with 10% serum for 16 h or 27 h. Immunoblots were probed with mouse anti-CRIK (BD-Transduction Laboratories) and rabbit anti-p27 (C19) antibodies. β-tubulin levels were used as loading control. (B) Citron-K localization during mitosis in immortalized MEFs. Cells were fixed in 1% paraformaldehyde for 3 min at 37°C and 5 min in methanol at -20°C. Citron-K was detected using goat anti-Citron (C20) antibody (red) and DNA and microtubules were stained with Hoechst H33342 (blue) and mouse α-tubulin antibody (clone DM-1, Sigma) (green), respectively. Citron-K is cytoplasmic during metaphase and anaphase, translocates to the cortex (zone of ingression) at the end of anaphase, accumulates at the cleavage furrow in telophase and remains at the midbody in cytokinesis (60X lens).

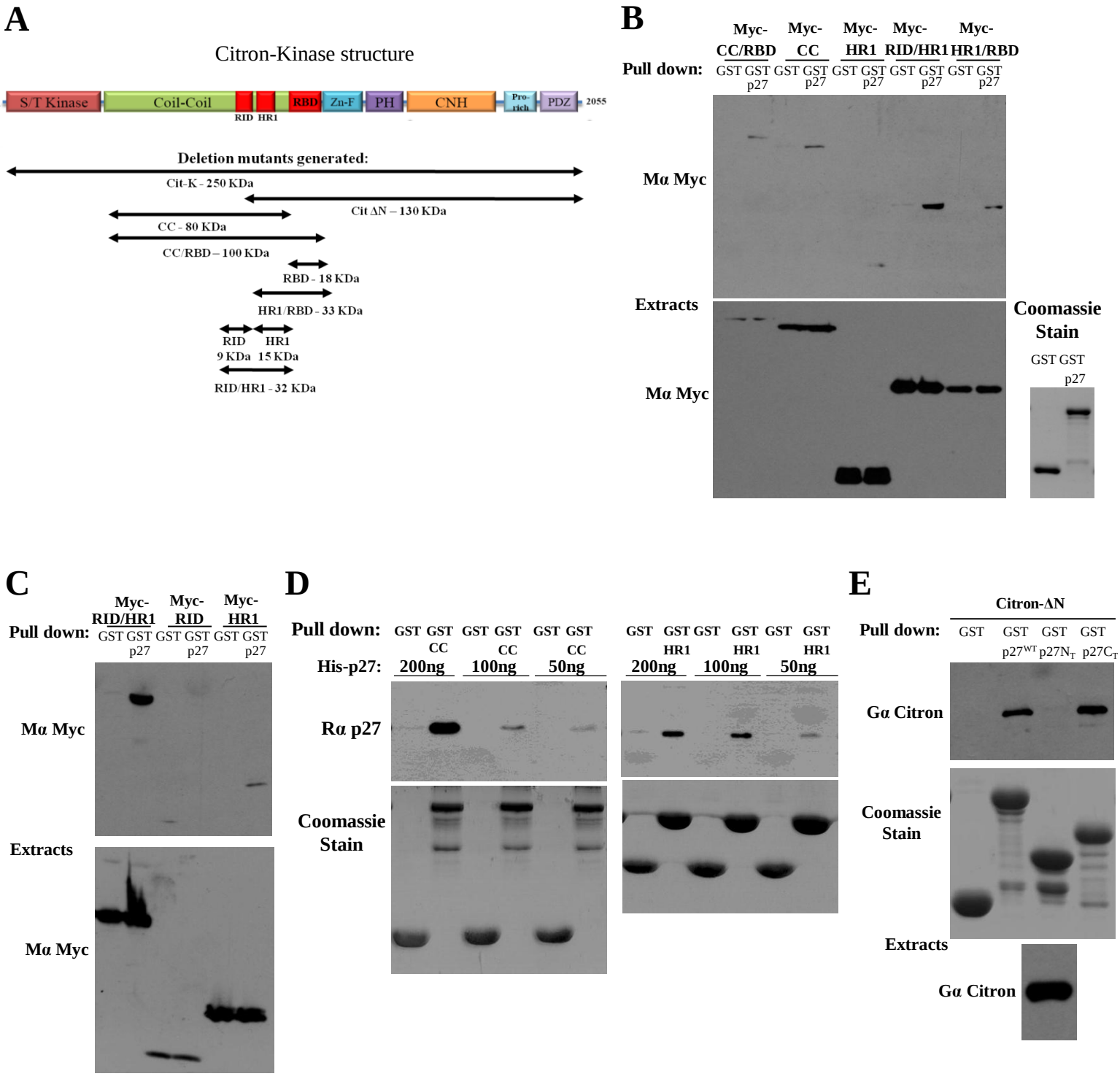

Figure S3: The C-terminal part of p27 interacts with the HR1 domain of Citron-K.

(A) Schematic representation of full length Citron-K and deletion mutants used in this study. (B-C) Pull-down assays using GST or GST-p27 beads on HEK293 extracts overexpressing different Citron-K mutants. The amount of protein recovered in pull-downs was detected by immunoblot using mouse anti-Myc (9E10) antibody. Immunoblot of respective extracts indicate the level of expression of the transfected proteins. The amount of GST or GST-p27 was visualized by Coomassie staining. (D) Pull-down assays using different amount of recombinant His-p27 with GST and GST-CC beads (left panel) or GST-HR1 beads (right panel). The amount of protein recovered in pull-downs was detected by immunoblot using rabbit anti-p27 (C-19) antibody. The amount of different GST beads used in the assay was visualized by coomassie staining. (E) HEK 293 cells were transfected with Cit-ΔN. Pull-down assays were performed using the following GST beads: GST, GST-p27WT, GST-p27N_T (aa 1-86) and GST-p27C_T (aa 87-198). The amount of Cit-ΔN recovered in pull-downs was detected by immunoblot with goat anti-Citron (C20). The amount of the different GST beads was visualized by coomassie staining. The level of Cit-ΔN present in the extract was determined by immunoblot with goat anti-Citron antibodies.

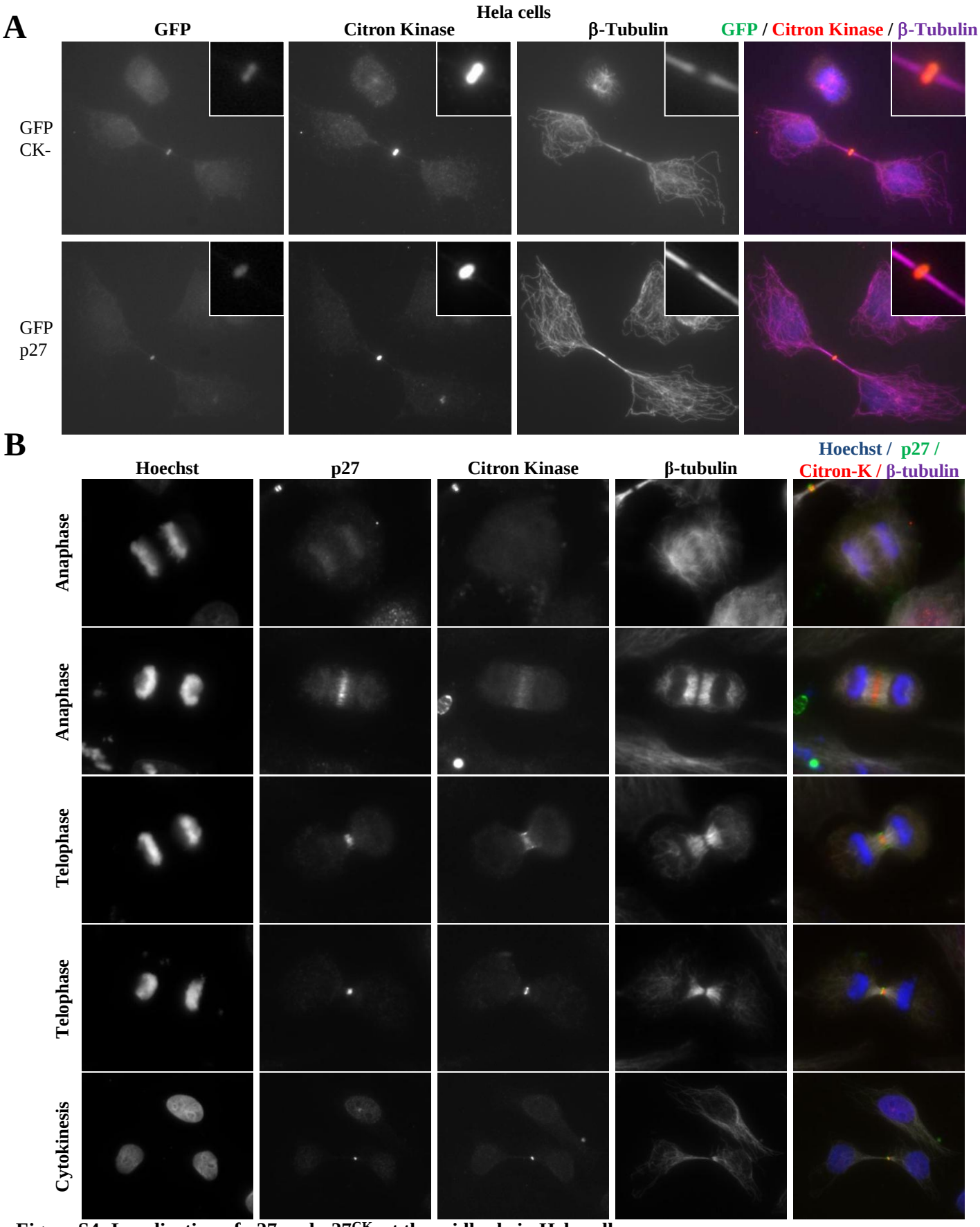

Figure S4: Localization of p27 and p27^{CK-} at the midbody in Hela cells.

(A) Hela cells were grown on coverslips and transfected with eGFP-p27 or eGFP-p27^{CK-}. After 24 h, cells were permeabilized with digitonin and fixed (see Methods for details). The localization of p27 or p27^{CK-} was visualized by eGFP fluorescence and cells were stained with goat anti-Citron (C20) antibody and mouse anti-β-tubulin (TUB2.1, sigma) (60X lens). (B) Localization of p27 and Citron-K throughout mitosis. Hela cells were stained with rabbit anti p27 (C19), goat anti-Citron and mouse anti-β-tubulin antibodies (60X lens). p27 appears at the spindle midzone in late anapahse and colocalize with Citron-K at the contractile ring and midbody in telophase and cytokinesis, respectively.

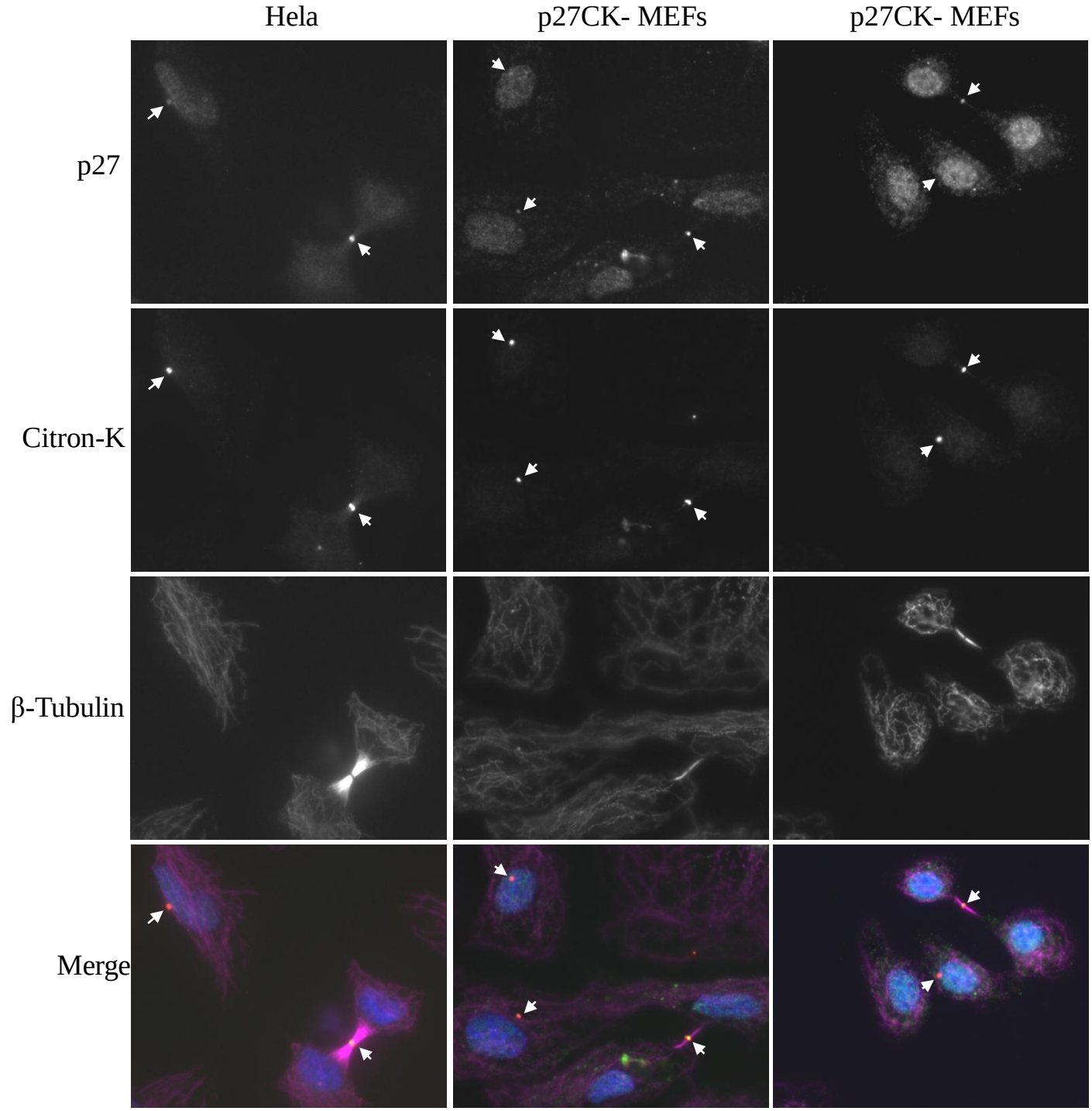

Figure S5: p27 localization to the midbody is specific to mitotic cells.

Some cells maintain a midbody remnant or midbody derivative in interphase. Citron-K remains present in these structures, while p27 is largely absent from the midbody in interphase cells compared to mitotic cells.

Hela cells and MEFs grown on coverslips were permeabilized with digitonin and fixed (see Methods for details). The localization of wild-type p27 or $p27^{CK-}$ was visualized with a rabbit anit p27 (C19) antibody (green) and cells were stained with goat anti-Citron (C20) antibody and mouse anti-β-tubulin (TUB2.1, sigma) (purple) (60X lens). Midbodies are indicated with arrowheads.

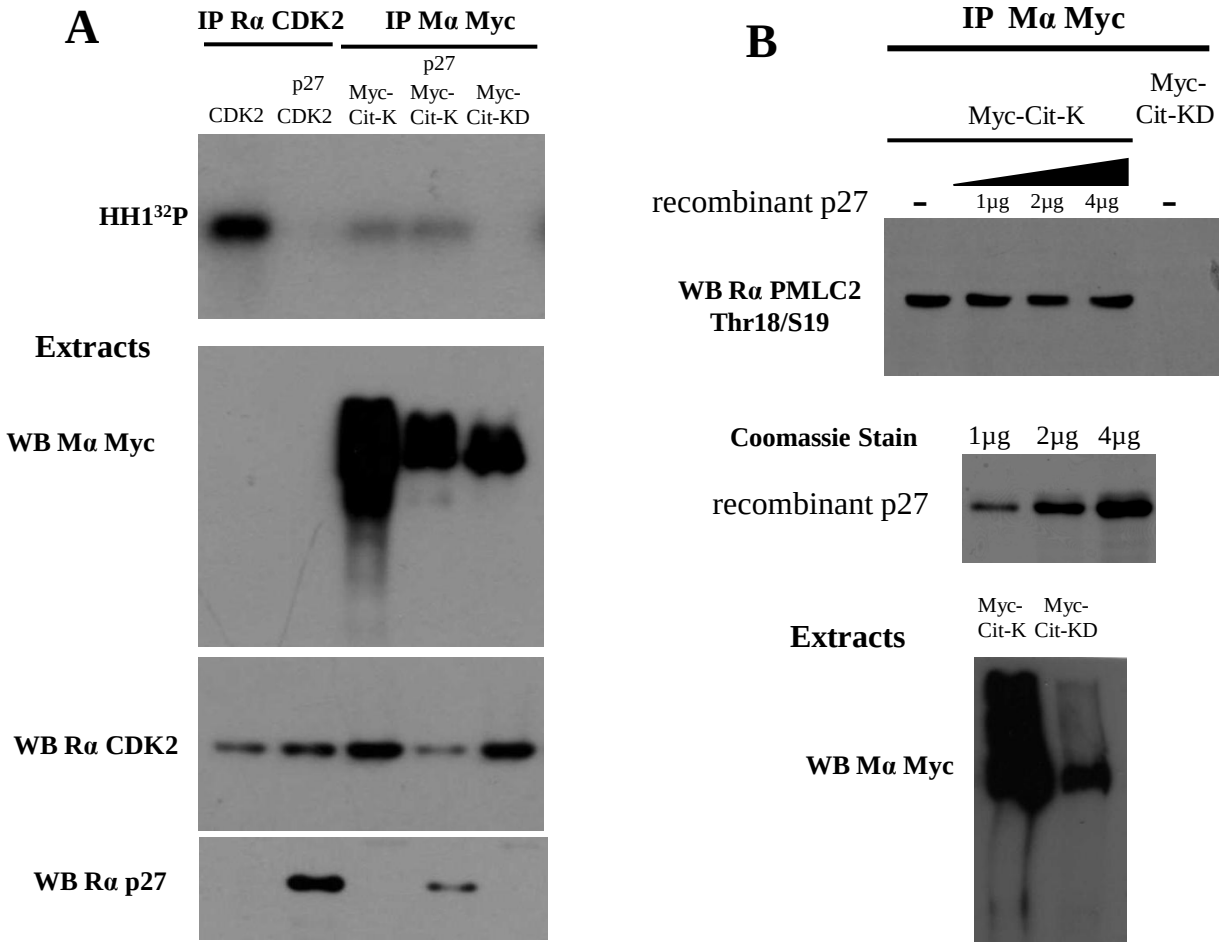

Figure S6: p27 does not inhibit the activity of Citron-K *in vitro*.

(A) HEK293 cells were transfected with the indicated proteins. Cell extracts were prepared and Citron-K (or the kinase-dead mutant, Cit-KD) or endogenous CDK2 were immunoprecipitated with mouse anti-Myc (9E10) or rabbit anti-CDK2 (M2, Santa Cruz Biotechnology), respectively. Immunoprecipitated proteins were subjected to kinase assays. Kinase assays were performed using 200µM ATPγ^{32}P and 1µg histone H1 as an exogenous substrate in kinase buffer (50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM dithiothreitol) for 15 min at 30°C. Results were analyzed by autoradiography. CDK2 kinase assays were performed to control the inhibitory activity of p27 in these experimental conditions. The specificity of the kinase activity detected in Citron-K immunoprecipitations was confirmed by the absence of signal when Citron-KD was transfected. The protein levels in cell extracts was visualized by immunoblot with anti-Myc (9E10), anti CDK2 (M2) and anti-p27 (C19) antibodies.

(B) HEK293 were transfected with Citron-K or Citron-KD. Cell extracts were immunoprecipitated with mouse anti-Myc and kinase assays were performed as in (A) in presence of increasing amounts of recombinant p27. Recombinant MRLC2 was used as exogenous substrate and results were analysed by immunoblot using rabbit anti-phosphoThr18/Ser19 MLC antibody (Cell Signalling Technology). The levels of His-p27 used in the assays were visualized by coomassie staining. Citron-K or Citron-KD transfected were detected by immunoblot with an anti-Myc antibody (9E10).

Uniprot Name	Description	Peptide sequence	Probability
Ctro_Mouse	Citron Rho-interacting kinase	VASSPAPPEGPSHPR	0.8559
Ctro_Mouse	Citron Rho-interacting kinase	WVTALESVVAGGR	0.8688

Table S1: Identification of Citron Kinase from a p27 interaction screen.
Two peptides from Citron Kinase were identified from mass spectrometry analysis of $p27^{CK-}$ interacting proteins. The peptide sequences and their probability scores determined by statistical analysis using PeptideProphet (Keller et al. 2002) were listed.

Identification of p27interacting proteins by affinity purification and mass spectrometry

Mouse p27 was fused at the C-terminus with two tandem copies of Tobacco Etch Virus (TEV) cleavage site (ENLYFQG) followed by a 23 amino acid peptide sequence recognizable by the E. coli biotin ligase, BirA. Construction of this biotin affinity tag has been described previously (Furuyama and Henikoff 2006). Biotin peptide-tagged p27 (wild-type or CK-) and BirA sequences were cloned into the pQCXIP and pQCXIH vectors (BD Biosciences), respectively. Stable MEF cells lines co-expressing biotin peptide-tagged p27 (wildtype or CK-) and the BirA protein were generated by sequential retroviral infection of $p27^{-/-}$ MEFs using first the p27 constructs (with puromycin selection) and then the BirA construct (with hygromycin selection). Cell lysates were prepared from exponentially growing MEFs, and p27 was purified from lysates using streptavidin beads followed by elution using the TEV protease. The TEV eluates were digested with trypsin and then subjected to LC-MS/MS analysis using an ion-trap mass spectrometer as described previously (Chi et al. 2008). The MS data were searched against the mouse NCI database using SEQUEST, and the search results were filtered using PeptideProphet (Keller et al. 2002) with a minimal probability score of 0.85. As a negative control, p27 containing a mutant biotin peptide tag (in which the target lysine is replaced by arginine) was used in parallel in the analysis.

References

Keller A, Nesvizhskii AI, Kolker E, Aebersold R. 2002. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem. 74:5383-92.

Furuyama T, Henikoff S. 2006. Biotin-tag affinity purification of a centromeric nucleosome assembly complex. Cell Cycle. 5:1269-74.

Chi Y, Welcker M, Hizli AA, Posakony JJ, Aebersold R, Clurman BE. 2008. Identification of CDK2 substrates in human cell lysates. Genome Biol. 9:R149.