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Chfr interacts and co-localizes with TCTP to the mitotic spindle

Running Title: Chfr binds TCTP and tubulin

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Abstract

Chfr is a checkpoint protein that plays an important function in cell cycle progression and tumour suppression, although its exact role and regulation are unclear. Previous studies have utilised over-expression of Chfr in order to determine the signalisation pathway of this protein in vivo. In this study, we demonstrate by using three different antibodies against Chfr, that the endogenous and highly overexpressed ectopic Chfr protein are localized and regulated differently in cells. Endogenous and lowly expressed ectopic Chfr, are cytoplasmic, and localize to the spindle during mitosis. Higher expression of ectopic Chfr correlates with a shift in the localization of this protein to the nucleus/PML bodies, and with a block of cell proliferation. In addition, endogenous and lowly expressed ectopic Chfr are stable throughout the cell cycle whereas, when highly expressed, ectopic Chfr is actively degraded during S-G2/M phases in an auto-ubiquitination, and proteasome-dependent manner. A two-hybrid screen identified TCTP as possible Chfr interacting partner. Biochemical analysis with the endogenous proteins confirmed this interaction, and identified β -tubulin as an additional partner for Chfr, supporting the mitotic spindle localisation of Chfr. The Chfr-TCTP interaction was stable throughout the cell cycle, but could be diminished by the complete depolymerisation of the microtubules, providing a possible mechanism where Chfr could be the sensor that detects microtubule disruption and then activate the prophase checkpoint.

Introduction

The prophase or antephasic checkpoint is a p38-dependent, early mitotic checkpoint that responds to a wide variety of cellular stresses. Upon checkpoint activation, early prophase cells transiently return to a late G2 state (Matsusaka and Pines, 2004; Mikhailov *et al.*, 2004). The ubiquitin ligase Chfr (Checkpoint protein with FHA and RING finger domains) was identified as crucial component of this checkpoint in specific response to microtubule

depolymerisation with nocodazole (Scolnick and Halazonetis, 2000). Yet, the exact role of Chfr is still unclear, however the E3 ubiquitin ligase activity of its RING finger domain appears to be required for a functional checkpoint (Bothos *et al.*, 2003; Chaturvedi *et al.*, 2002; Kang *et al.*, 2002). It has been proposed that Chfr mediates the prophase arrest via the degradation of the Plk1 kinase, however, no degradation of Plk1 has been observed in cells under checkpoint conditions (Matsusaka and Pines, 2004), although Chfr preferentially forms Lys-63 polyubiquitin chains (Bothos *et al.*, 2003), suggesting Chfr may mediate the checkpoint via ubiquitin signalling, rather than degradation of substrates.

The Chfr gene is expressed ubiquitously in normal tissues, but in human cancer cell lines and in tumours, its promoter is frequently hyper-methylated leading to a loss of expression (Erson and Petty, 2003; Satoh *et al.*, 2003). Mutations in the Chfr gene have been identified in a variety of human cancers including lung, nasopharyngeal, gastric and breast tumours, leading to the suggestion that Chfr is a tumour suppressor gene candidate (Cheung *et al.*, 2003; Corn *et al.*, 2003; Erson and Petty, 2003; Mariatos *et al.*, 2003; Mizuno *et al.*, 2002). In support of this, knockdown of Chfr in immortalised mammary epithelial cells results in a malignant phenotype with cells displaying increased growth rates, invasiveness, and colony formation, while knockout mice are cancer-prone (Mariatos *et al.*, 2003; Privette *et al.*, 2007; Yu *et al.*, 2005).

Despite its potential importance in human cancer, very little is known about the regulation of Chfr. Overexpressed Chfr localizes at the PML bodies and a loss of this localization correlates with a loss of Chfr functionality, thus, it has been proposed that PML-Chfr interaction could regulate Chfr activity (Daniels *et al.*, 2004). In addition, there is a ubiquitin-dependent degradation of Chfr during M and G1 phases of the cell cycle (Chaturvedi *et al.*, 2002). However, these studies utilised over-expression of Chfr, and currently, there is no data on the regulation of the endogenous form. We demonstrate here, using three different

antibodies, that endogenous Chfr is primarily cytoplasmic, stable throughout the cell cycle, and partially localised to the mitotic spindle. When expressed at low levels, ectopic Chfr displays the same expression and localization pattern of the endogenous protein. In contrast, highly expressed ectopic Chfr is localized in the nucleus, often at PML-like bodies and is actively degraded in an autoubiquitination-dependent manner by the proteasome. In accordance with its localization to the spindle, endogenous Chfr also binds β -tubulin and interacts with the Translationally Controlled Tumour Protein (TCTP), with both proteins co-localising at the mitotic spindle. Finally, depolymerisation of the microtubules with nocodazole disrupts Chfr-TCTP interaction. Thus, Chfr is present at the mitotic spindle, and could play a role as a sensor for microtubule disruption, providing a logical explanation for its requirement in the prophase checkpoint response to nocodazole.

Results

Endogenous Chfr is ubiquitinated and phosphorylated in a MPF-dependent manner in Xenopus egg extracts and oocytes

To study the regulatory mechanisms involved in the control of the mitotic stress checkpoint, the *Xenopus* homologue of Chfr was cloned and three different antibodies produced, one directed against the total protein (Anti-Chfr) and the other two directed against peptides to the first 12 N-terminal (Anti-Nter) and the last 12 C-terminal (Anti-Cter) amino-acids. All three antibodies recognise a band in interphase (INT) and metaphase II-arrested *Xenopus* egg extracts (CSF) that co-migrates with *in vitro* translated Chfr (Rt) although, only the antibodies directed against the total protein and the N-terminal peptide were capable to detect the low levels of Chfr present in XL2 cells (Figure 1a). Moreover, all three antibodies completely immunodepleted Chfr from CSF, interphase *Xenopus* egg extracts and XL2 cell lysates (Figure 1a and data not shown), and therefore, the antibodies specifically recognise Chfr.

Chfr immunodepletions performed in CSF extracts produced a smeared signal above the focused endogenous Chfr band in the immunoprecipitate. It is possible that this smeared form of Chfr could correspond to the ubiquitination of this protein in the mitotic extracts. Therefore, a immunoprecipitation using antibodies directed against the full-length protein was performed with 20 ml of CSF extracts, and the smeared bands were excised and sequenced by MALDI-MS-MS (Figure 1b). The mass spectrometry results showed that the focused band corresponded exclusively to Chfr, while Chfr and ubiquitin were present in all of the sequenced smeared bands, indicating that endogenous Chfr is continuously ubiquitinated in CSF extracts.

The over-expressed form of Chfr can target itself for degradation in mitotic cells through autoubiquitination (Chaturvedi *et al.*, 2002), however no change was observed in the levels of endogenous Chfr in CSF extract, and the addition of the protein synthesis inhibitor cycloheximide, or the proteasome inhibitor MG132 had no effect on Chfr levels (Figure 1c). Thus, the ubiquitination of endogenous Chfr in these extracts is not involved in its proteolysis. Similar results were obtained in U2OS cells stably transfected with Chfr, where no Chfr degradation was detected throughout the cell cycle. Moreover, this stable pool of Chfr was phosphorylated during mitosis (Bothos *et al.*, 2003). It is possible that this mitotic phosphorylation of Chfr is dependent on cyclin B-cdc2. To test this hypothesis, we used G2-arrested *Xenopus* oocytes in which maturation is induced by the addition of progesterone. In these oocytes, progesterone promotes an initial activation of cyclin B-cdc2 and thus, maturation or germinal vesicle breakdown (GVBD). Maturation is followed by a partial proteolysis of cyclin B and a consequent decrease of cyclin B-cdc2 activity. Re-synthesis of cyclin B then ensues, the mitotic kinase is reactivated and oocytes arrest at metaphase II. Addition of Cycloheximide after GVBD prevents cyclin B re-synthesis, and the subsequent re-activation of cyclin B-cdc2, therefore providing a model for assessing the dependence of

Chfr phosphorylation on cyclin B-cdc2 activity. G2-arrested oocytes were divided into two groups, the first was supplemented with progesterone, and the second was supplemented with progesterone, and 30 minutes after GVBD, with cycloheximide. Three oocytes per time-point were removed and Chfr was analysed by western blot. A clear retardation in the electrophoretic mobility of Chfr induced by the phosphorylation of this protein (Supplementary data S1) was observed just after GVBD when cyclin B-cdc2 became activated (noted by the phosphorylation shift seen for cyclin B at GVBD), and remained in this form throughout the experiment (Figure 1d). However, when cyclin B-cdc2 reactivation was prevented by the addition of Cycloheximide, the electrophoretic mobility of Chfr returned to its original non-phosphorylated state. In addition, Chfr levels did not decrease after Cycloheximide treatment, confirming that Chfr is stable in maturing oocytes and is phosphorylated directly or indirectly by cyclin B-cdc2.

Endogenous Chfr is stable throughout the cell cycle and present in the mitotic spindle.

The stability and localization of endogenous Chfr was then analysed in XL2 and XTC cells respectively. *Xenopus* XL2 cells were synchronized at G1/S boundary by aphidicolin block and then released into the cell cycle. Samples were taken at various intervals for western blot and Flow Cytometry Analysis (FACS). Approximately 70% of cells synchronously entered the cell cycle, with peak mitosis at 12h post release (Figure 2a). Samples corresponding to G1, S, G2 and M phase showed no change in the levels of the Chfr protein. Immunofluorescent staining of *Xenopus* XTC cells mirrored the western blot data, with no obvious change in the total levels of Chfr throughout the cell cycle, with a majority of the staining localised to the cytoplasm (Figure 2b). Sub-cellular fractionation of XL2 cells confirmed this localisation with a significant proportion of endogenous Chfr present in the soluble cytosolic fraction, while no protein was detected in the extracted nuclear fraction (Figure 2c). More

importantly, there was a partial but clear localization of endogenous Chfr in the mitotic spindle (Figure 2b). This localization was first detected at prophase, increased during metaphase and early anaphase, decreased at late anaphase and disappeared completely by telophase. Similar staining was observed with the anti-Cter Chfr antibody (data not shown).

Exogenous Chfr is nuclear and degraded in an auto-ubiquitination dependent manner.

The data presented here on the endogenous form of Chfr is in contradiction with the previous published data on the over-expressed form of this protein. Therefore, we over-expressed *Xenopus* wild type YFP-Chfr in XL2 cells and analysed the localization and the stability of this protein. In accordance with previous reports (Daniels *et al.*, 2004), over-expressed Chfr displayed a clear nuclear staining, characterised by the accumulation in large and small focal structures that likely correspond to the PML bodies (Figure 3a). Thus, ectopic and endogenous Chfr proteins localize in opposite cellular compartments. Moreover, no mitotic YFP-Chfr transfected cells were observed (data not shown), thus the cell cycle distribution of YFP-Chfr expressing population was analysed. Transfected XL2 cells were synchronized, and analysed by 2-dimensional flow cytometry. In agreement with the fluorescence microscopy results, no YFP-Chfr expressing cells contained a 4n DNA, indicating either that YFP-Chfr is degraded during the S/G2 phases of the cell cycle, or that YFP-Chfr overexpression blocks cell cycle progression in G1 phase (Figure 3b).

The degradation of the ectopic expressed Chfr protein is dependent on its ring-finger domain and associated auto-ubiquitination activity (Chaturvedi *et al.*, 2002). Therefore to assess if ectopic Chfr blocks cell cycle progression or is degraded, synchronised HeLa cells were transfected with either the YFP-wild-type (WT) or the YFP-Ring Finger (Δ RFD) mutant of Chfr and the cell cycle distribution of the overexpressed cells was analysed. Cells transfected with the Δ RFD mutant contained a two-fold number of YFP positive cells, and a four-fold

level of expression of this protein per cell in comparison to WT transfected cells (data not shown). Western blots confirmed this observation with approximately 6 times less WT Chfr compared to the Δ RFD mutant in G1/S cell lysates (Figure 3c). In addition, there was a clear decrease in the total levels of WT protein during S phase and to a lesser extent G2/M, which could be reversed by the S-phase addition of the proteasome inhibitor MG132. Furthermore, addition of MG132 to asynchronous cells transfected with WT protein for 24 hours restored the level of expression of the WT protein to similar levels of the Δ RFD mutant (Figure 3d). FACS analysis of the over-expressed populations mirrored the western blot data. Transfection of WT construct induced a clear accumulation of approximately 80% of YFP positive cells in G1. The addition of Nocodazole failed to alter this pattern, however S-phase addition of MG132 resulted in the accumulation of WT-Chfr cells in G2/M with a 4n DNA content. In contrast, Δ RFD-transfected cells presented a similar pattern to non-transfected cells even after treatment with nocodazole and MG132, although a slight accumulation in G1 was also observed (Figure 3e). These results demonstrate that the WT protein is degraded throughout the cell cycle by the proteasome in an auto-ubiquitination dependent manner. Therefore, it is possible that the accumulation at G1 observed in WT-transfected cells is the result of degradation of YFP-WT Chfr during S and G2/M phases, however the possibility cannot be excluded that in addition to degradation, the over-expression of the WT-Chfr may also block cell cycle progression in G1 phase.

Low level Exogenous Chfr is stable throughout the cell cycle and is present in the mitotic spindle.

In order to investigate if over-expression of WT Chfr blocked cell cycle progression, time-lapse video microscopy of synchronised HeLa cells transfected with YFP-Chfr was performed. 50 random YFP positive cells were followed for 24 h after their release from

Thymidine (G1/S). Two different localisations of WT-Chfr were observed. First, strongly expressing cells (24 %) displayed a nuclear staining with the protein shifting between a diffuse nuclear staining to localised foci (Figure 4a and supplementary data S2). Moreover, no mitotic cells were observed in this group, with a two-fold increase in expression of ectopic Chfr in the interphase population compared to mitotic cells, indicating that high over-expression of Chfr might block cell cycle progression (Figure 4c, Int). Second, and more commonly (76 %), a weak diffuse total cytoplasmic staining was observed. Of the 50 analysed cells, 6 cells underwent mitosis, and all contained a weak diffuse staining, with no nuclear foci observed throughout the time-course (Figure 4b). The low expression of Chfr in mitotic cells remained stable (Figure 4c, mitotic) and a distinct localisation at the mitotic spindle, similar to the one obtained for the endogenous protein was observed (Figure 4b, and supplementary data Figure S3). These results indicate that when expressed at low levels, exogenous Chfr is not degraded, and does not affect cell cycle progression. MG132 treatment of interphase cells presenting a weak diffuse staining shifted the balance towards a nuclear, foci localisation (Figure 4d), and prevented the decline in expression of interphase cells over time (Figure 4c, MG132). Thus preventing the proteasome degradation of Chfr promotes the nuclear and PML-like foci localisation of the protein.

More detailed analysis of the earlier FACS data confirmed the live cell imaging results. Synchronised HeLa cells were harvested as they underwent mitosis, and processed by FACS. For analysis, cells were sub-divided into high, medium, low and non- Chfr over-expressing fractions. The percentage of cells in G1, S and G2/M was then quantified. Both the high and medium over-expressing Chfr cells remained primarily in G1 phase, whereas only the low-expressing cells were present in G2/M phase (Figure 4e).

In summary, it is clear that there is a threshold for Chfr within cells. Below this threshold, Chfr is stable, does not perturb cell cycle progression, is localised to the cytoplasm and the

mitotic spindle. Above this threshold, Chfr is nuclear often localizing to PML-like foci, actively degraded, and blocks cell cycle progression at G1.

Chfr binds β -tubulin and the Translationally Controlled Tumour Protein (TCTP) in Xenopus egg extracts and in XL2 cells

Since Chfr detects mitotic stress during prophase, we hypothesised that its spindle localization might be important for its function. To further characterise the Chfr signalisation pathway and spindle localisation, a yeast two-hybrid screen was performed to identify novel Chfr-interacting proteins. Full-length amino acids of Chfr were used as baits to screen a cDNA library obtained from 6 to 8-week-old human embryo. One of the positive clones isolated was the Translationally Controlled Tumour Protein (TCTP). Mating assays subsequently confirmed an interaction between LexA-Chfr and TCTP. Rabbit polyclonal antibodies were produced against bacterially expressed full-length Xenopus TCTP-GST in order to further investigate the endogenous interaction between Chfr and TCTP. The affinity-purified antibodies recognised a single band that migrated at the expected molecular weight of approximately 25 kDa in CSF, interphase Xenopus egg extracts and XL2 cells (Figure 5a). Unfortunately, despite multiple rounds of immunoprecipitation, only a very low quantity of TCTP was present in the IP, with the bulk of the protein remaining in the supernatant, (Figure 5a and data not shown).

Gel filtration performed on interphase extracts showed that a small amount of endogenous TCTP co-eluted with Chfr. The peak fraction of Chfr eluted in fractions corresponding to 140-240 kDa, whereas the majority of TCTP eluted in fractions corresponding to 43 kDa. However, a small, but repetitive fraction of TCTP co-eluted with the peak of Chfr (Figure 5b). The endogenous interaction was further analysed by immunoprecipitation with anti-Chfr or anti-TCTP antibodies in CSF and XL2 extracts. Due to the low immunoprecipitation capacity

of the anti-TCTP antibodies (Figure 5a), no Chfr was detected in the anti-TCTP immunoprecipitate (data not shown), therefore the reverse experiment was performed. A weak but consistent band corresponding to TCTP was obtained in the Chfr immunoprecipitate from CSF and XL2 extracts (Figure 5c). This band was specific as similar results were obtained with the full-length, anti-Cter or anti-Nter Chfr antibodies. Moreover, the interaction could no longer be detected after the complete depletion of Chfr by several rounds of immunoprecipitation from the extracts (Figure 5c).

Given the mitotic spindle localisation of Chfr, the ability to interact with tubulin was also assessed. Similar to TCTP, a small reproducible quantity of β -tubulin was present in Chfr immunoprecipitations, which disappeared after depletion of Chfr from the extract (Figure 5c), indicating that this interaction is specific.

TCTP localises to the Mitotic Spindle

In order to investigate if TCTP could co-localize with Chfr *in vivo*, immunofluorescence of TCTP in XTC cells was performed. TCTP has been reported to co-localizes with tubulin during interphase (Gachet *et al.*, 1999), however, we failed to observe this localization, with TCTP primarily localised to an undefined cytoplasmic structure (Figure 5d). During mitosis, a clear staining of the mitotic spindle was observed. This staining started at prophase, increased during metaphase and anaphase and completely disappeared at telophase. Thus, both Chfr and TCTP are present at the mitotic spindle with similar kinetics.

The Chfr-TCTP interaction is independent of the cell cycle, but dependent on microtubules

The mitotic stress checkpoint responds to a depolymerisation of microtubules by mitotic poisons. TCTP and tubulin both co-immunoprecipitate with Chfr, and are located at the mitotic spindle. It is possible that this association could be involved in the activation of this

checkpoint. In order to investigate this hypothesis, we first determined the interaction between Chfr and TCTP throughout a normal cell cycle. XL2 cells were synchronized by aphidicolin block, and immunoprecipitation with anti-Chfr antibodies is performed on samples corresponding to the various phases of the cell cycle. A clear association, that remained constant throughout the cell cycle, was observed between Chfr and TCTP indicating that this association is not cell cycle regulated (Figure 6a). To analyse the interaction during the prophase checkpoint, nocodazole (1 μ M) was added during S phase to synchronized XL2 cells. No difference in the association was observed between TCTP and Chfr in the presence or absence of nocodazole despite the activation of the Chfr checkpoint, noted by the delay in the mitotic index in nocodazole treated cells (Figure 6b).

The strength and stability of the Chfr- β -tubulin-TCTP interactions in soluble XL2 extracts was then determined. Binding of TCTP to Chfr was resistant up to 500 mM NaCl washing, but was sensitive to detergent (1% IGEPAL-C630), while β -tubulin was resistant to both 500 mM NaCl and detergent, suggesting that tubulin binds more tightly to Chfr than TCTP, and thus may mediate the interaction.

Therefore to determine if the Chfr-TCTP interaction is dependent on the presence of stable microtubules, interphase extracts were treated on ice for 30 min in the absence (Con) or in the presence of 10 μ M Nocodazole (Noc) to completely depolymerise the microtubules (Rieder and Cole, 2000), and then subjected Chfr immunoprecipitations. Comparable levels of Chfr were present in both control and nocodazole treated extracts and precipitates, with a clear association between Chfr and both TCTP and β -tubulin in the control extracts. Treatment with nocodazole significantly decreased the interaction between Chfr, β -tubulin, and TCTP (Figure 6d). Therefore, microtubules might play a significant role in mediating the interaction between Chfr and TCTP.

Discussion

Chfr is a checkpoint protein that transiently arrests cells in early prophase in the presence of microtubule poisons (Scolnick and Halazonetis, 2000). The Chfr gene is ubiquitously expressed in normal tissues, but in human cancer cell lines and tumours, its promoter is frequently hypermethylated (Erson and Petty, 2003; Satoh *et al.*, 2003). Despite its potential importance in cancer, the function of Chfr is poorly understood, possibly due to its low cellular levels, and the poor availability of sensitive, robust antibodies. Consequently, all current data on Chfr has been obtained using the over-expressed protein with no information available for the endogenous protein. In this study we produced three different Chfr antibodies that specifically recognise and immunoprecipitate Chfr either in *Xenopus* egg extracts or in XL2 cells in culture and analysed for the first time the endogenous Chfr protein. Endogenous Chfr can be ubiquitinated under certain conditions, is stable throughout the cell cycle, and is phosphorylated during mitosis in a cyclin B-cdc2-dependent manner. In addition, the immunofluorescence and sub-cellular fractionation of cells show that endogenous Chfr localises in the cytoplasm during interphase, while during mitosis it partially migrates to the mitotic spindle where it is present until late anaphase. This localization of endogenous Chfr protein is in contrast with previous reports, which described the ectopic protein as nuclear and localised to PML foci (Chaturvedi *et al.*, 2002; Daniels *et al.*, 2004). In agreement, we found that high over-expression of the ectopic protein results in a nuclear localisation, with a distinct punctuate like staining that likely correspond to PML bodies. However, this highly over-expressed nuclear Chfr was degraded during the cell cycle in an autoubiquitination-dependent manner, and caused a G1 cell cycle arrest. Notably, overexpression of the yeast homologue of Chfr in *S. cerevisiae* can induce both a G1 and G2 arrest (Loring *et al.*, 2008).

At lower levels, exogenous Chfr behaved similar to the endogenous protein, with both

proteins stable throughout the cell cycle and localising to the cytoplasm during interphase, and the spindle during mitosis. Therefore, there appears to be a threshold for Chfr expression within cells, endogenous or exogenous, which is permissive to normal cell cycle progression. Above this level, Chfr is redistributed to the nucleus, is actively degraded by the proteasome in an auto-ubiquitination dependent manner, and blocks cell cycle progression.

To identify novel Chfr-interacting proteins that could co-localise to the mitotic spindle, a two-hybrid screen was undertaken. Through this screen, TCTP was identified as a novel interacting partner for Chfr. TCTP is a ubiquitous highly expressed protein that has been implicated in a wide variety of cellular functions, including microtubule stabilisation (Gachet *et al.*, 1999; Yarm, 2002), anti-apoptotic function, Na/K-ATPase inhibition (Jung *et al.*, 2004) or as a guanine nucleotide exchange factor (Hsu *et al.*, 2007). Moreover, TCTP has been implicated in cancer with knockdown of TCTP enabling tumour reversion (Tuynder *et al.*, 2004; Tuynder *et al.*, 2002), while over-expression can protect cells from apoptosis (Liu *et al.*, 2005; Yang *et al.*, 2005). The specificity of Chfr-TCTP interaction was confirmed by immunoprecipitation of endogenous Chfr from *Xenopus* extracts and cell lines, with a small but consistent quantity of TCTP present. Furthermore, this association did not vary throughout the cell cycle, and both proteins were present at the mitotic spindle with similar kinetics. Interestingly, TCTP has previously been shown to bind with and co-elute on gel filtrations with tubulin (Gachet *et al.*, 1999). Therefore, there seemed a strong possibility that Chfr may also interact with tubulin. Accordingly, immunoprecipitation data confirmed that a small quantity of β -tubulin specifically binds Chfr. Both the TCTP-Chfr and tubulin-Chfr interactions were resistant to salt, but TCTP was highly sensitive to detergent, suggesting that the interaction between Chfr and tubulin was stronger, and possibly mediating the Chfr-TCTP interaction. In support of this, depolymerisation of the microtubules by high dose nocodazole, greatly reduced the Chfr- β -tubulin-TCTP interaction.

The Chfr-dependent prophase checkpoint responds specifically to microtubule damage by delaying entry into mitosis. We hypothesize that Chfr is the sensor for microtubule depolymerisation. First, it could be localised by its association with TCTP at the mitotic spindle. Then disruption of the spindle could then liberate TCTP and Chfr, allowing Chfr to interact with additional substrates such as the mitotic kinase's Aurora A and Plk, thereby preventing the onset of mitosis. However, no decrease in the total Chfr-TCTP interaction was detected during the checkpoint response in cells. Although it is possible that only the spindle localised form of Chfr is affected by checkpoint activation, and thus our experiments may not be sensitive enough to detect this dissociation. Unfortunately, we failed to successfully knockdown TCTP via siRNA in *Xenopus* cell lines, and thus have been unable to determine if TCTP binding to Chfr protects the latter from being degraded. Therefore, more research is needed, preferably in mammalian cell lines with functional Chfr, where the ability to synchronise and manipulate gene expression via siRNA and stable-inducible expression is more feasible. Unfortunately, there are no antibodies currently available capable of detecting the endogenous mammalian Chfr protein, limiting the advancement of this research field.

In summary, the above data demonstrate for the first time that endogenous Chfr is stable throughout the cell cycle and partially localizes to the mitotic spindle with the tumour protein TCTP and tubulin. All three proteins are present in a complex that is partially dependent on the presence of microtubules, providing a mechanism where Chfr could be the sensor for microtubule disruption, and when dissociated from the spindle it is then able to prevent mitotic entry through interaction with its mitotic substrates.

Materials and methods

c-DNA cloning, immunization procedures, protein purification and antibodies.

Xenopus Chfr and TCTP cDNA were amplified from total mRNA of Xenopus metaphase II- arrested eggs and subcloned blunt into the Stu I site of a PCS2+ vector. For immunization, Xenopus Chfr and TCTP were amplified by PCR, subcloned in the BamHI-XhoI site for Chfr and EcoRI-Sall site for TCTP of pGEX4T1. Fusion proteins were expressed in Escherichia coli. Inclusion bodies were prepared and subjected to SDS PAGE, and electroeluted according to standard procedures. Proteins were dialysed against NaCl 500 mM, NaHCO₃ 100 mM buffer and used to immunize rabbits. Immune sera were precleared in a GST-immobilized column and subsequently affinity purified on immobilized GST-Chfr and GST-TCTP columns respectively. Anti-Nter and anti-Cter antibodies were generated against the first 12 N-terminal and last 12 C-terminal peptides, coupled to thyroglobulin for immunization and to immobilized bovine serum albumin for affinity purification as previously described (Abrieu *et al.*, 2001).

For cell transfection, Chfr was amplified by PCR from PCS2-Chfr plasmid and subcloned in the pYFP-C1 vector in the BglII-Sall site.

Cell culture and synchrony

HeLa cells, were grown in DMEM medium (Gibco BRL, N.Y), supplemented with 3 mM 4-(2-hydroxyethyl) piperazineethane sulphonic acid (HEPES) and 10% (v/v) Fetal Calf Serum (Invitrogen). Thymidine block/release synchronizations were performed as previously described (Gabrielli *et al.*, 1996). XL2 cells were cultured in L-15 media supplemented with

10% FCS, L-glutamine, and HEPES. Cells were synchronised by blocking for 30 h with 2 g/ml aphidicolin, and releasing into fresh media following 3 washes (Uzbekov *et al.*, 1998).

Flow Cytometry

Flow cytometry (FACS) was performed as previously described (Burgess *et al.*, 2006). The stained cells were acquired on a FACS Calibur (Becton Dickinson, NJ) using Cell Quest Pro software. Cell cycle distributions of YFP positive cells were analysed using Cell Quest Pro software, the YFP FACS fluorescence signal was divided in three different areas corresponding to G1, S and G2/M with respect to the DNA content, and the cells in each area were counted and expressed as the percentage of total YFP positive population.

Live Cell Imaging

HeLa cells seeded in 6 well plates were transfected using JetPei as per the manufacture's protocol, with YFP-WT Chfr, and then subsequently synchronised in G1/S with 2.5 mM thymidine. Upon release cells were filmed with a Leica DMIRE2 inverted microscope fitted with a Leica 20X N PLAN L 0.4 LMC lense, and Micromax YHS 1300-4 camera, controlled by Metamorph 7.1 software (Molecular Devices). Images, DIC and YFP fluorescence, were taken every hour for 25 h, with the total fluorescence measured for each cell with Metamorph 7.1 software.

Site-directed mutagenesis.

Deletions were obtained by using the QuickChange site-directed mutagenesis kit according to the manufacturer's protocol (Stratagene, Amsterdam, The Netherlands).

Two-hybrid analysis

A cDNA library was constructed with human mRNAs isolated from three complete 6 to 8-week-old human embryo (Zhou et al., 2002). cDNA library was then transformed into MATa Y187 yeast strain.

The human Chfr bait plasmid was constructed in frame with a downstream GAL4 DNA binding domain in plasmid pGBT9, and then transformed into MATa CG1945 yeast cells. Yeast two-hybrid screening was conducted as previously described (Fromont-Racine et al., 1997). 215 positive clones on the histidine depleted plates and 45 blues in the B-Gal test were obtained. A PCR reaction directly on the yeast clones and sequenced the product.

Immunoprecipitation and Xenopus XL2 and egg extracts

Immunoprecipitations were carried out using 20 μ l of magnetic Protein A-Dynabeads (Invitrogen) and 3 μ g of each antibody. Beads were washed and incubated for 30 min at room temperature with 20 μ l of Xenopus egg extracts.

For hypotonic lysis, cells were swollen for 10 min on ice in hypotonic lysis buffer (20 mM Tris, 1 mM EDTA, 1 mM EGTA), lysed by 30 strokes with a 1 ml syringe and 26-gauge needle, spun at 2500 rpm for 10 minutes and the supernatant collected (soluble cytosolic fraction). The nuclear pellet was washed once with hypotonic buffer and extracted with the same buffer containing 400 mM NaCl and 0,25 % IGEPAL CA-630 (Sigma). All buffers were supplemented with fresh 1 mM NaF, NaVO₄, and PMSF, and complete Protease Inhibitor Cocktail Tablets (Roche), and in all cases, Bradford (Sigma) protein estimations were performed to ensure equal protein loading. CSF and interphase egg extracts were prepared from Xenopus eggs as previously described (Murray and Kirschner, 1991).

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Titles and legends to figures

Figure 1. Endogenous Chfr is ubiquitinated and phosphorylated in an MPF-dependent manner in mitotic *Xenopus* egg extracts. **(a)** Western blot analysis (WB) of Chfr programmed reticulocyte lysate (2 μ l), interphase (INT) and CSF *Xenopus* egg extracts (2 μ l), as well as of total XL2 cell lysate (40 μ g) was performed with antibodies directed against the full length Chfr protein (Anti-Chfr), the first 12 N-terminal amino acids (Anti-Nter) and the last 12 C-terminal amino acids (Anti-Cter). CSF and interphase *Xenopus* egg extracts (20 μ l) as well as XL2 cell lysate (300 μ g of total protein) were immunoprecipitated with either anti-Chfr (IP-Chfr), anti-Cter (IP-Cter) or with anti-Nter (IP-Nter) antibodies. 2 μ l of CSF and interphase extracts and of the supernatant, as well as the whole amount of the immunoprecipitate, were analysed by western blot. 30 μ g of total XL2 cell lysate and supernatant, as well as an equivalent to 100 μ g of the immunoprecipitate, were analysed by western blot with anti-Chfr antibodies. **(b)** 20 ml of CSF extracts were immunoprecipitated with anti-Chfr antibodies. The immunoprecipitate was divided into three parts. One third was used for western blot analysis with anti-Chfr antibodies and the rest was stained with Coomassie Blue. The indicated bands were excised and treated to be sequenced by MALDI-MS-MS. **(c)** CSF extracts were incubated with either cycloheximide (CSF+CHX) or MG132 (CSF+MG132) to a final concentration of 100 μ g/ml and 1 mM respectively and 2 μ l of the mix were removed at the indicated time points and used to analyse Chfr levels by western blot. **(d)** G2-arrested oocytes were treated with progesterone and supplemented (+CHX) or not (-CHX) with cycloheximide 30 minutes after GVBD. Three oocytes per point were taken and lysed. The volume corresponding to one oocyte was then used to examine Chfr and cyclin B by western blotting. Asterisk denotes a non-specific band recognised by the anti-cyclin B antibody.

Figure 2. Endogenous Chfr stability and localization in *Xenopus* cells. **(a)** *Xenopus* XL2 cells were synchronized at G1/S by 30 h aphidicolin block. Cells were taken at the indicated times after release and analysed by FACS and by western blot to determine cell synchronization and to examine the levels of Chfr. **(b)** XTC cells were fixed with 3.7% formaldehyde, permeabilised with 0.25 % Triton X100 and blocked with PBS-5% foetal bovine serum. Subsequently coverslips were incubated with anti-Chfr antibody (affinity purified, 4 μ g/ml) and anti- β -tubulin (mAB, 1:10). Anti-mouse Alexa 488 and anti-rabbit Alexa 555-conjugated 2° antibodies (1/600 and 1/1000, respectively, Molecular Probes, Leiden, The Netherlands) and DAPI (10 μ g/ml) were used. Scale bar, 5 μ m. **(c)** Asynchronous XL2 cells (300 μ g) were lysed as described in Materials and methods and the cytosolic soluble fraction (Cy) and the nuclear fraction (Nu) were immunoprecipitated with anti Chfr antibodies. The immunoprecipitates were then submitted to western blot to analyse Chfr and β -tubulin levels in each fraction.

Figure 3. Ectopic Chfr stability and localization in XL2 and HeLa cells. **(a)** Asynchronous cells were transfected with full length YFP-Chfr for 24 h before analysis by immunofluorescence. **(b)** XL2 cells were transfected with the YFP or YFP-Chfr constructs, synchronized by aphidicolin block and subsequently recovered at 10 h after block release to analyse DNA content of total and YFP positive cells by FACS. Grey line profile indicates gated over-expressing cells **(c)** Asynchronous HeLa cells were transfected with a wild-type-YFP (WT) or a ring finger-YFP mutant (Δ RFD) of Chfr and synchronised by thymidine block at G1/S phase. Samples were then taken at the indicated times to measure the levels of the overexpressed YFP proteins by western blot. Protein levels were quantified by densitometry, normalized with respect to β -tubulin levels and plotted at the different times after thymidine release. **(d)** HeLa cells were transfected with YFP-WT or YFP- Δ RFD Chfr and treated with

(MG +) or without (MG -) the proteasome inhibitor MG132 (12.5 μ M) for 24 hours. The levels of the two overexpressed proteins, as well as the levels of β -tubulin were subsequently analysed by western blot. (e) The percentage of YFP positive cells at each phase of the cell cycle was obtained by analysing FACS results and were compared to the cell cycle distribution of the total cell population.

Figure 4. Low level Exogenous Chfr is stable throughout the cell cycle and is present at the mitotic spindle. (a) Synchronised HeLa cells transfected with WT YFP-Chfr were followed by time-lapse video microscopy for 25 h. Shown is a typical high over-expressing cell that remained in interphase for the duration of the time-course with the shift from a diffuse to foci nuclear staining. (b) A typical low Chfr expressing mitotic cell. Arrows indicate the spindle localisation of Chfr in the, with the expected orientation of the condensed DNA at metaphase plate clearly visible in the corresponding DIC field. (c) The total YFP-Chfr mean fluorescence for 3 cells, was calculated for either cells that progressed through mitosis (mitotic), remained in interphase for the 25 h time period (interphase) or were treated with MG132. (d) The diffuse and speckled foci localisation of WT Chfr was analysed for interphase, mitotic and MG132 treated cells (n=25 for interphase and MG132, n=6 for mitotic). (e) The peak mitotic fraction (10 h) of synchronised HeLa cells transfected with Chfr-YFP was analysed by 2-D FACS. Transfected cells were gated according to their level of expression (none, low, medium, high) and the cell cycle distribution determined for each sub-fraction.

Figure 5. TCTP binds to Chfr in Xenopus egg extracts and XL2 cells. (a) Affinity-purified anti-full length TCTP antibodies (Anti-TCTP) were used to probe CSF and interphase Xenopus egg extracts (2 μ l) as well as XL2 cells (40 μ g total protein). 20 μ l of CSF extract was immunoprecipitated with anti-TCTP antibodies. An equal amount of CSF and

supernatant (2 μ l) as well as the total immunoprecipitate (corresponding to 20 μ l of extract) was loaded in the gel and probed with anti-TCTP antibodies. **(b)** Side-by-side analysis by western blot of consecutive fractions (30 μ l loaded), after chromatography of a CSF extract by gel filtration on Superdex 200 column. Upper panel: blot probed with affinity-purified antibodies against Chfr. Lower panel: blot probed with affinity-purified antibodies against TCTP. **(c)** XL2 cells (300 μ g total protein) or CSF extracts (20 μ l) were immunoprecipitated with either full length Chfr (Chfr IP), anti-N terminal Chfr (Nter IP) or anti-C terminal Chfr antibodies (Cter IP). An equal amount of supernatants and CSF extracts (2 μ l) and XL2 cell lysate (40 μ g) and the total amount of the immunoprecipitate were loaded and probed against Chfr and TCTP. To confirm the specificity of the interaction, Chfr immunoprecipitations were performed on 20 μ l of interphase extract (Int), and on 20 μ l of Chfr depleted interphase extract (Dep). For western blot analysis of β -tubulin and TCTP samples were re-suspended in Laemmli buffer without β -Mercapto-ethanol ($-\beta$ -M) and no boiling was performed, in order to prevent the migration of IgG. Under these conditions, TCTP and β -tubulin present an abnormal electrophoretic mobility. For Chfr visualisation samples were boiled in the presence of β -M. **(d)** XTC cells were treated as described in Figure 2b except that anti-TCTP instead of anti-Chfr primary antibodies were used. Scale Bar, 5 μ m.

Figure 6. Ectopic Chfr does not bind endogenous TCTP. **(a)** XL2 cells were synchronized by aphidicolin block. After block release, cells (300 μ g total protein) were taken at the indicated times and used for anti-Chfr immunoprecipitation. The total immunoprecipitates were loaded and used to analyse by western blot the levels of Chfr and TCTP. **(b)** XL2 cells were synchronized by aphidicolin block and treated with Nocodazole (1 μ g/ml) 4 hours after release. The mitotic index was measured at different times post aphidicolin release in control and Nocodazole treated cells. A sample of 300 μ g of total protein content of XL2 lysate was

obtained at 10 hours after aphidicolin release in cells treated (Ct) or not with Nocodazole (Noc) and was submitted to immunoprecipitation with anti-Chfr antibodies to analyse the levels of Chfr and TCTP in the immunoprecipitates. (c) XL2 cells (300 µg total protein) were immunoprecipitated with anti-Chfr antibody and the immunoprecipitate was washed with either hypotonic buffer (20 mM Tris, 1 mM EDTA and 1 mM EGTA), or the same buffer containing either 150, 500 mM NaCl, 1% IGEPAL-C630 or 0,1% SDS. After washing 3 times the immunoprecipitates were probed with anti-Chfr, anti-TCTP and anti-β tubulin. (d) 30 µl of *Xenopus* interphase extracts were treated with (Noc) or without (Con) 10 µM Nocodazole on ice for 30 min. Chfr immunoprecipitations were then performed on 20 µl of the treated extracts. For visualisation of β-tubulin, TCTP and Chfr, samples were treated as per Figure 5c.

Supplementary Data

Figure S1. Retardation of the electrophoretic mobility of Chfr in CSF extracts is due to protein phosphorylation. CSF extracts (2 µl), containing high cyclin B/cdk1 activity, were treated (+) or not (-) with λ Phosphatase (λ -P) for 60 minutes and then loaded side-by-side with the same volume of Interphase extract (Int), with low cyclinB/cdk1 activity and submitted to western blot to detect Chfr.

Figure S2. Time-lapse video microscopy of synchronised HeLa cells expressing high levels of WT Chfr-YFP. The un-altered absolute fluorescence and the DIC images are shown for two cells that remain in interphase for the duration of the time-course.

Figure S3. Time-lapse video microscopy of synchronised HeLa cells expressing low levels of WT Chfr-YFP. The un-altered absolute fluorescence and the DIC images are shown for a low expressing cell undergoing cell division. The inset image at 12 h is the optimised mitotic figure clearly showing a distinct localisation of Chfr to the mitotic spindle.

Figure 1

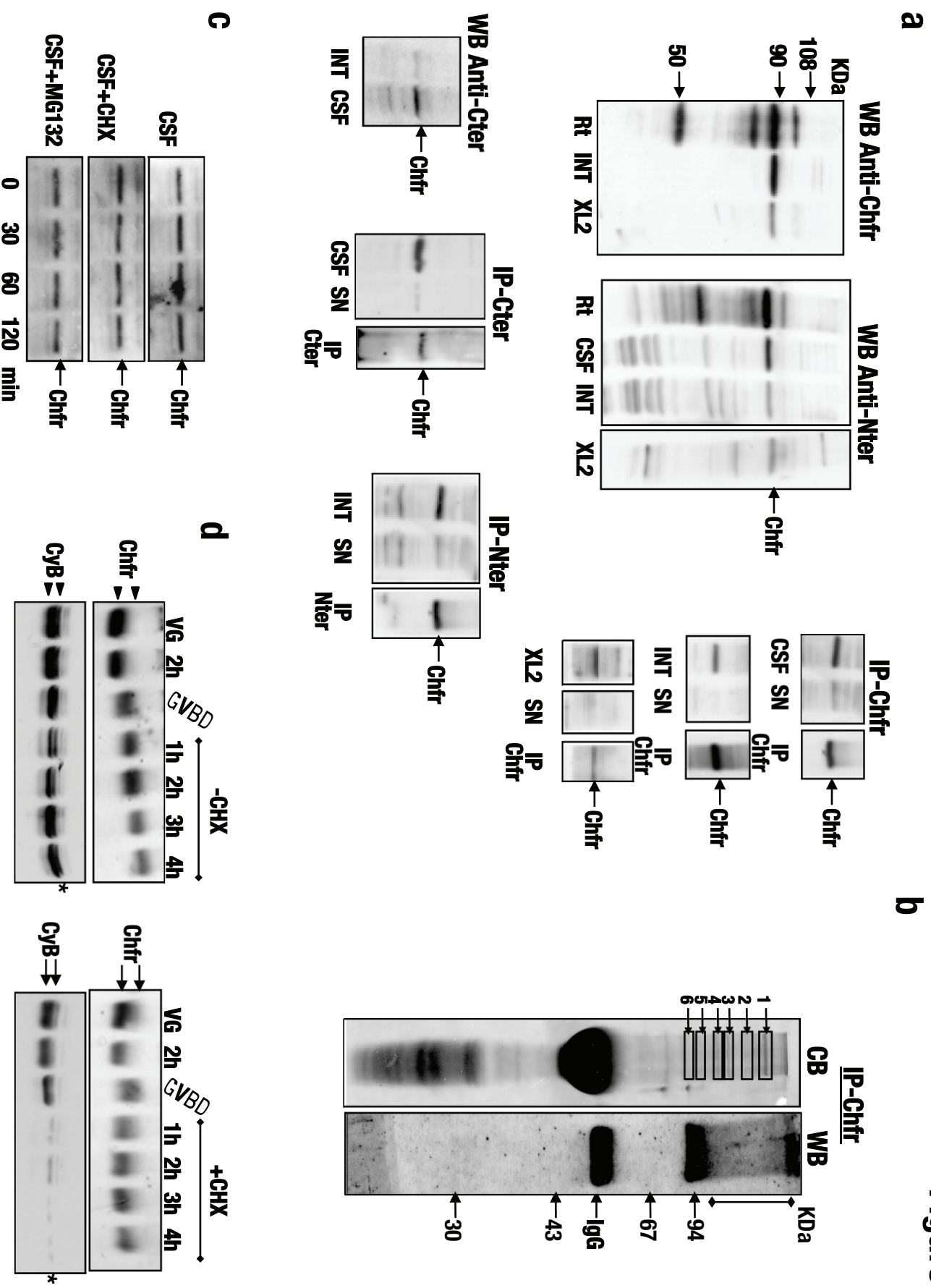
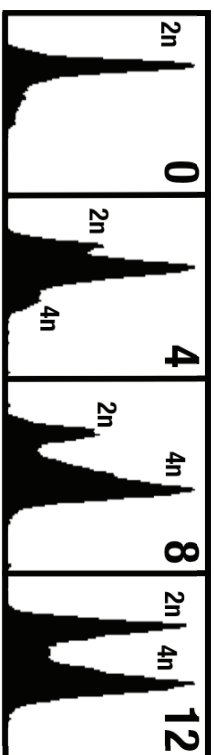
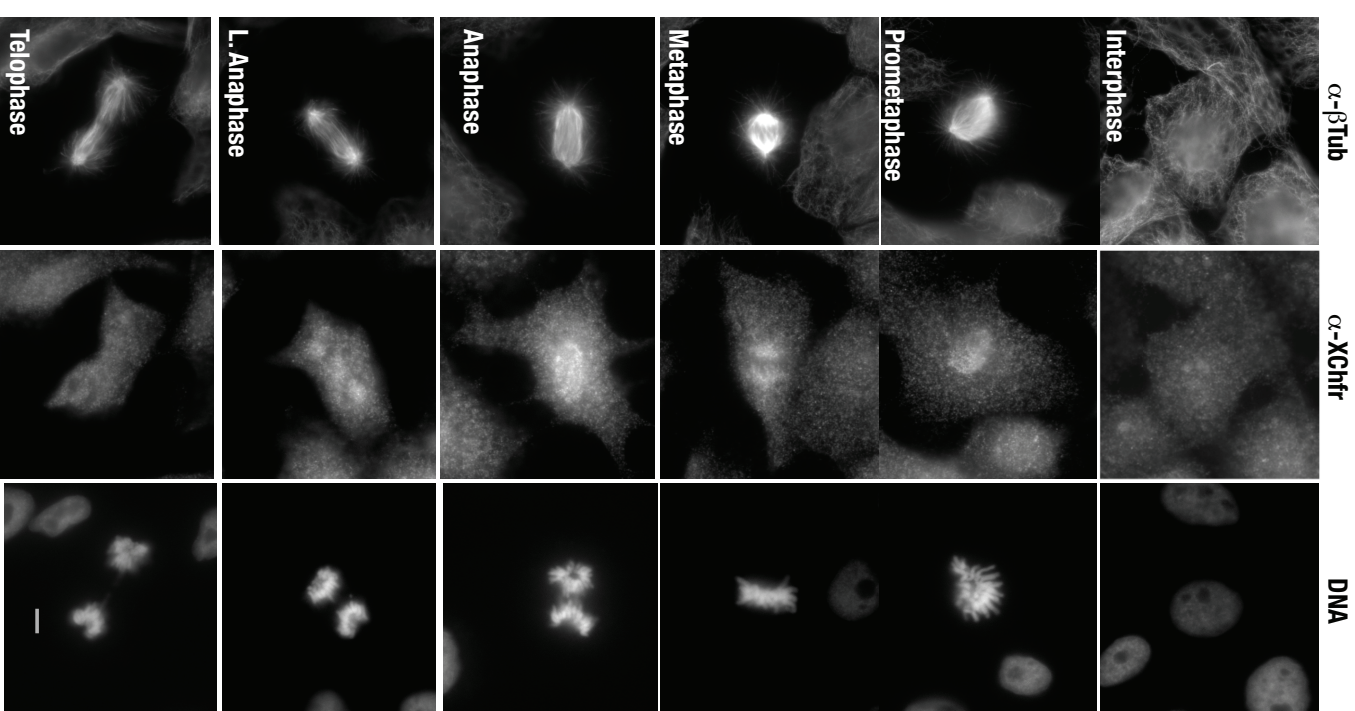


Figure 2

a



b



c

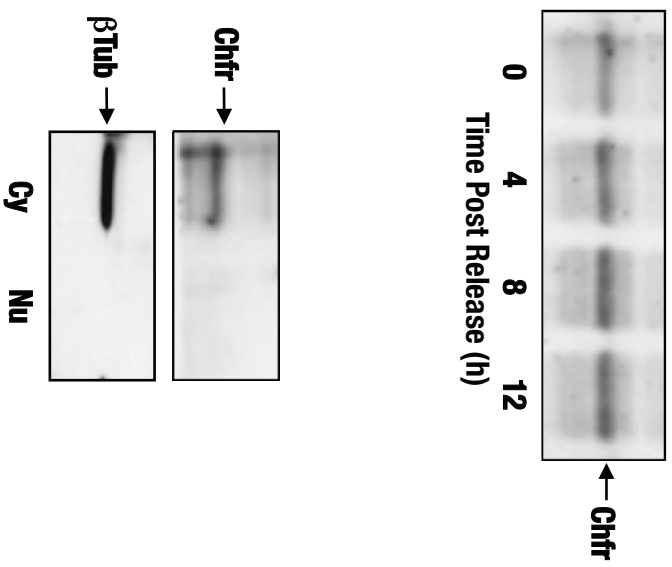
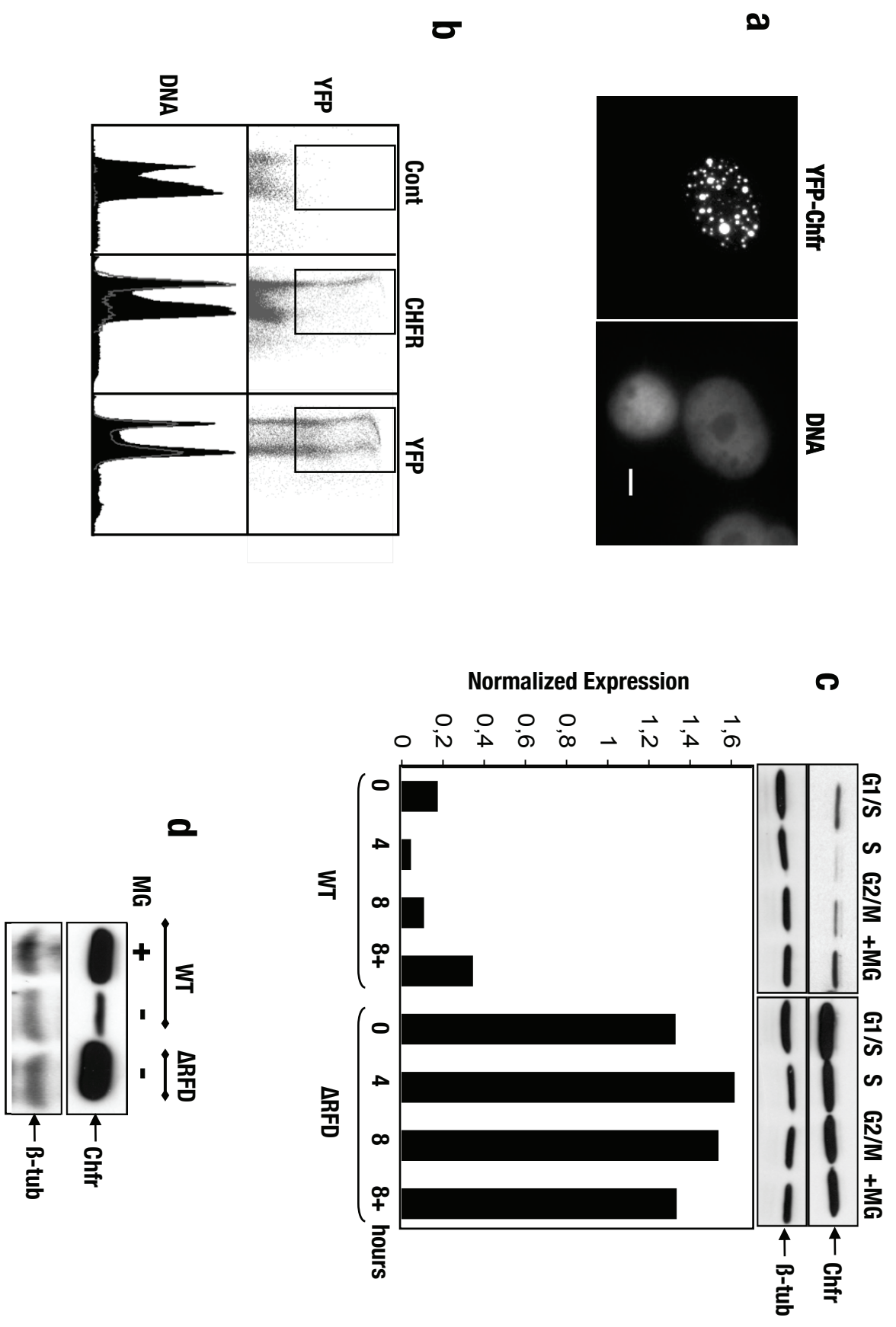


Figure 3



6

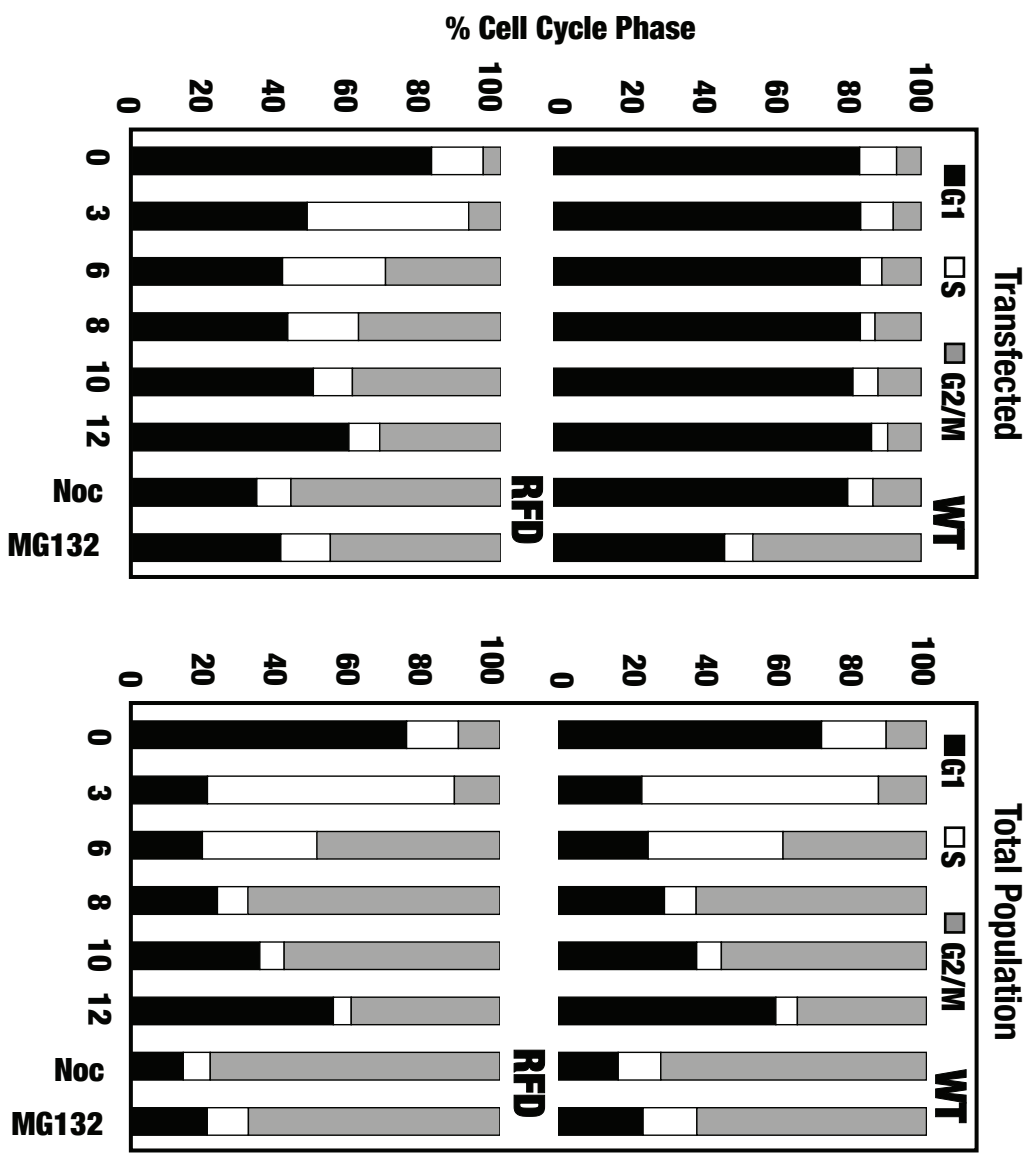


Figure 3

a

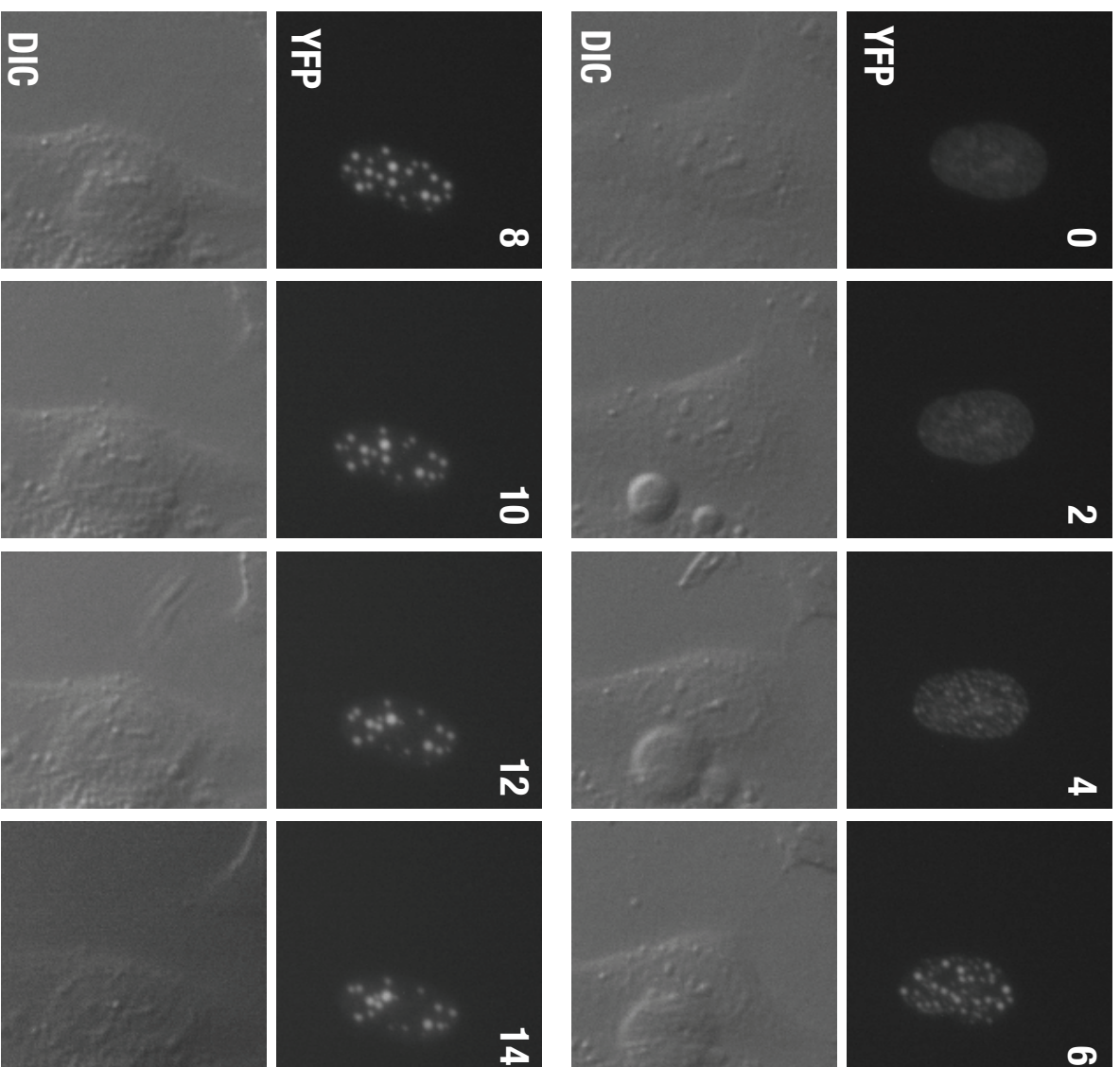


Figure 4

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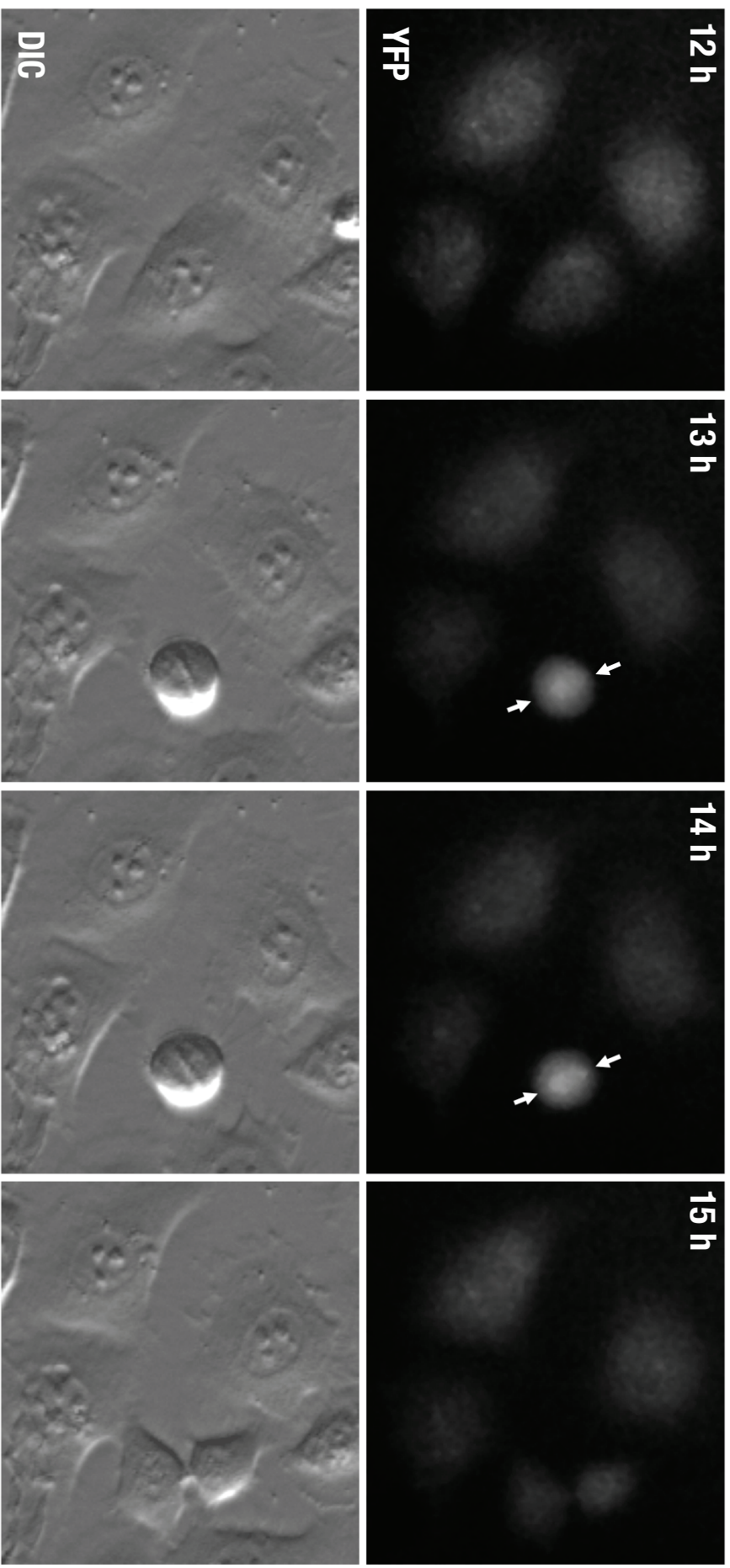


Figure 4

Figure 4

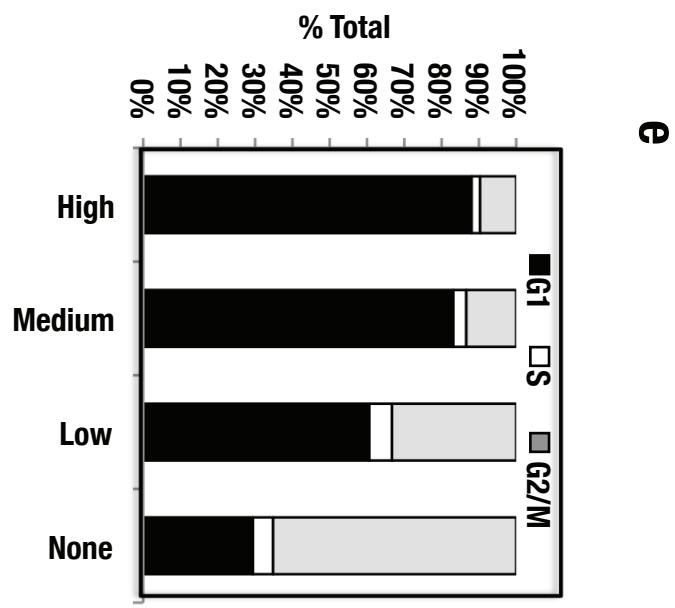
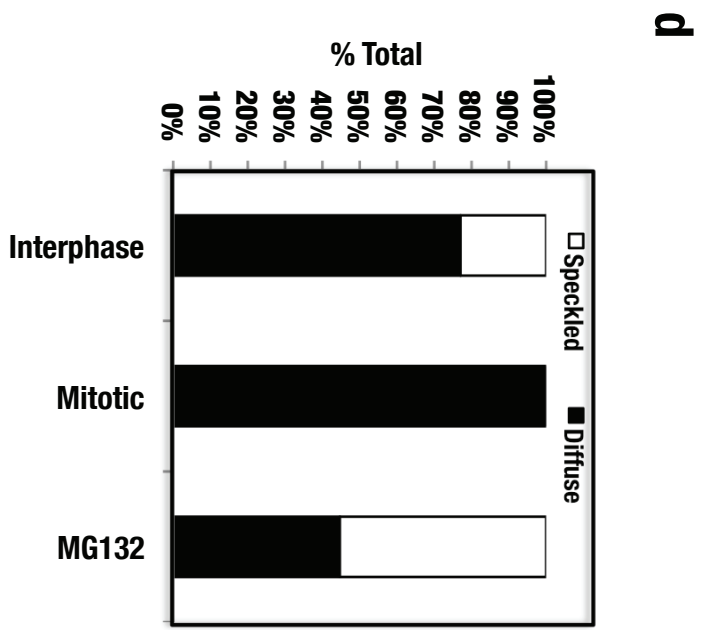
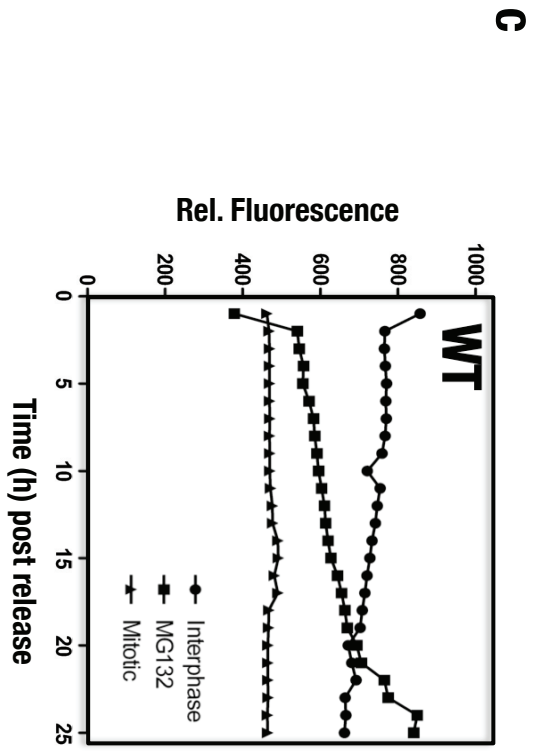
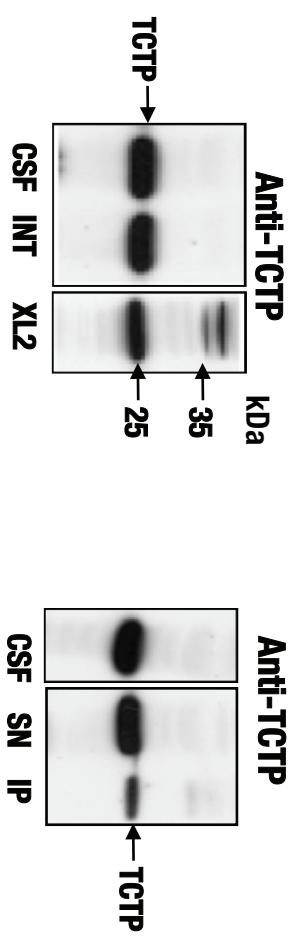


Figure 5

a



b

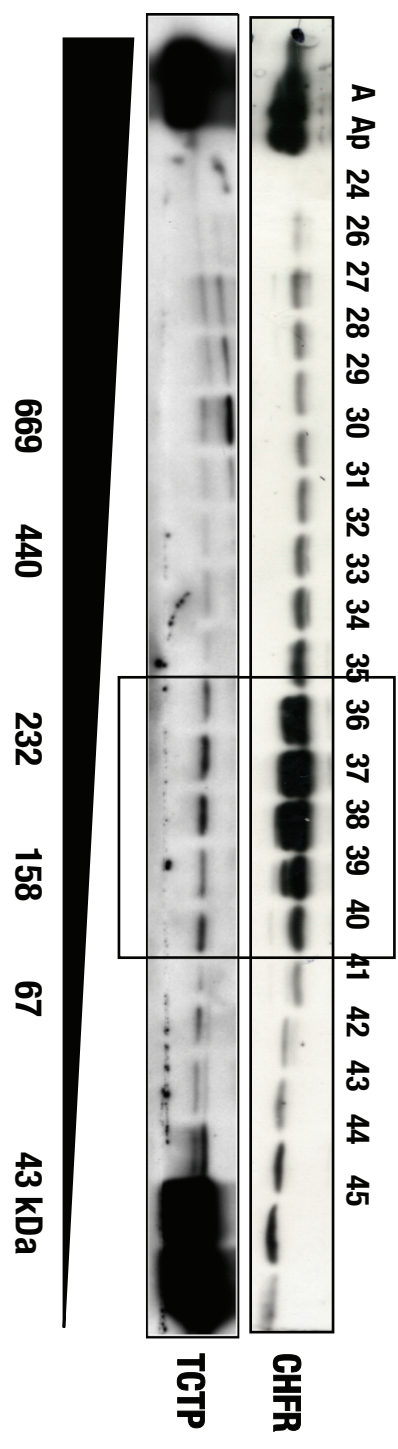
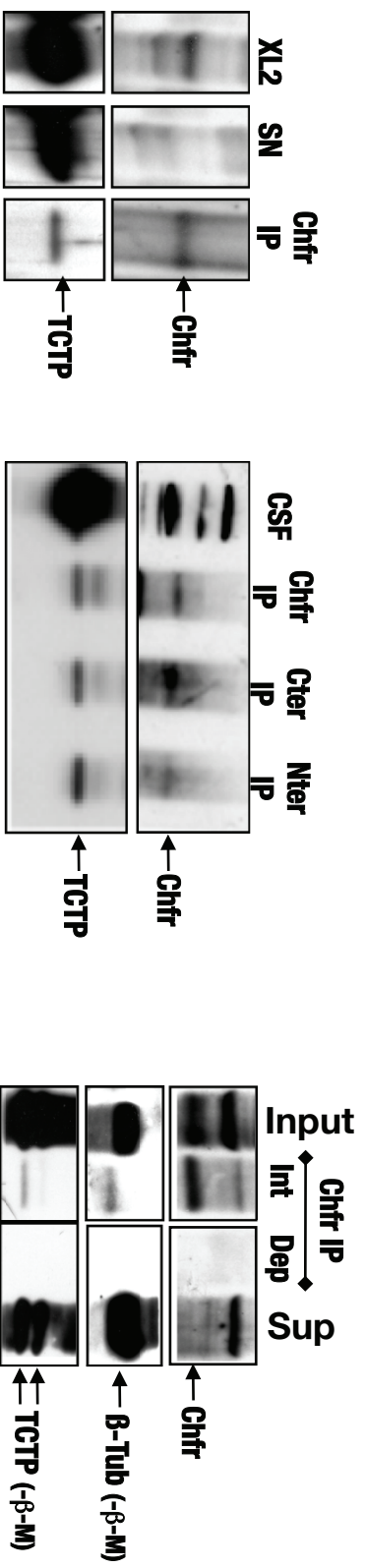


Figure 5

c



d

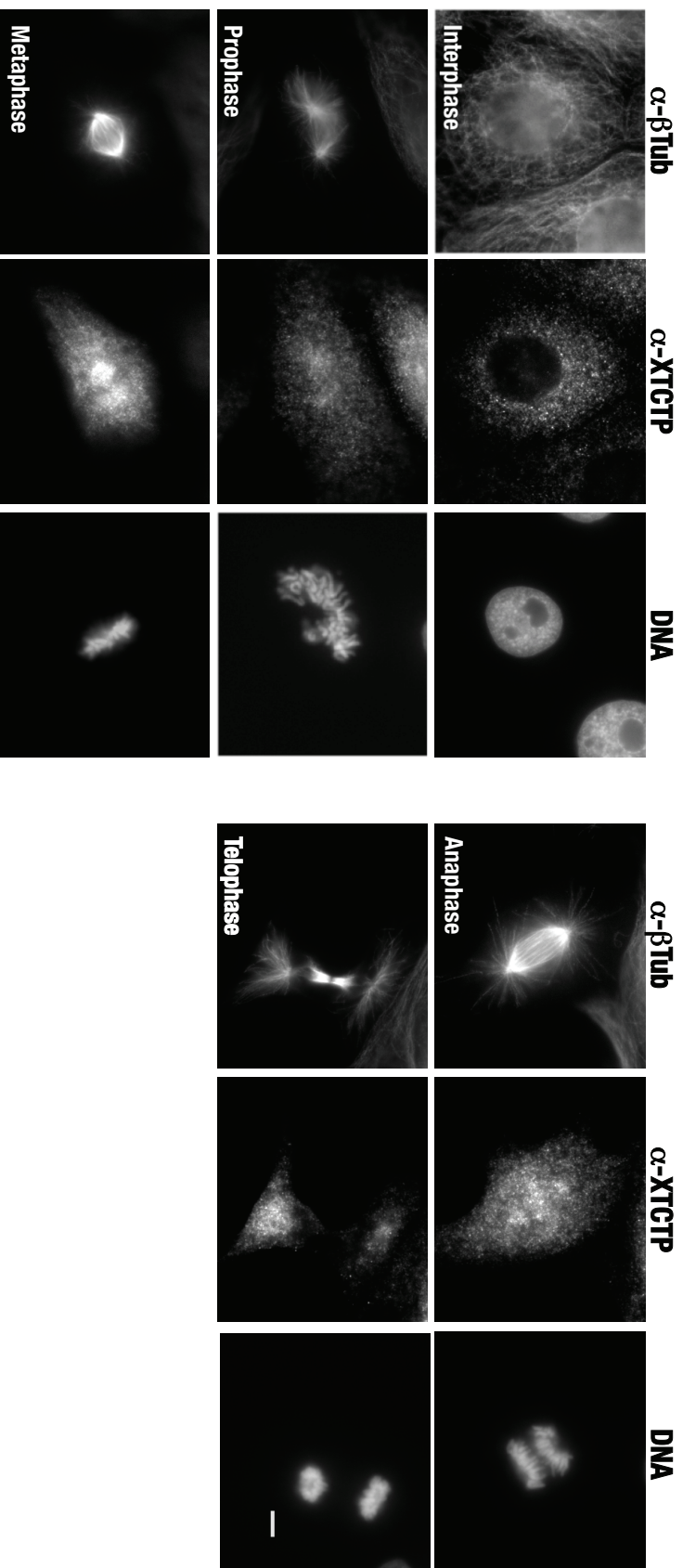


Figure 6

