Regulation and Function of the Mitotic Checkpoint Protein CHFR

by

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DEDICATION

Dedicated to my parents, Maria and Ken Keller

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I have been blessed to have many people in my life who have supported me through my studies and my life. First of all, my family: my parents, Maria and Ken Keller, have always supported me in any endeavor. They taught me to take responsibility for things around me, and to do my best even when failure seems imminent. These things were critical to the completion of this degree. My sister, Robin, has always been there for me, and I am so proud and lucky to have such a talented, smart, and strong sister. My brother, Adam, never fails to impress me with his intellect, as well as his thoughtfulness. My grandparents, Warren and Grace Keller, and Paulino and Ignazia Liuzzo have been and continue to be an inspiration to me. And of course, I'd like to acknowledge my fiancé, Chris, who has been a great source of support and calm when things have seemed out of control.

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GLOSSARY OF ACRONYMS USED

APC	Anaphase Promoting Complex
BCC	Breast Cancer Cell
BUBR1	Budding Uninhibited by Benzimidazoles Related 1
CDC20	Cell Division Cycle 20
CDK1	Cyclin Dependent Kinase 1
CHFR	Checkpoint with Forkhead-associated and RING domains
DMBA	Dimethylbenz(a)anthracene
ERα	Estrogen Receptor-α
EZH2	Enhancer of Zeste Homolog 2
FHA	Forkhead-associated domain
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HDAC1	Histone Deacetylase 1
HLTF	Helicase-like Transcription Factor
IHMEC	Immortalized Human Mammary Epithelial Cell
IL-8	Interleukin 8
KEN	a three residue domain : lysine-glutamic acid-asparagine (K-E-N)
Kif22	Kinesin Family Member 22
MAD2	Mitotic Arrest-deficient 2
MEF	Mouse Embryonic Fibroblast
MMS2	MMS (Methyl Methanesulfonate) sensitivity 2
miRNA	microRNA (also designated miR)
mRNA	messenger RNA
NSCLC	non-small cell lung cancer
NF-κB	nuclear factor κB
PAI-1	plasminogen activator inhibitor 1
PAR	Poly(ADP-ribose)

PARP	Poly(ADP-ribose) polymerase
PBZ	Poly(ADP-ribose)-binding Zinc Finger
PLK1	Polo-like Kinase 1
RING	Really Interesting New Gene
RNF8	Ring Finger Protein 8
shRNA	short hairpin RNA
siRNA	small interfering RNA
SIRT2	Sirtuin 2
Stil	SCL/TAL1 Interrupting Locus
TCTP	Translationally Controlled Tumor Protein
UBC	Ubiquitin Conjugating Enzyme
UTR	Untranslated Region

ABSTRACT

Regulation of mitosis through mitotic checkpoints is critical to prevent propagation of DNA damage and to ensure proper DNA content of the resulting daughter cells. Loss of these checkpoint functions may lead to neoplasias or cancers. The protein checkpoint with forkhead associated and RING domains (CHFR) has been implicated as a tumor suppressor in a multitude of cancers. Originally identified as a major component of the antephase checkpoint, CHFR has recently been associated with the spindle assembly checkpoint through its interaction with MAD2. To further understand the role of CHFR in this checkpoint, we deleted key functional domains from the CHFR protein and investigated the effects on MAD2 binding and function. We found that the C-terminal cysteine-rich domain of CHFR is required for the CHFR/MAD2 interaction. In addition, this domain is important for MAD2 localization, interaction with CDC20, and prevention of chromosome segregation defects. These data indicate an important role for CHFR in the function of MAD2 and the spindle assembly checkpoint.

CHFR loss is observed in a wide array of cancers, supporting its role as a tumor suppressor. Most often, CHFR is lost via hypermethylation of the *CHFR*

gene promoter. However hypermethylation is not observed in the majority of breast cancers. Using a panel of breast cancer lines we explored the role of microRNA in reducing CHFR levels. We found a correlation between expression of miR-26 and decreased transcription of *CHFR*, suggesting that this miRNA could target the *CHFR* mRNA to reduce protein levels. These data suggest that miR-26 could be useful in the future as a biomarker indicating CHFR protein loss.

CHAPTER 1

Introduction

Identification of cancer related genes and proteins that can serve as diagnostic, prognostic, and/or therapeutic biomarkers is essential to improve targeted management of cancer. Because of the diversity of molecular pathways by which cancers arise, even within cancers of similar cellular origin, many markers, or combinations of markers will likely be required to accurately profile each case of cancer for accurate diagnosis and effective treatment. CHFR (Checkpoint with Forkhead-associated domain and Ring finger), a cell cycle checkpoint regulator, has been attracting increased attention as a potential tumor suppressor and predictor of chemotherapy response. CHFR was first reported in 2000 as the result of a screen to identify cell cycle checkpoint proteins containing forkhead-associated (FHA) domains [1]. The identification of CHFR was most significant in that it defined a novel cell cycle checkpoint occurring at metaphase entry in response to microtubule stress brought on by nocodazole treatment [1]. CHFR was expressed in all normal human tissues tested, further supporting its important role in cell cycle control [1]. In addition, the authors found mutation or loss of *CHFR* expression in 50% of their tested cancer cell lines, implicating CHFR as a tumor suppressor [1]. Interestingly, the mitotic index of cell lines

lacking CHFR was significantly higher than the lines that retained CHFR expression, and re-introduction of *CHFR* into CHFR-lacking lines significantly reduced the mitotic index in these cells [1]. Since its discovery, CHFR has been the focus of extensive research to determine the cellular and clinical impact of its function, as well as the mechanism by which CHFR regulates the cell cycle.

Decreased CHFR expression has been observed in a wide array of cancer types ranging from breast to urogenital tumors, suggesting that it has tumor suppressor-like properties [2]. In addition, the *CHFR* gene is located on chromosome 12q24, a site identified as a location of frequent chromosome rearrangements and allelic imbalance in some cancers [3]. Most often, reduced CHFR expression results from increased methylation of its gene promoter [2] but some cancers demonstrate CHFR loss without evidence of promoter hypermethylation [4]. Loss of CHFR expression has been shown to confer cellular characteristics associated with cancer, such as an accelerated growth rate, enhanced cell motility, increased invasiveness, and higher rates of aneuploidy [2, 3, 5], which likely result, at least in part, from failed checkpoint arrest in cells that may already lack normal apoptotic mechanisms. Accordingly, *Chfr* knockout mice have increased incidence of spontaneous and DMBA (7,12-dimethylbenz(a)anthracene)-induced tumors [5]. The predisposition of *Chfr*$^{-/-}$ mice to tumors indicates that CHFR indeed functions as a tumor suppressor *in vivo*, and underscores the importance of CHFR in cancer progression. Further understanding of CHFR regulation and function will likely lead to more detailed

diagnoses and better cancer treatments. To this end, we undertook several studies of CHFR, two of which are described in this thesis. In one, we explored a novel mechanism for the regulation of *CHFR* expression in breast cancer lines where CHFR is lost. In the other, we chose to look closely at the functional domains of the CHFR protein and their role in the mitotic checkpoint function of CHFR.

CHFR Protein Structure

CHFR contains five identified domains as illustrated in Figure 1.1. The combination of FHA and RING domains is found in only one protein other than CHFR, namely RNF8 (Ring Finger Protein 8) [6]. The cysteine-rich domain, including the PBZ (Poly(ADP-ribose)-binding Zinc Finger)domain, is unique to CHFR among FHA-RING proteins [2, 6]. The known functions for each domain are discussed below.

The forkhead-associated domain

CHFR was initially identified in a screen for proteins containing the forkhead-associated (FHA) domain [1]. The FHA domain is known to bind to phosphorylated threonine residues, and is found in proteins with several cellular functions, notably several cell cycle checkpoint proteins [6-8]. The FHA domain of CHFR has been shown to be critical for its checkpoint function, as a FHA deletion allele behaves as a dominant negative, increasing mitotic index in cells

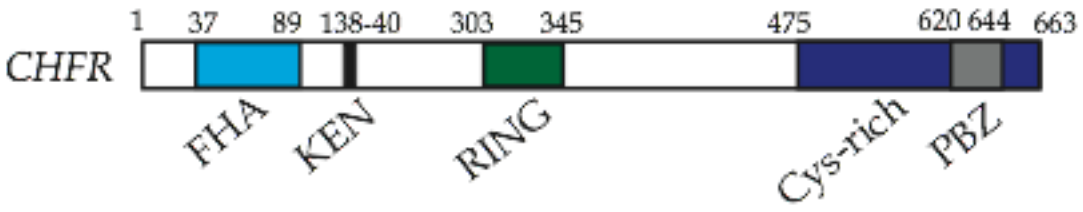

Figure 1.1. The domains of CHFR. CHFR protein contains several domains. The FHA domain is a phospho-threonine binding domain. The KEN box domain may be involved in cell cycle stage-dependent degradation. The RING domain is critical for the E3 ubiquitin ligase function of CHFR. The cysteine-rich domain has been identified as the site of CHFR target protein binding, and contains the PBZ domain, a domain shown to bind PAR and possibly involved in CHFR substrate recognition.

that have been under mitotic stress [1, 9]. A splice variant of *CHFR* in which the exons encoding the FHA domain are deleted is expressed in very low levels in normal human tissues, but is highly expressed in cancer cell lines [10] (Figure 1.2). In addition, the FHA domain has been reported to be essential for proper nuclear localization of CHFR [11]. Structural analyses of the FHA domain of CHFR indicate that it is likely to bind phosphorylated substrates as predicted [12], however no binding partner has yet been identified. As the function of this domain is critical to the checkpoint function of CHFR, the target of the FHA domain on CHFR is an important unknown in the understanding of CHFR function.

The KEN box domain

CHFR contains a putative KEN box domain, defined as the amino acid sequence lysine-glutamic acid-asparagine (K-E-N) [13, 14]. This putative motif is especially interesting for CHFR studies because it has been identified as a targeting signal for the anaphase promoting complex (APC) and is enriched in cell cycle proteins [13, 14]. The APC is a ubiquitin ligase thought to promote mitotic progression by degradation of its target proteins [14]. As there is some evidence that CHFR protein levels change throughout the cell cycle [15]. The presence of the KEN box domain on CHFR raises the possibility of cell stage-specific degradation as a mechanism of CHFR regulation. However, an interaction between APC and CHFR has not yet been demonstrated, and some

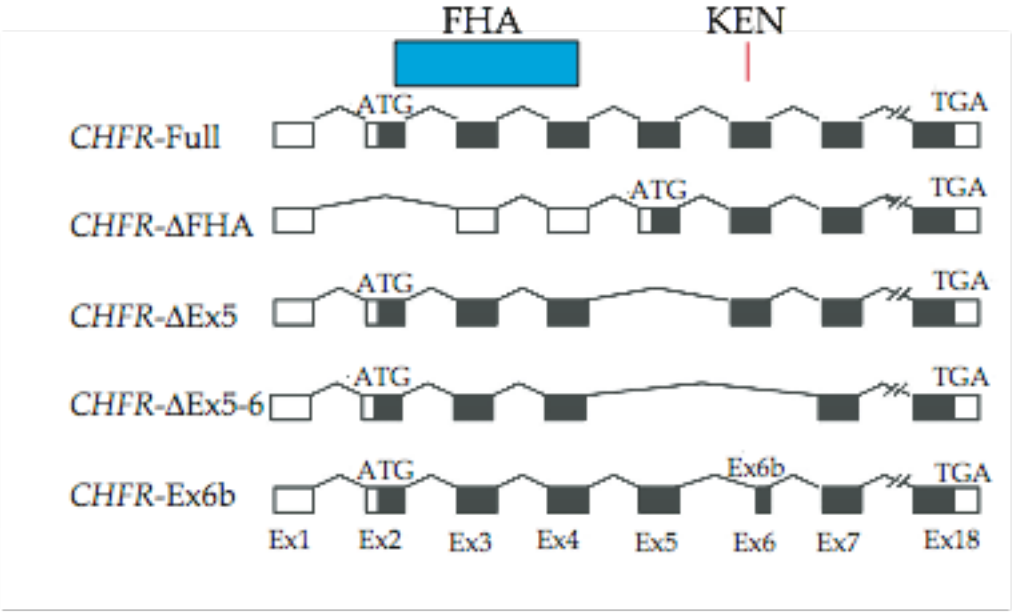

Figure 1.2. *CHFR* splice variants. Full length *CHFR* is illustrated, with splice variants of *CHFR* below it. Locations of FHA and KEN box domains, which are lost in some splice variants, are indicated above. RING, cysteine-rich and PBZ domains are preserved in all of the splice variants. Adapted from Toyota *et al* [10]

evidence suggests that KEN box domains following the KENxP motif are the relevant domains for APC targeting [16]. However, CHFR does not fit this extended motif (the sequence in CHFR is KENVF), and two splice variants of CHFR (ΔEx6 and Ex6b) do not include the KEN box (Figure 1.2). More work is required to determine the contribution of this motif to the cellular function of CHFR.

The RING domain

CHFR contains a RING finger (RING) domain, a domain commonly found in ubiquitin ligase proteins, which is required for CHFR E3 ubiquitin ligase activity [15, 17]. As with many E3 ligase proteins, CHFR is reported to interact with multiple E2 ubiquitin conjugating enzymes. UBC4 and UBC5 were identified as E2 conjugating enzymes with which CHFR could auto-ubiquitinate, and UBC4 also functioned with CHFR to ubiquitinate PLK1 (Polo-like Kinase 1), which was then degraded [17]. CHFR was also shown to act in complex with UBC13-MMS2 *in vitro*, forming noncanonical Lys63-linked ubiquitin chains [18]. Lys63-linked ubiquitin chains generally do not target proteins for degradation, but rather act as signaling modifications [18]. In addition, the RING domain, and therefore the ubiquitin ligase activity of CHFR, is required for the checkpoint function of CHFR, as deletion of the RING domain resulted in reduced viability of cells treated with taxol to induce microtubule stress [15]. Thus, the RING

finger of CHFR represents a critical domain for CHFR molecular and checkpoint functions.

The cysteine-rich domain

The C-terminus of the CHFR protein contains a large cysteine-rich domain which is unique to CHFR among FHA-RING containing proteins [1]. This domain appears to confer substrate specificity to CHFR as it has been identified as the domain required for its interaction with ubiquitination targets Aurora A, Kif22, HDAC1 and HLTF [5, 19-21]. The cysteine-rich domain contains zinc-binding motifs including a PBZ often found in DNA-damage response and checkpoint proteins [22, 23]. This domain is further described below.

The PBZ domain

The cysteine-rich domain of CHFR contains a poly(ADP ribose)-binding zinc finger (PBZ domain) [22, 23]. Poly(ADP ribose), or PAR, is a protein modification that is enriched at the mitotic spindle and at sites of DNA damage [24]. PAR is involved in the proper formation of the mitotic spindle, as well as mitotic spindle function [25, 26]. Mutation of the PBZ domain disrupts the early mitotic checkpoint function of CHFR [22]. Additionally, chemical inhibition of poly(ADP ribose) polymerase also disrupted the CHFR checkpoint, suggesting that this checkpoint function depends on CHFR recognition of ADP-ribosylated substrates [22]. Of interest, mutation of the PBZ domain abolished the dominant

negative activity of FHA-deleted CHFR [22]. This effect lends support to the hypothesis that the PBZ domain is the critical domain for CHFR substrate targeting, specifically after PAR modification. As stated above, several CHFR substrates have been found to interact with CHFR via the cysteine-rich domain [5, 19-21]. The PBZ domain alone has not been evaluated for CHFR substrate targeting; thus it is possible that the binding of CHFR to these substrates is entirely dependent on the PBZ domain. In this scenario, regulation of PAR polymerases (PARPs) could control CHFR activity in the mitotic checkpoint. Currently, no PAR-modified substrates of CHFR have been identified, but the role of the PBZ domain in CHFR function is an interesting question warranting further study.

CHFR Cellular Functions

CHFR is an E3 ubiquitin ligase protein responsible for an early mitotic checkpoint [1, 2]. E3 proteins are critical in the ubiquitination signaling process as they provide substrate specificity, and function to recruit E2 ubiquitin conjugating proteins to the intended target. The function of CHFR-dependent ubiquitination appears to be multifaceted. Many CHFR ubiquitination substrates have been shown to be downregulated as a result, suggesting that they are targeted for degradation by this modification [5, 17, 19-21]. However, evidence in human cells as well as yeast indicate that CHFR can function with UBC13-MMS2 to form lysine-63-linked polyubiquitin chains, which are generally

associated with non-degradative (noncanonical) ubiquitin signaling [18, 27]. Evidence from yeast is consistent with this dual function of CHFR. Yeast cells contain two homologs of human *CHFR*, Chf1 and Chf2 (a.k.a. Dma1 and Dma2), and these proteins have been shown to function with Ubc13-Mms2 to form lysine-63-linked ubiquitin chains, as well as with Ubc4 to catalyze canonical lysine-48-linked as well as lysine-63-linked ubiquitin chains [27, 28]. These data suggest a dual role for CHFR-controlled ubiquitination in both degradative and non-degradative regulation.

The range of CHFR functions is not limited to its ubiquitination activity. CHFR has been implicated in several cellular processes, including the antephase and spindle assembly checkpoints. In fact, CHFR was first identified as a checkpoint protein essential for its role in causing chromosome condensation delay due to microtubule stress before the entry into mitosis [1]. This work established CHFR as a component of the so-called novel "antephase" checkpoint which monitors microtubule dependent events between prophase and metaphase [1]. More recent studies have implicated CHFR in the spindle assembly checkpoint between metaphase and anaphase [5, 20, 29], which can also be triggered by microtubule-targeting drugs. Given this, it is hypothesized that CHFR functions in two distinct cellular checkpoints during mitosis (Figure 1.3) where CHFR functions to halt the cell cycle in response to microtubule damage or spindle defects, ensuring proper chromosome segregation and cell

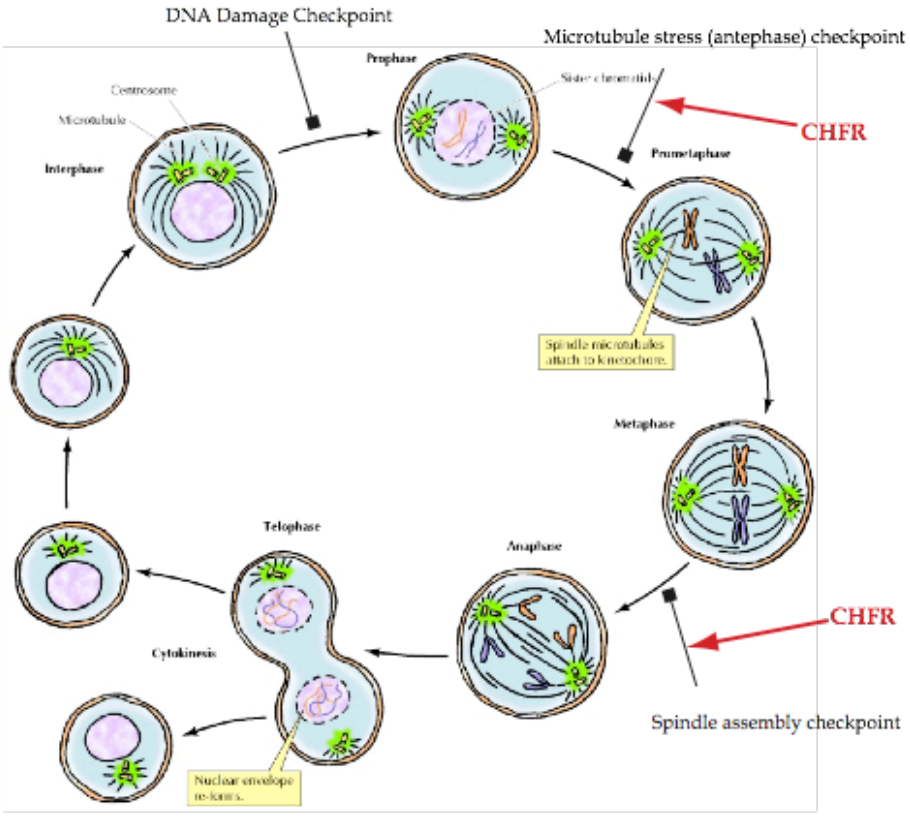

Figure 1.3. CHFR in the mitotic checkpoints. CHFR was originally identified as a major component of the antephase checkpoint occurring prior to metaphase in response to microtubule stress. Recent work has identified a role for CHFR later in mitosis at the spindle assembly checkpoint, which arrests cells prior to chromosome separation in anaphase. Adapted from: The Cell: A Molecular Approach. 2nd ed. [38]

division [1, 2]. In addition, CHFR activity has been implicated in processes leading to metastasis and angiogenesis [21, 30].

CHFR in the Antephase Checkpoint

The antephase checkpoint occurs at the end of G2, prior to prometaphase when cells are committed to mitosis, and is activated in response to microtubule stress [31, 32]. This checkpoint was defined by the discovery of CHFR, and cell lines lacking functional CHFR fail to activate this checkpoint [1, 31]. There is some evidence that this checkpoint occurs in response to a failure of the centromeres to properly separate [1], a critical step for assembly of a mitotic spindle. Extensive study of CHFR has revealed several CHFR targets implicated in mitotic progression in cells.

Polo-like kinase-1 (Plk1) was the first ubiquitination target of CHFR identified [17]. Plk1 is a critical mitotic protein, which regulates cell cycle and mitotic entry [33]. Early studies identified Cyclin B1/Cdk1 activity as key activators of mitotic entry regulated by the CHFR checkpoint [1, 34]. PLK1 is known to promote mitotic entry through activation of its targets CDC25C and Cyclin B/Cdk1, and these targets are inhibited by CHFR [17, 35, 36]. Furthermore, CHFR was shown to exclude Cyclin B1 from the nucleus, and expression of Cyclin B1 lacking nuclear export signal could bypass the early mitotic checkpoint [34]. CHFR could ubiquitinate Plk1 in *Xenopus* extracts, targeting Plk1 for degradation and delaying mitotic onset as measured by

histone H1 phosphorylation [17]. Since then, two publications have shown an inverse relationship between CHFR levels and Plk1 levels in mammalian cell lines, including *Chfr* knockout mouse embryonic fibroblasts (*CHFR*$^{-/-}$ MEFs) [5, 37]. While Plk1 represents an interesting target for CHFR control of mitotic onset, more evidence is needed to strengthen the tie between these two proteins.

CHFR's dual checkpoint function may be carried out, at least in some cases, by CHFR-mediated ubiquitination/degradation of AurA (Aurora A kinase), a protein that drives mitotic progression [5, 39]. AurA is ubiquitinated by CHFR both *in vitro*, and *in vivo* ubiquitination of AurA is abolished in *Chfr* knockout cells [5]. While, the effect of CHFR on AurA levels has been questioned [34], *Chfr* knockout MEFs (Mouse Embryonic Fibroblasts) show a clear increase in AurA levels relative to MEFs derived from wild type littermates [5]. Aurora A is critical to the early stages of mitosis, and plays a role in mitotic entry as well as centrosome maturation and separation [40, 41]. AurA is required for the recruitment of several centrosome components, thought to be required for both centrosome function and migration to opposite poles [40]. In addition, Aurora A is critical for centrosome duplication and is also involved in the spindle assembly checkpoint [39].

Loss of AurA inhibits proper bipolar spindle formation and results in mitotic arrest, while overexpression of AurA has been shown to induce multipolar spindle formation and is linked to oncogenic phenotypes such as aneuploidy [42, 43]. Similar phenotypes are seen when *CHFR* expression is

reduced, which increases AurA levels in the cell [5, 29]. AurA phosphorylates Cdc25B, a phosphatase required for Cyclin B/Cdk1 activation, and likely promotes mitotic entry in part through this modification [44]. AurA has also been shown to directly phosphorylate and activate polo-like kinase-1, another CHFR target, to promote mitotic entry [45, 46]. In addition to its G2/M functions, AurA appears to have some role in the spindle assembly checkpoint, as the checkpoint is bypassed in cells overexpressing AurA [47].

CHFR in the Spindle Assembly Checkpoint

The spindle assembly checkpoint delays progression through metaphase in response to unattached or improperly attached kinetochores. Critical components of this checkpoint include the MAD and BUB proteins, which serve to delay sister chromatid separation until proper spindle attachment is achieved [48]. MAD2 (Mitotic Arrest Deficient 2) is of particular importance in that it targets CDC20, a component of the anaphase promoting complex (APC) to inactivate the APC and delay anaphase in response to unattached kinetochores [49, 50].

The involvement of CHFR in the spindle assembly checkpoint is a fairly recent discovery. The interaction between CHFR and MAD2 implicated CHFR in this checkpoint, although several known CHFR targets may be involved at this stage of mitosis (such as AurA, mentioned above [47]). Cells with reduced CHFR expression do not delay metaphase in response to spindle defects, but continue

mitosis resulting in mitotic defects such as failed nuclear separation, lagging chromosomes, and failed cytokinesis [1, 5].

MAD2, a protein that is recruited to unattached kinetochores to act in the spindle-assembly checkpoint during mitosis, has recently been shown to bind to CHFR protein in immortalized mammary epithelial cells [29, 51]. Loss of CHFR results in mislocalization of MAD2 and BUBR1, both of which bind to kinetochores and are critical for the spindle assembly checkpoint [29]. CHFR reduction disrupts the CDC20/MAD2 complex, which is hypothesized to free CDC20 to activate the anaphase-promoting complex, leading to progression through mitosis [29].

CHFR has also been found to bind to TCTP (Translationally Controlled Tumor Protein), α-tubulin, and the chromokinesin Kif22, all thought to be components of the mitotic spindle [20, 29, 52]. TCTP has been connected to several cellular processes, most interesting regarding the mitotic spindle is its microtubule stabilizing effect [53]. It has been suggested that this binding of CHFR to TCTP or tubulin could be important for CHFR to sense microtubule stress and trigger the checkpoint [29, 52]. Studies of TCTP indicate that it may be involved in stabilizing the mitotic spindle microtubules, and regulation of TCTP may be necessary for returning the microtubules to a more dynamic state after metaphase [54].

The role of α-tubulin in the mitotic spindle is clear, as α-tubulin is a major component of spindle microtubules. As CHFR was originally identified as a

sensor of microtubule stress, the interaction between CHFR and α-tubulin could indicate the mechanism by which CHFR senses microtubule dysfunction. In fact, the interaction between CHFR and α-tubulin was observed in MCF10A cells only after nocodazole treatment [29]. siRNA reduction of CHFR resulted in an increase in α-tubulin levels and a decrease in ubiquitinated α-tubulin, indicating that CHFR may play a role in α-tubulin stability [29]. In addition, reduction of CHFR increased the amount of acetylated α-tubulin, which is associated with more stable microtubules, and suggests a role for CHFR in regulating spindle microtubule dynamics [29, 55].

Kif22 is a chromokinesin that is involved in the alignment of chromosomes within the spindle [56]. CHFR targets Kif22 for degradation, and overexpression of Kif22 causes multipolar spindles and aneuploid cells, suggesting that CHFR regulation of Kif22 contributes to regulation of the mitotic spindle [20]. The interactions between CHFR and α-tubulin, TCTP, and Kif22 suggest that CHFR has a physical interaction with the mitotic spindle, and that CHFR may regulate the mitotic spindle checkpoint directly, through Kif22, in addition to the checkpoint delay brought on by inhibition of the APC through MAD2.

CHFR in Migration, Motility and Angiogenesis

In addition to the widely recognized role of CHFR in mitotic checkpoint control, CHFR is known to interact with proteins involved in migration,

metastasis and angiogenic pathways. siRNA reduction of CHFR in immortalized human mammary epithelial cell lines has been shown to increase cell motility and invasion into matrigel, while expression of CHFR in CHFR-lacking breast cancer lines has the opposite effect [3]. Support for the role of CHFR in these phenotypes can be found in studies identifying cellular targets of CHFR, specifically helicase-like transcription factor (HLTF) and histone-deacetylase 1 (HDAC1) [19, 21]. HLTF regulates the transcription of the gene PAI-1, which controls cell motility [19, 57]. CHFR targets HLTF for degradation, and cells overexpressing HLTF show significantly higher motility, which can be reduced by CHFR expression [19]. Similarly, HDAC1 is thought to affect transcription of multiple genes involved in cell motility, and shRNA reduction of HDAC1 in cancer lines reduced the invasiveness of cells into matrigel [21]. These two ubiquitination targets implicate CHFR in pathways leading to motility and invasion, suggesting that CHFR could contribute to metastasis.

Finally, CHFR has been shown to inhibit the transcription factor NF-κB, leading to suppression of several genes [30]. One NF-κB target, IL-8, is associated with cell motility as well as tumor angiogenesis, and a xenograft model demonstrated that CHFR expression reduced angiogenesis in tumor tissues [30]. These studies demonstrate that CHFR likely plays an extensive and complex role in cancer progression, and further understanding of CHFR and its targets will lead to a better understanding and treatment of cancers in the future.

CHFR and Cancer

CHFR as a tumor suppressor

CHFR was initially characterized as a checkpoint protein that was lost in four of the eight human cancer cell lines initially tested (including colon cancer, osteosarcoma, and neuroblastoma cancer lines) [1]. Cell cycle checkpoint function insures proper segregation of chromosomes, and misregulation of mitotic checkpoints is understood to cause aneuploidy, a characteristic of many cancers [58]. In addition, reduction of endogenous CHFR in immortalized mammary epithelial cell lines resulted in the onset of tumor-like phenotypes such as aneuploidy, as well as increased mitotic index, growth rate, invasiveness, motility and soft agar colony formation [29]. In cancer cell lines that express little or no CHFR, restoration of CHFR expression reduced the mitotic index, invasiveness, motility, and growth rate of the cells [29].

CHFR is connected to cancer-related phenotypes through its downstream targets and interacting proteins (Figure 1.4). The CHFR targets connected to the antephase and mitotic spindle checkpoints, such as AurA, PLK1, MAD2 and CyclinB1/Cdc2, are important for growth control and prevention of aneuploidy [17, 42, 58-60]. HLTF has been shown to modulate expression of PAI-1 to regulate cell migration [19], and HDAC1 is associated with motility and invasiveness of cells, linking it to metastasis in breast cancer [21, 61]. Finally, NF-κB is implicated in enhancing progression through the cell cycle, promoting

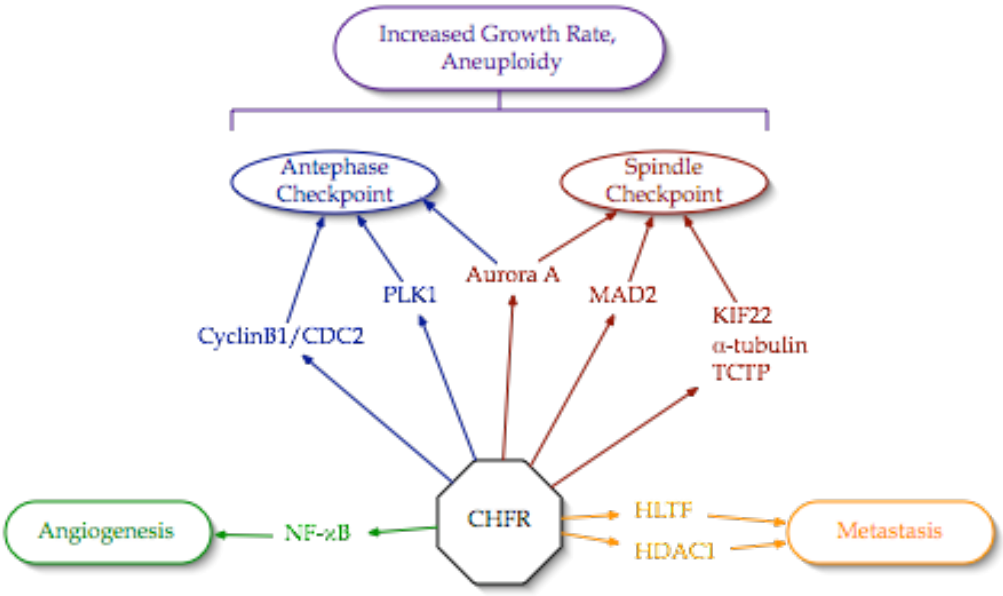

Figure 1.4. The role of CHFR in the cell cycle and neoplastic processes. CHFR loss has been implicated in several cancer progression-related phenotypes. CHFR has been shown to target HDAC1 and HLTF for degradation, linking CHFR loss to metastasis. CHFR expression in some cancer lines reduced NF-κB, and reduced angiogenesis in a xenograft model. In addition, several downstream effectors of CHFR involved in mitotic checkpoint processes have been identified. PLK1, Aurora A, and KIF22 are ubiquitination targets of CHFR[5, 17, 20], MAD2, α-tubulin and TCTP have been shown to interact with CHFR, and CyclinB1/CDC2 appears to be regulated by CHFR expression. Loss of the antephase and spindle checkpoints results in more rapid growth and increased aneuploidy when CHFR is lost.

angiogenesis and preventing apoptosis through its transcriptional regulation of a wide array of genes [30, 62].

The strongest data in support of CHFR as a tumor suppressor protein comes from analysis of the *Chfr* knockout mouse. *Chfr*$^{-/-}$ mice had increased incidence of skin tumors after exposure to the carcinogen DMBA, as well as spontaneous development of lymphomas and epithelial tumors of major organs [5]. Embryonic fibroblasts isolated from these mice also displayed chromosome instability, consistent with previous knockdown studies of *CHFR* [3, 5]. In addition, the *Chfr*$^{-/-}$ MEFs were able to form colonies in culture, a characteristic of cellular transformation [5]. For these reasons, CHFR is considered a tumor suppressor protein likely to play an important role in the progression of many cancer types.

While mutation of *CHFR* is not found in many cancer cells, loss of CHFR protein is a frequent occurrence [2, 63]. One polymorphism (V539M) located in the cysteine-rich domain was reported to be strongly associated with colorectal cancer risk [64]. Loss of CHFR protein expression has been reported in a wide range of cancer types, including breast, lung, colorectal, and ovarian cancers [2]. The majority of these cancer types show evidence that this loss is due to *CHFR* promoter hypermethylation, however breast cancers appear to be the exception [2, 4, 65]. Clearly, CHFR loss represents an important step in the progression of several cancers, and therefore could have clinical significance as a prognostic and therapeutic biomarker.

CHFR as a biomarker of malignancy

While decreased and/or lost CHFR expression is implicated in cancer progression, it also represents a promising biomarker for cancer treatments. The status of CHFR, as well as related proteins, in a given tumor may be informative for microtubule targeting drug therapies. Cell lines in which CHFR expression has been inhibited show reduced survival and increased apoptosis when treated with microtubule-targeting drugs [29, 66, 67]. Additionally, many studies have shown correlations between loss of CHFR expression and tumor progression phenotypes, such as tumor size, invasion, differentiation and tumor stage, in cancers of different origins (Table 1.1). CHFR loss is associated with increased malignant potential and poor outcomes [2]. CHFR loss has been associated with advanced tumor stage in hepatocellular carcinomas [68] and head and neck squamous cell carcinomas [69], but is found in all stages in gastric and esophageal cancers [70].

One of the challenges in treating cancers is that many chemotherapeutics are effective in only a subset of patients. In order to provide the best outcomes, it is important to have methods to determine the best course of treatment for each individual case. Because cancers arise from a variety of genetic changes, treatment choices may be optimized based on the genetic features of the cancer cells. This represents a powerful tool by which physicians can choose the most effective treatment for their patients. A strong correlation between CHFR loss and sensitivity to microtubule drugs such as paclitaxel has been demonstrated in

NSCLC (non-small cell lung cancer) [71], suggesting that *CHFR* promoter methylation could be used as a predictive marker of drug response. However, work by Yoshida *et al* suggests that, in advanced and recurrent gastric cancer, *CHFR* promoter methylation does not predict response to paclitaxel [72]. This may indicate that gastric cancers often contain other mutations that counteract the paclitaxel sensitivity conferred by CHFR loss. Thus, additional markers may be required to determine proper drug response. Recently, Takeshita *et al* presented evidence that the combination of *CHFR* methylation status, EGFR mutation status and patient smoking habit can more effectively predict tumor response to paclitaxel treatment in NSCLC [71]. Therefore, CHFR represents an important marker for sensitivity to microtubule-specific chemotherapeutics.

CHFR promoter methylation alone may not be informative enough as a predictor of chemoresponsiveness in all cancers. The invasiveness of the procedure necessary to determine *CHFR* promoter methylation status in lung cancers (and likely other solid tumors) make CHFR-based predictions less than ideal in NSCLC [71]. In addition, *CHFR* promoter methylation does not appear to be the cause of CHFR protein loss in breast cancers [2, 4, 65]. This indicates the importance of finding alternate indicators for drug response. Other related genes, such as SIRT2, a tubulin deacetylase that is involved in the same antephase checkpoint processes as CHFR and lost in gliomas [86], or PLK1, which is lost in many cancer types [17], may be predictive of chemotherapeutic

Cancer origin	Loss of CHFR associated with:
Breast	Increased tumor size [3] Estrogen receptor negative [3]
Gastric	Poor differentiation [73, 74]* Increased sensitivity to paclitaxel (in culture) [75, 76]† Microsatellite instability [77] Increased tumor size [74]
Lung	Smoking habit [78, 79] Increased tumor size [78] Poor differentiation [78, 79] Lymphatic invasion [79] Poor prognosis [78, 79]
Peripheral nerve sheath	High mitotic count [80] Poor prognosis [80]
Colorectal	Microsatellite instability [81] Increased recurrence [82]
Liver	Advanced stage [68] Infiltrative growth [68]
Endometrial	Increased sensitivity to taxanes [83]
Cervical	Increased sensitivity to taxanes (in culture) [84]
Head and neck	Late stage [69]

Table 1.1. CHFR loss associated with human cancer phenotypes. Listed are cancer types in which CHFR loss has been observed, and the clinically relevant phenotypes associated with CHFR loss.

*Disputed in [85] †Disputed in [72]

response to paclitaxel similarly to *CHFR* methylation status. While CHFR appears to be a promising biomarker for cancer progression and chemotherapeutic response, significant work is still needed to fully understand the prognostic and chemotherapeutic impact of CHFR loss in cancers.

Investigating the Regulation and Function of CHFR

In order to further the understanding of CHFR, we have explored both the function of CHFR in regulating mitotic checkpoints and a potential novel mechanism for regulating CHFR protein expression. First, we investigated the interaction between CHFR and MAD2 in the mitotic spindle checkpoint. As both CHFR and MAD2 have been implicated in cancers [2, 87, 88], understanding the relationship between these proteins, physical interactions as well as functional, could lead to better understanding of the processes leading to cancer and influence treatments based on the molecular signature of each cancer. We focused on the major functional domains of CHFR in order to gain insight into the mechanism by which CHFR interacts with MAD2 and regulates the spindle assembly checkpoint, and also how that mechanism relates to previously identified functions of CHFR in the antephase checkpoint and toward other substrates. First, we confirmed the interaction between CHFR and MAD2 in a human cell line as well as mouse embryonic fibroblasts. We then identified the cysteine-rich domain of CHFR as required for interaction with Mad2, while the RING domain was dispensable for this interaction. In addition, interaction with

CHFR appears to be required for proper MAD2 localization and spindle assembly checkpoint function, lending support to the evidence that CHFR functions in the spindle assembly checkpoint in addition to the antephase checkpoint. Interestingly this function does not appear to depend on the RING or FHA domains, which have been identified as critical to the antephase checkpoint and for ubiquitination of downstream substrates (discussed above). These results suggest that CHFR has multiple functions, some dependent on the E3 ubiquitin ligase function while some, as in the case of MAD2, independent of this function.

We also examined the possible role of microRNA regulation in CHFR expression changes, specifically focusing on breast cancer lines in which *CHFR* mRNA and protein levels do not correlate as expected. CHFR loss has been demonstrated to result in cancer progression phenotypes in breast and other cancers, and restoration of CHFR expression in BCC lines lacking CHFR ameliorates several of these phenotypes [2, 3, 29]. CHFR loss is observed in many breast cancer lines, indicating that loss of CHFR may be a major event in breast tumorigenesis [3]. Furthermore, no explanation currently exists for the mechanism by which CHFR is lost in breast cancers. Understanding this mechanism may lend insight into tissue-specific events unique to breast tissue and/or breast cancer, and allow better understanding of the differences between breast cancers and other cancers. Promoter methylation of *CHFR* is currently being explored as a biomarker for CHFR loss in other cancers, and has, in some

cases been correlated to chemotherapeutic response to paclitaxel [89]. In the same way, understanding the mechanism underlying CHFR loss in some breast cancers may be useful in biomarker development. In addition, new treatment options may be possible targeting the cause of CHFR loss, to restore CHFR expression in tumor cells. We show evidence that one microRNA, miR-26, could be inhibiting translation of *CHFR* mRNA, implicating the microRNA as a mechanism of CHFR regulation. As miR-26 has been previously implicated in several cancer types, it may prove to be important for cancer progression through CHFR loss.

Notes

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CHAPTER 2

CHFR binds to and regulates MAD2 in the spindle assembly checkpoint through its cysteine-rich domain

Abstract

CHFR has been implicated as a tumor suppressor in a multitude of cancers. It was originally identified as a major component of the antephase checkpoint. Recently, CHFR was reported to interact with MAD2, an important component of the spindle assembly checkpoint, where *CHFR* knockdown resulted in mislocalization of MAD2 and disruption of the MAD2/CDC20 interaction. To further understand how CHFR interacts with MAD2, we deleted key functional domains of CHFR, and investigated the effect on MAD2 binding and function. Here we show that deletion of the cysteine-rich domain of CHFR is required for the CHFR/MAD2 interaction as well as proper localization of MAD2 in the cell. Furthermore, the cysteine-rich domain deletion exhibits impaired ability to promote the MAD2/CDC20 interaction, leading to an increase in mitotic defects relative to wild type CHFR. These data support a critical role for CHFR in the MAD2 spindle checkpoint. Furthermore, these data establish the cysteine-rich domain of CHFR as the essential domain for the

CHFR/MAD2 interaction and for promoting interaction between MAD2 and CDC20 to inhibit the anaphase-promoting complex.

Introduction

CHFR (Checkpoint with FHA and Ring finger) is an E3 ubiquitin ligase that functions as a mitotic checkpoint protein and has been implicated as a tumor suppressor in a wide array of cancer types [1, 2, 15]. *Chfr*$^{-/-}$ mice developed spontaneous lymphomas and epithelial tumors, and formed skin tumors in response to a DMBA treatment that did not induce tumor formation in wild type mice [5]. In addition, *Chfr* knockout (*Chfr*$^{-/-}$) mouse embryonic fibroblasts (MEFs) display mitotic defects including failed nuclear segregation, lagging chromosomes, and failed cytokinesis, and become aneuploid over time in culture [5]. A similar aneuploidy and mitotic defect phenotype was observed in immortalized breast epithelial cells when *CHFR* was knocked down by siRNA [29]. Aneuploidy is often observed as a consequence of mitotic checkpoint defects. In fact, CHFR was initially identified as an antephase checkpoint protein essential for triggering the mitotic stress checkpoint in response to nocodazole treatment [1, 15]. Subsequent studies have identified Aurora A and Kif22 as ubiquitination targets of CHFR, implicating CHFR in the spindle-assembly checkpoint occurring later in mitosis [5, 20].

Mitotic arrest deficient 2 (MAD2) has been identified as a key protein responsible for detecting proper spindle attachment to kinetochores, and triggers

delay through inhibition of the anaphase-promoting complex (APC) when attachments are incomplete [51]. This inhibition occurs through binding of MAD2 to CDC20, which inhibits its activation of the APC [90]. While this checkpoint has been extensively studied, full understanding of the mechanism by which MAD2 and CDC20 interact to trigger the spindle checkpoint remains elusive. MAD2, like CHFR, has been linked to cancer phenotypes and is often overexpressed in human tumors [88, 91]. Overexpression of MAD2 occurs in multiple tumor types and is associated with chromosome instability and poor prognosis [88, 91, 92]. Overexpression of MAD2 in mice produced an array of tumor types, including lung adenoma, hepatoma, and intestinal tumors [88]. Furthermore, MEF cells derived from these mice displayed chromosome segregation defects and increased aneuploidy relative to wild type cells [88].

Recently, MAD2 was identified as a CHFR-interacting protein by yeast-two-hybrid, and this interaction was verified using cultured cells [29]. Notably, knockdown of *CHFR* via siRNA resulted in mislocalization of MAD2 during mitosis, and inhibited the MAD2/CDC20 interaction [29]. *CHFR* knockdown also resulted in mislocalization of BUBR1 [29], which cooperates with MAD2 to inhibit the APC [90]. These data implicate CHFR in regulation of the MAD2/CDC20 interaction, and may point to a complex role of CHFR in the spindle checkpoint, but further analyses supporting an interaction between CHFR and MAD2 have not yet been reported.

To better understand the role of CHFR in the MAD2-dependent spindle checkpoint, we deleted key CHFR domains and examined the effect on MAD2. We find that the FHA and Ring domains are not required for MAD2 binding to CHFR, while the C-terminal cysteine-rich domain is required for this interaction. Furthermore, the FHA and Ring domain deletions had no effect on MAD2-CDC20 binding, while deletion of the cysteine-rich domain inhibited this interaction. Finally, deletion of the cysteine-rich domain resulted in mislocalization of MAD2 in mitotic cells, leading to improper chromosome migration. Together, this data suggest that CHFR binding to MAD2 is important for proper cellular localization of MAD2 during mitosis and effective activation of the mitotic spindle checkpoint.

Materials and Methods

Plasmids and Antibodies

CHFR deletion constructs were created using the QuikChange site-directed mutagenesis kit (Stratagene). MAD2 antibodies were obtained from BD Biosciences (blotting) and Santa Cruz (Immunoprecipitation). CHFR antibody was a gift from the Yu lab (University of Michigan); CDC20 antibody was from BD biosciences. Anti-Flag and anti-HA antibodies were from Sigma and Covance, respectively. Fluorescent secondary antibodies were purchased from Jackson ImmunoResearch.

Cell Culture and Immunoprecipitation

HEK293T cells were obtained from ATCC and cultured in DMEM containing 10% FBS. Immortalized mouse embryonic fibroblasts were a gift from the Yu lab (University of Michigan). Transfection of HEK293T cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. MEF cells were transfected by electroporation (BioRad Gene Pulser XCell). Transfected cells were treated with nocodazole for 18 hours, then lysed with NETN buffer [5], spun at 4 °C and the supernatant was collected. For immunoprecipitation, lysate was incubated overnight with Protein A (Invitrogen), Protein G (Invitrogen) or anti-Flag beads (Sigma), and the indicated antibodies. After binding, beads were washed three times with NETN buffer and subjected to Western blot.

Immunofluorescence

After electroporation, MEFs were plated to glass coverslips and allowed to recover for 24 hours, then treated with nocodazole for 18 hours. After treatment, cells were fixed in cold methanol for 10 minutes, then stained with the indicated antibodies at 1:1000 dilution in 8% goat serum/PBS for one hour. Secondary antibodies were applied at 1:600 dilution for 30 minutes, followed by 1 minute of DAPI/PBS.

Results

The Cystein-rich domain is required for CHFR-MAD2 binding.

The interaction between CHFR and MAD2 was originally reported in 2008 by Privette *et al [29].* We verified this interaction by immunoprecipitation using antibodies against endogenous CHFR and MAD2 in HEK293T cells (Figure 2.1). To further examine this interaction we utilized three deletion constructs of CHFR. CHFR has several reported domains including the forkhead associated (FHA), the RING finger (RING) and the cysteine-rich (Cys) domains (Figure 2.2) [1]. The FHA domain is thought to be a phosphothreonine-binding domain, although no binding target for the CHFR FHA domain has been reported [6]. The RING domain is required for the ubiquitin ligase activity of CHFR [17], and is required for the mitotic stress checkpoint [15]. Finally, the Cys domain of CHFR has been identified as a protein-binding domain, required for the interaction of CHFR with its ubiquitination targets Aurora A and Kif22 [5, 20]. Using co-immunoprecipitation with individual deletion constructs, we investigated which domain is required for MAD2 binding. As shown in Figure 2.3, only the CHFRΔCys mutant protein failed to pull down HA-tagged MAD2, while CHFRΔFHA and CHFRΔRing deletions had no effect on HA-MAD2 binding. This indicated that the Cys domain was likely the domain of MAD2 binding, consistent with previous findings that the Cys domain is important for CHFR protein-protein interactions.

Figure 2.1. CHFR binds to MAD2 in HEK293T cells. HEK293T cell lysates were immunoprecipitated using anti-Chfr antibodies and blotted for MAD2 (top), and immunoprecipitated using anti-MAD2 antibodies and blotted for CHFR (bottom).

Figure 2.2. CHFR deletion mutants. This illustration depicts the CHFR deletion mutants utilized in this study.

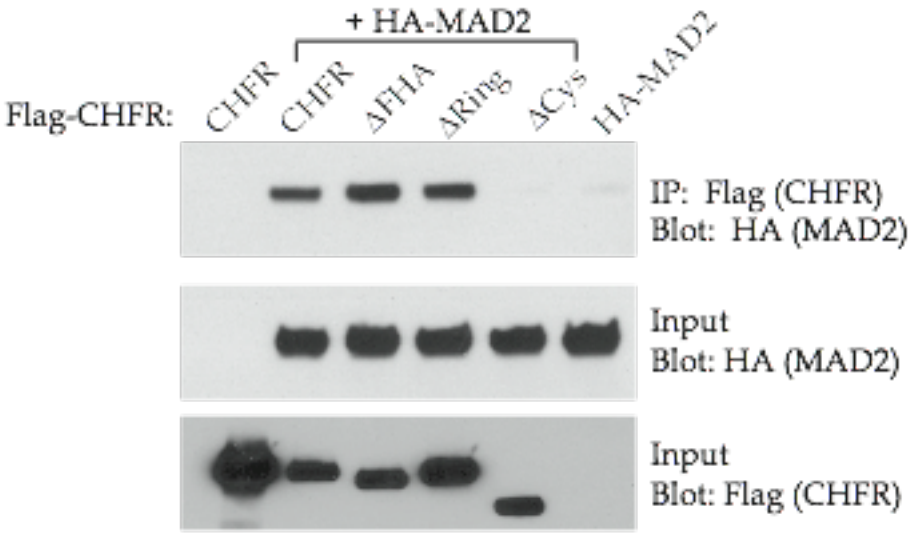

Figure 2.3. The MAD2 interaction with CHFR requires the cysteine-rich domain. *Chfr*$^{-/-}$ MEF cells were transfected with the indicated Flag-*CHFR* constructs and HA-MAD2 where indicated. Immunoprecipitations using anti-Flag (top) and blotted with anti-HA are shown at the top. The bottom two lanes show input protein levels. The *CHFR*ΔCys construct is unable to pull down any visible MAD2 protein, indicating that the Cys domain is critical for MAD2 binding.

CHFRΔCys expression cannot rescue abnormal Mad2 localization in Chfr$^{-/-}$ MEFs.

Previous work has shown that siRNA reduction of *CHFR* in human cell lines results in mislocalized MAD2 and disruption of the MAD2-dependent spindle-assembly checkpoint [29]. We were able to confirm this phenotype using immunofluorescence of endogenous Mad2 in immortalized *Chfr*$^{-/-}$ mouse embryonic fibroblasts (MEFs). While Mad2 in wild type MEFs displayed the characteristic punctate staining by immunofluorescence, *Chfr*$^{-/-}$ MEFs showed more diffuse Mad2 localization (Figure 2.4). Transfection of GFP-tagged *CHFR*, as well as GFP-*CHFR*ΔFHA and GFP-*CHFR*ΔRing constructs into *Chfr*$^{-/-}$ MEFs restored the punctate staining, while GFP-CHFRΔCys was unable to restore punctate Mad2 staining in mitotic cells (Figure 2.5). These data suggested that Chfr plays a role in Mad2 localization, which is dependent on binding via the cysteine-rich domain of Chfr.

CHFRΔCys does not rescue segregation defects in Chfr$^{-/-}$ MEFs.

MAD2 functions in the cell as part of the spindle assembly checkpoint, to ensure proper chromosome segregation during mitosis. Considering the Mad2 localization changes we observed in transfected *Chfr*$^{-/-}$ MEFs, we wondered if the localization of Mad2 correlated to a spindle-assembly checkpoint phenotype. Thus, we investigated if the *CHFR*ΔCys construct, and other deletion constructs, could rescue the chromosome segregation defects seen in *Chfr*$^{-/-}$ MEFs. DAPI

staining of *Chfr*$^{-/-}$ and wild type MEFs indicates that *Chfr*$^{-/-}$ MEFs have a higher incidence of lagging chromosomes, in agreement with previous reports using primary MEF cells [29] (Figure 2.6). Transfection of *Chfr*$^{-/-}$ MEFs with *CHFR*, *CHFR*ΔFHA, and *CHFR*ΔRing constructs were able to reduce the incidence of lagging chromosomes, while cells expressing CHFRΔCys had an incidence of mitotic defects similar to untransfected *Chfr*$^{-/-}$ cells (Figure 2.7).

The Cystein-rich domain of CHFR is important for Mad2-Cdc20 binding.

The mitotic defect seen in *Chfr*$^{-/-}$ MEFs and maintained when those cells expressed CHFRΔCys suggests that the cysteine-rich domain of CHFR may be critical for proper Mad2 function. To further investigate the effect of CHFRΔCys on Mad2 function, we used co-immunoprecipitation to assay the binding of Mad2 with Cdc20 in MEFs expressing *CHFR* constructs. In agreement with previous data using siRNA reduction of *CHFR* [29], we observed a decrease in Mad2-Cdc20 binding in *Chfr*$^{-/-}$ MEFs relative to wild type MEFs (Figure 2.8). Notably, expression of CHFR in *Chfr*$^{-/-}$ MEFs increased the interaction between Cdc20 and Mad2 (Figure 2.9). Expression of both *CHFR*ΔFHA and *CHFR*ΔRing constructs were co-immunoprecipitated with Mad2 at levels similar to wild type, while CHFRΔCys was unable to increase the Mad2-Cdc20 interaction (Figure 2.9). This suggests that the cysteine-rich domain is necessary for CHFR to enhance Mad2-Cdc20 binding, possibly through control of Mad2 localization during mitosis.

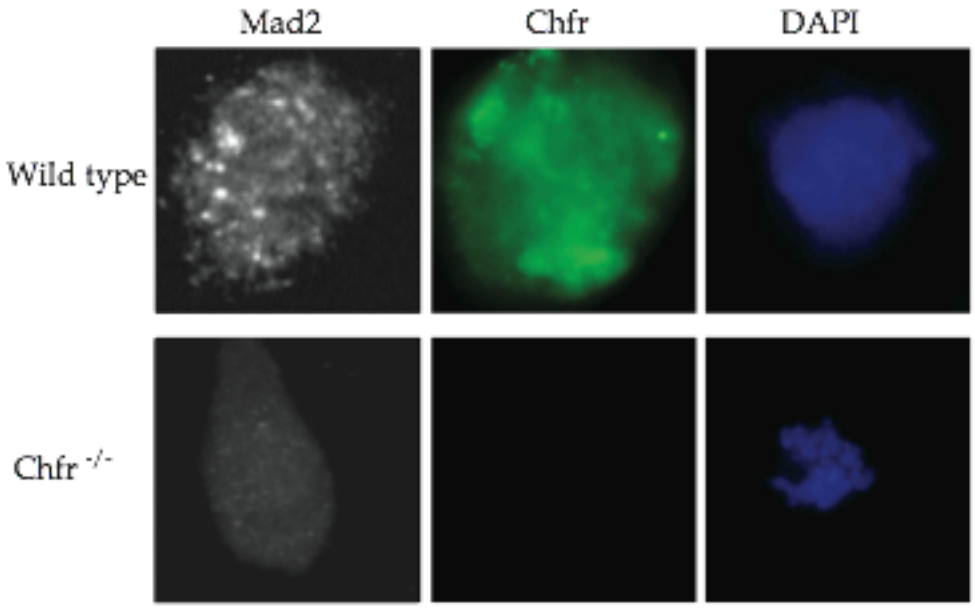

Figure 2.4. Localization of Mad2 in wild type and *Chfr*$^{-/-}$ MEFs. MEF cells were subjected to indirect immunofluorescence using anti-MAD2 antibodies. Mitotic *Chfr*$^{-/-}$ cells display a more diffuse Mad2 staining than the wild type cells.

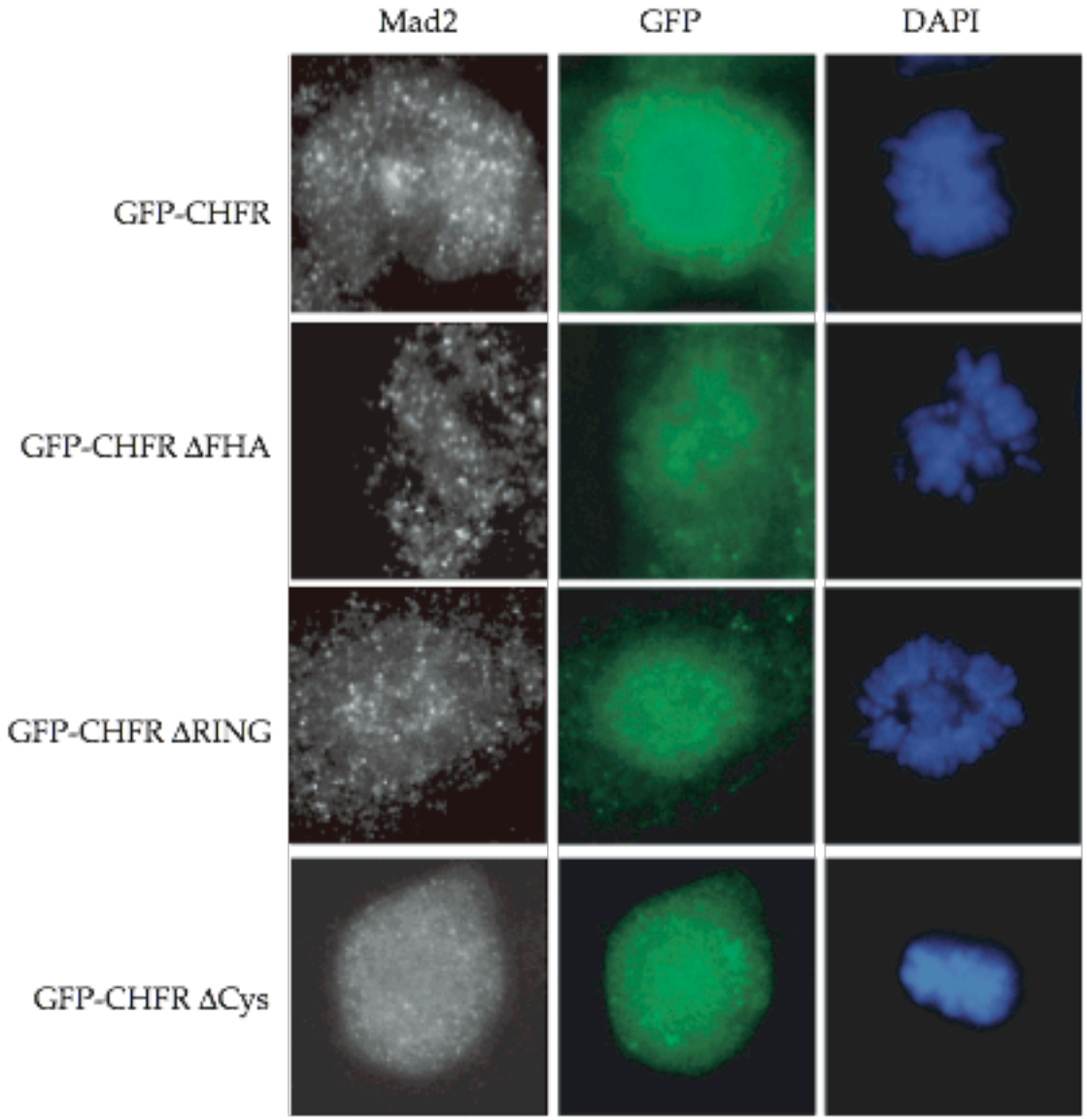

Figure 2.5. The cysteine-rich domain of CHFR is required for proper Mad2 localization. *Chfr*$^{-/-}$MEFs were transfected with GFP-tagged *CHFR* constructs (indicated at the left), and subjected to indirect immunofluorescence for Mad2 staining. While wild type CHFR, as well as the FHA and RING domain deletions could restore clear punctate staining to the MEF cells, GFP-CHFRΔCys failed to restore punctate staining.

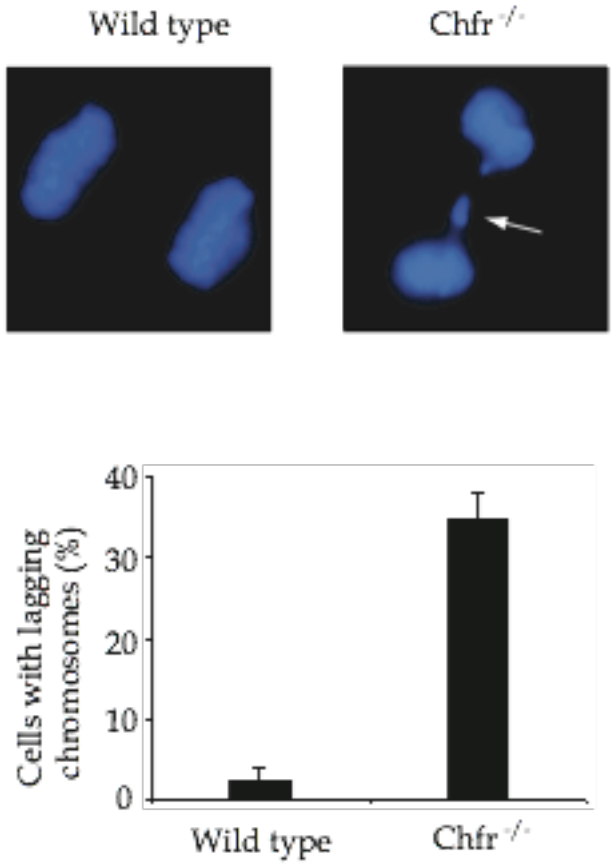

Figure 2.6. *Chfr*$^{-/-}$ MEFs display a higher rate of chromosome segregation defects. Wild type and *Chfr*$^{-/-}$ MEFs were enriched in mitosis and observed for lagging chromosomes (indicated by arrow). DAPI staining was used to observe lagging chromosomes during mitosis, the incidence of this defect is indicated in the histogram at the bottom.

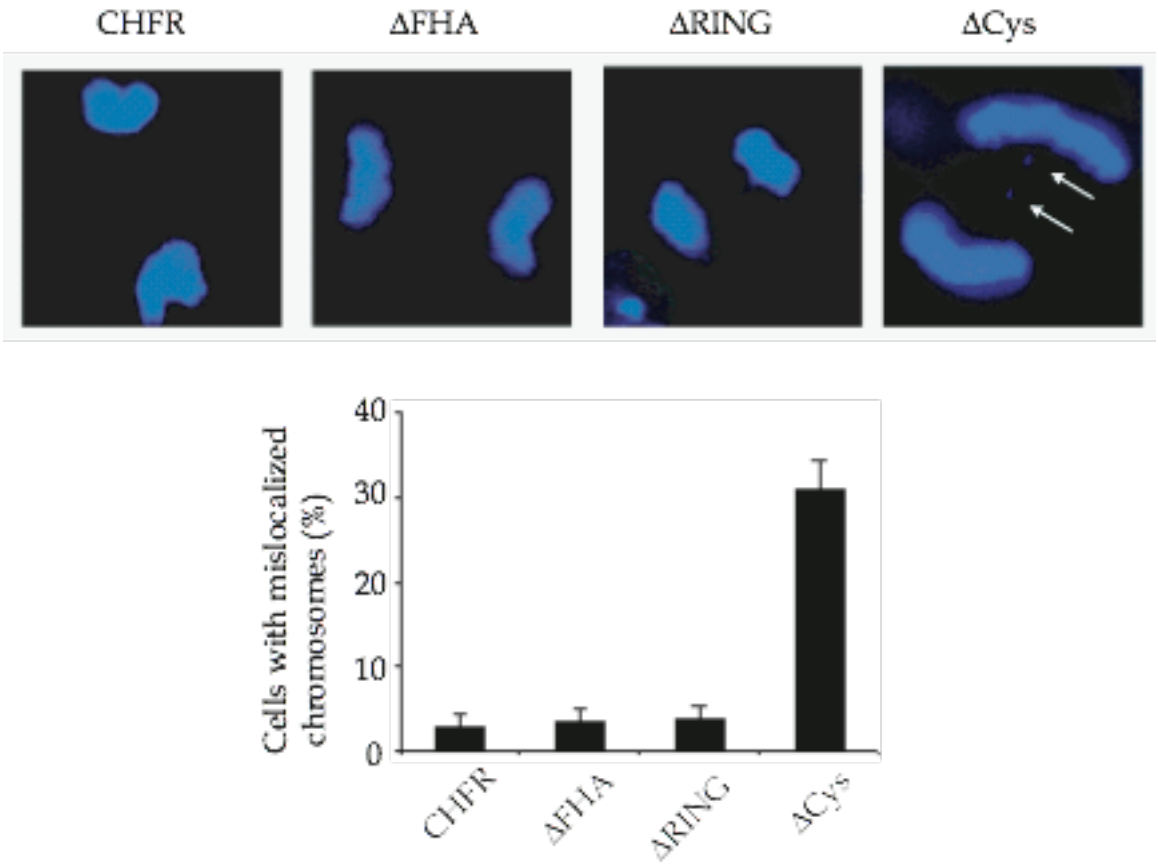

Figure 2.7. The cysteine-rich domain is required to rescue the chromosome segregation phenotype in *Chfr*$^{-/-}$ MEFs. *Chfr*$^{-/-}$ MEFs were transfected with GFP-*CHFR* constructs indicated on the left. DAPI staining was used to observe lagging chromosomes during mitosis (indicated with arrow). Incidence of lagging chromosomes in mitotic cells is quantitated in the histogram at the bottom.

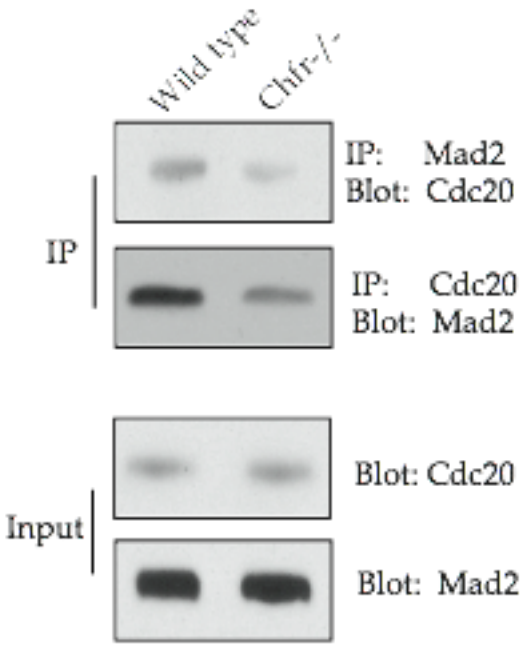

Figure 2.8. Mad2 interaction with Cdc20 is impaired in *Chfr*$^{-/-}$ MEFs. Wild type and *Chfr*$^{-/-}$ MEFs were lysed and the lysate subjected to immunoprecipitation using anti-Mad2 and blotted for Cdc20 (first row), or immunoprecipitation using anti-Cdc20 antibodies and blotted for Mad2 (second row) Bottom half indicates input levels of both proteins. *Chfr*$^{-/-}$ MEF lysates showed reduced Cdc20 co-immunoprecipitating with Mad2, and vice versa.

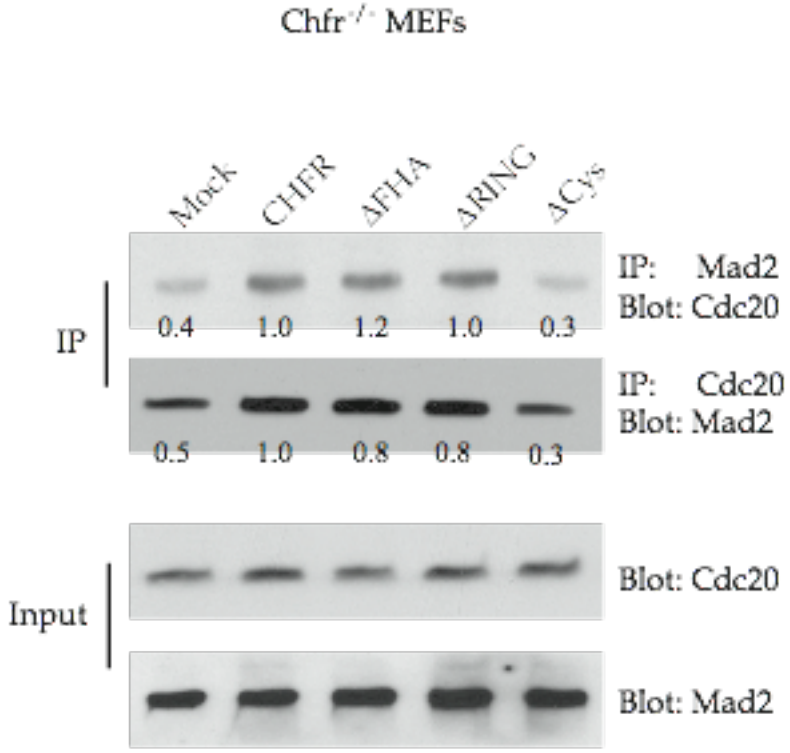

Figure 2.9. The cysteine-rich domain is required for restoring the Mad2/Cdc20 interaction to *Chfr*$^{-/-}$ MEFs. *Chfr*$^{-/-}$ MEFs were transfected with *CHFR* deletion constructs indicated at the top. Lysates from these cells were subjected to immunoprecipitation as in Figure 2.8. While ΔFHA and ΔRING expressing cells display Mad2 and Cdc20 pulldown comparable to wild type expressing cells, the ΔCys transfected cells retained the reduced pulldown between the two proteins.

Discussion

The MAD2 spindle assembly checkpoint is the focus of a wide number of studies. While the complex mechanism by which MAD2 performs its function is not fully understood, the interaction between MAD2 and CDC20 has been established as a critical component of this pathway [49, 51]. MAD2 activity is regulated by a change in conformation between closed (active, C-MAD2) and open (inactive, O-MAD2) forms [93-95]. C-MAD2 is bound to MAD1 at an unattached kinetochore, and is thought to serve as a template for conversion of other O-MAD2 to C-MAD2 at the kinetochore (Figure 2.10) [96, 97]. The newly converted C-MAD2 then forms a diffusible complex with CDC20, BUBR1, BUB3 and the APC [51, 58, 96, 98]. C-MAD2 in this complex may also act as a template for conversion of cytoplasmic O-MAD2 to C-MAD2 [96]. This complex impairs the CDC20-dependent activation of the anaphase-promoting complex and is abolished when the spindle-assembly checkpoint is satisfied [51, 58, 98]. MAD2 phosphorylation may play a role in regulating its binding to APC and MAD1, as phosphorylation of MAD2 reduced the interactions *in vivo* [99]. Interestingly, an inhibitor of the spindle assembly checkpoint, $p31^{comet}$ binds to C-MAD2 on the same surface as O-MAD2, and can compete with O-MAD2 for binding to C-MAD2, suggesting that $p31^{comet}$ inhibits the checkpoint by preventing conversion of MAD2 to its active form [96, 100-102].

Our data support the hypothesis that CHFR may play a role in localization of MAD2 to one or more of its functional complexes, and loss of CHFR or the

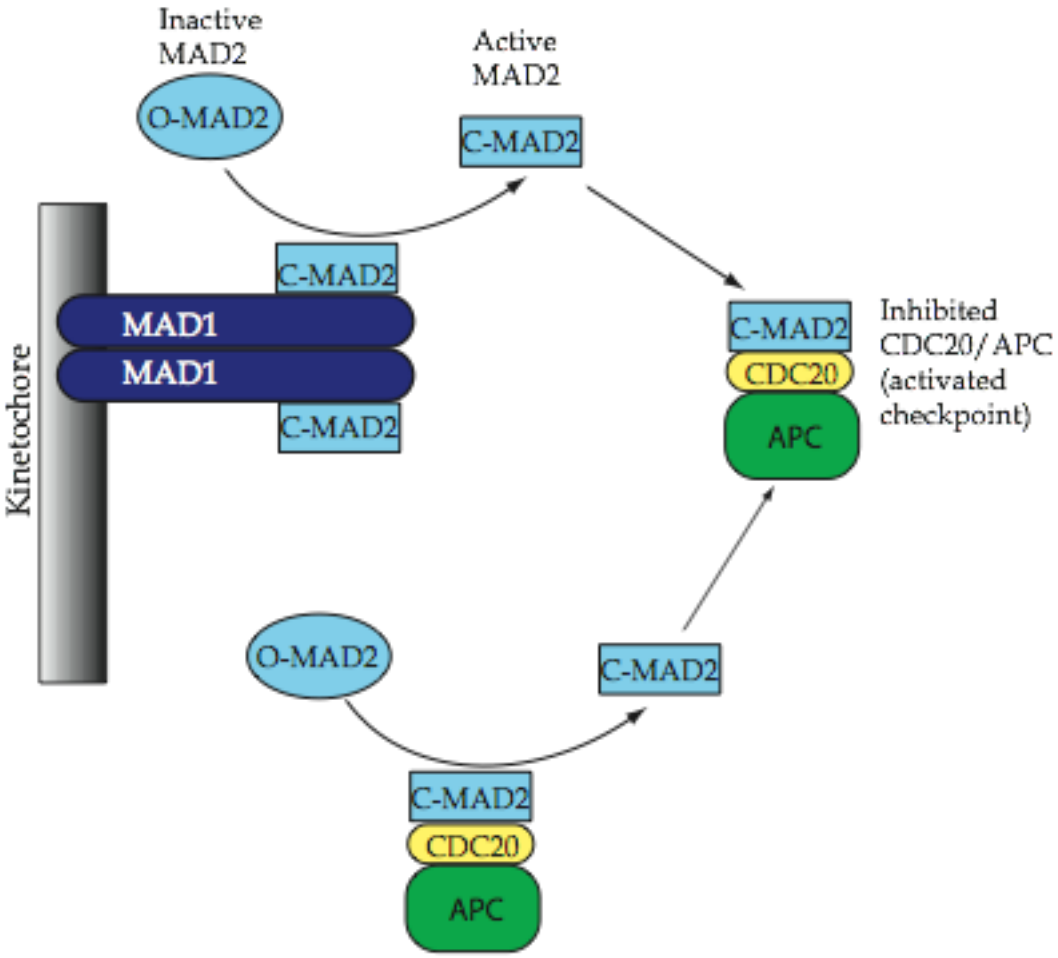

Figure 2.10. Model of MAD2 conformational switching. MAD2 is known to switch from the inactive, open form (O-MAD2) to the active, closed form (C-MAD2), and is thought to catalyze this conformational change through interaction of O- and C-MAD2. Current models consider C-MAD2 binding to MAD1 at the unattached kinetochore to be the first step, after which O-MAD2 will be converted to C-MAD2 through interaction with the MAD2/MAD1 complex. Additionally, cytoplasmic C-MAD2 may amplify the C-MAD2 levels by converting free O-MAD2 to C-MAD2 away from the kinetochores.

cysteine-rich domain of CHFR can disrupt the MAD2 spindle-assembly checkpoint pathway. In the absence of functional CHFR, MAD2 may not be efficiently transported to the kinetochores for activation (Figure 2.11). This disruption could impair the production of active MAD2, resulting in reduced inhibition of the anaphase-promoting complex, effectively repressing the spindle-assembly checkpoint and promoting anaphase progression. While CHFR has been defined as an E3 ubiquitin ligase [1], we could not see any measurable change in Mad2 or Cdc20 protein levels in cells expressing CHFRΔRing, a deletion that abolishes the ubiquitin ligase activity of CHFR (Figure 2.9) [15]. This suggests that the effect of CHFR on Mad2 activity is not dependent on ubiquitination. However, our results suggest an important role for Chfr in Mad2 localization and activation.

The cysteine-rich domain appears to be a major protein-protein interaction domain for CHFR, as it has been identified as the domain required for interactions with Aurora A, Kif22, and HDAC1 in addition to MAD2 [5, 20, 21]. The cysteine-rich domain also contains the PAR (poly(ADP-ribose))-binding zinc finger (PBZ) motif, which binds to PAR-bound proteins [22]. Interestingly, PAR-proteins accumulate on the mitotic machinery, and PAR addition to proteins is critical for spindle function [22, 23, 25]. It is possible that the cysteine-rich domain deletion is unable to localize to PAR-enriched sites, which then results in reduced binding to MAD2. Alternatively, CHFR binding to MAD2 could precede localization to the mitotic machinery, resulting in mislocalization of

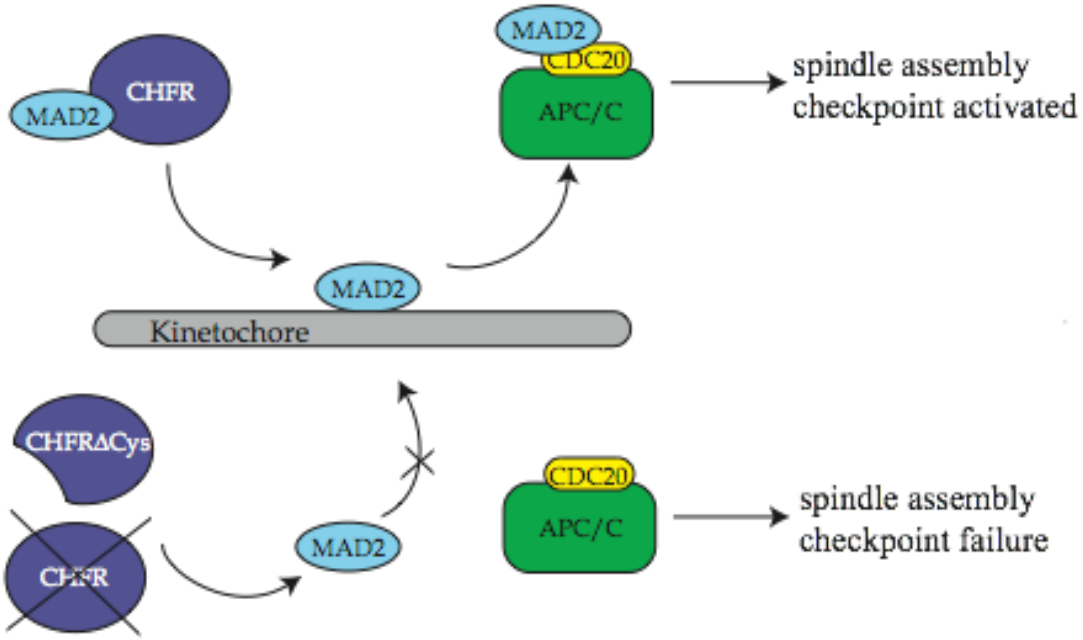

Figure 2.11. A model for CHFR regulation of MAD2 in the spindle assembly checkpoint. In the presence of CHFRΔCys, or in the absence of CHFR protein, MAD2 fails to localize to the kinetochore and does not bind CDC20 to inhibit the APC and trigger the spindle assembly checkpoint.

MAD2 when the PBZ domain is deleted. Extensive future work will be required to decipher the exact mode of action of CHFR with MAD2.

The interaction between CHFR and MAD2 represents another layer of complexity in the mitotic checkpoint function of CHFR, and may have important implications in cancer treatment. Both MAD2 and CHFR have been implicated as tumor suppressors, and changes in expression of both proteins have been linked to tumorigenesis [5, 87]. CHFR expression is reduced by promoter methylation in a wide array of cancer types [2]. A small number of *CHFR* mutations have been identified in cancer cells [2, 63]. One polymorphism (V539M) located in the cysteine-rich domain was reported to be strongly associated with colorectal cancer risk [64]. Surprisingly, MAD2 overexpression was reported to lead to tumorigenesis in mice and was shown to correlate to shorter survival in lung and bone cancers, suggesting that MAD2 has multiple functions in mitosis, which are sensitive to changes in MAD2 dosage [88, 92, 103]. Clearly both MAD2 and CHFR represent interesting candidates for cancer biomarkers. Further understanding of the interaction between CHFR and MAD2 may lead to increased accuracy in cancer prognoses as well as more fine-tuned cancer treatments in the future.

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Notes

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CHAPTER 3

CHFR protein levels may be regulated by microRNA in breast cancer lines

Abstract

CHFR protein levels are decreased in many breast cancer lines as well as multiple other cancers [2, 4]. Reduced transcription of *CHFR* due to promoter hypermethylation has been observed in many cancers, however this explanation does not appear to apply to breast cancers [4]. Preliminary experiments indicated that several breast cancer lines with low CHFR protein levels contained high levels of *CHFR* mRNA, suggesting a post-transcriptional regulation of *CHFR* expression is likely to be occurring in these cells. Several putative microRNA (miRNA) target sites are present in the 3'UTR of *CHFR*, leading to the hypothesis that miRNA binding may be responsible for the discord between the *CHFR* mRNA and protein levels. Several of the miRNAs predicted to target *CHFR* have been associated with cancers or cancer phenotypes. Utilizing a panel of breast cancer cell lines, we found that expression of miR-26 was high in each of the cell lines with high *CHFR* mRNA and low CHFR protein levels. Experiments using miR-26 inhibitors suggest a slight increase in CHFR protein

levels when miR-26 was inhibited, although these results were not significant. Further investigation is needed to determine the impact of microRNA inhibition on *CHFR*, and if microRNA inhibition is an alternate mechanism by which CHFR expression is lost in cancer cells.

Introduction

CHFR is a tumor suppressor protein that is implicated in multiple cancer-progression pathways [2, 3, 5, 89]. While significant effort has gone into identifying targets of CHFR, little is known about how CHFR activity itself is regulated. CHFR degradation via autoubiquitination is observed in cells, and deubiquitination of CHFR by USP7 can stabilize CHFR protein [15, 104]. However, there are conflicting reports regarding if CHFR protein levels are modulated between stages of the cell cycle [20, 52]. Recently, Stil (SCL/TAL1 interrupting locus) was identified as the first known inhibitor of CHFR [37]. Castiel *et al* showed that Stil$^{-/-}$ MEFs express increased levels of CHFR, and that Stil increases the autoubiquitination and degradation of CHFR [37]. Stil is reportedly overexpressed in lung cancers [105], and thus Stil expression changes could account for some loss of CHFR expression in cancers.

CHFR protein levels are reduced in a spectrum of cancer types, and in most cases evidence of *CHFR* gene promoter methylation has been found [2]. However, breast cancers appear to be the exception to this trend. A screen of 24 breast cancer cell lines found only two lines with evidence of *CHFR* promoter

methylation [4]. An extensive study of 110 primary breast carcinoma samples found only one case in which *CHFR* promoter methylation was present [65]. Finally, a recent study of ductal carcinoma *in situ* and adjacent invasive ductal cancer from 33 breast cancer patients found little evidence of *CHFR* promoter methylation in either tissue type (3% in ductal carcinoma *in situ*, none in invasive ductal cancer) [106]. Several alternate pathways could account for reduction in CHFR protein expression, including reduced mRNA or protein stability, or reduced gene transcription. Of particular interest is post-transcriptional regulation by microRNA-based inhibition. Many microRNA genes (designated "*mir*") are located in regions of genome instability in cancers, and changes in miRNA expression have been implicated in cancer progression [107, 108]. In breast cancer, miRNAs have been reported to suppress metastases and 35 *mir* genes were identified on genomic regions commonly amplified or lost in breast cancers [107, 109-111]. We therefore hypothesized that microRNA expression could account for the discord observed between *CHFR* mRNA and protein levels in breast cancer cell lines, and could also represent a novel mechanism of CHFR regulation.

Materials and Methods

Cell culture and transfection

Cell lines were acquired from ATCC and grown as recommended. 50 nM antisense microRNA inhibitors (anti-miR miRNA inhibitors, Applied

Biosystems) were transfected into HEK293 cells using Dharmafect 2 reagent (Dharmacon) according to manufacturer's protocol. Cells were lysed for qPCR and Western blot 48 hours post-transfection.

Western blot

Lysate samples were run on a 10% SDS-PAGE gel, and then transferred to PVDF. Proteins were immunoblotted with anti-CHFR (Abnova) and anti-GAPDH (Sigma) as a loading control. Protein quantities were estimated using ImageJ software (http://rsbweb.nih.gov/ij/).

Semi-quantitative RT PCR and qPCR

RNA was isolated using the miRNeasy kit from Qiagen. Semi-quantitative PCR was performed as previously described, using a touchdown PCR program with three cycles at 62°, three cycles at 60°, and eighteen cycles at 58°, [3]. For *CHFR* and *GAPDH* measurement, cDNA was produced using the Omniscript RT kit (Qiagen). Quantitative RT-PCR was performed using Taqman gene expression assays (Applied Biosystems) for *CHFR* (Hs00946136_m1) and GAPDH (Hs99999906_m1) according to the manufacturer's protocol. For microRNAs, RT was performed using Taqman microRNA Reverse Transcription Kit (Applied Biosystems), with microRNA-specific primers, followed by qPCR using Taqman MicroRNA Assays (Applied Biosystems) according to the manufacturer's protocol.

Results

Discordance between mRNA and protein levels of CHFR breast cancer cell lines

Reduced expression of CHFR is seen frequently in breast cancers as well as other cancers [2]. However, unlike in other cancers in which promoter methylation and a few rare mutations have been found, no cause has yet been identified for the reduced CHFR levels in breast cancer [2, 4, 65]. To address this question, we utilized a panel of nine breast cancer cell (BCC) lines, as well as three immortalized mammary epithelial cell (IHMEC) lines, and examined the *CHFR* mRNA and protein levels in each cell type. Semi-quantitative RT-PCR analysis indicated that every cell line, with the exception of the BCC DU4475, contained some *CHFR* mRNA (Figure 3.1). Western blot analysis of CHFR protein showed much more variation (Figure 3.2), and suggested that mRNA levels in these lines do not correlate well to protein levels. *CHFR* mRNA was also assayed using the more sensitive method of real-time PCR, the results of which were similar to the semi-qPCR results (Figure 3.3). Side-by-side comparison of *CHFR* mRNA and protein measurements is indicated in Figure 3.4. These results indicate that *CHFR* mRNA and protein levels do not correlate well in BCC lines.

The CHFR 3′UTR contains putative miRNA binding sites

Growing evidence implicates miRNA inhibition in the regulation of gene expression, specifically by interfering with mRNA stability or translation [107].

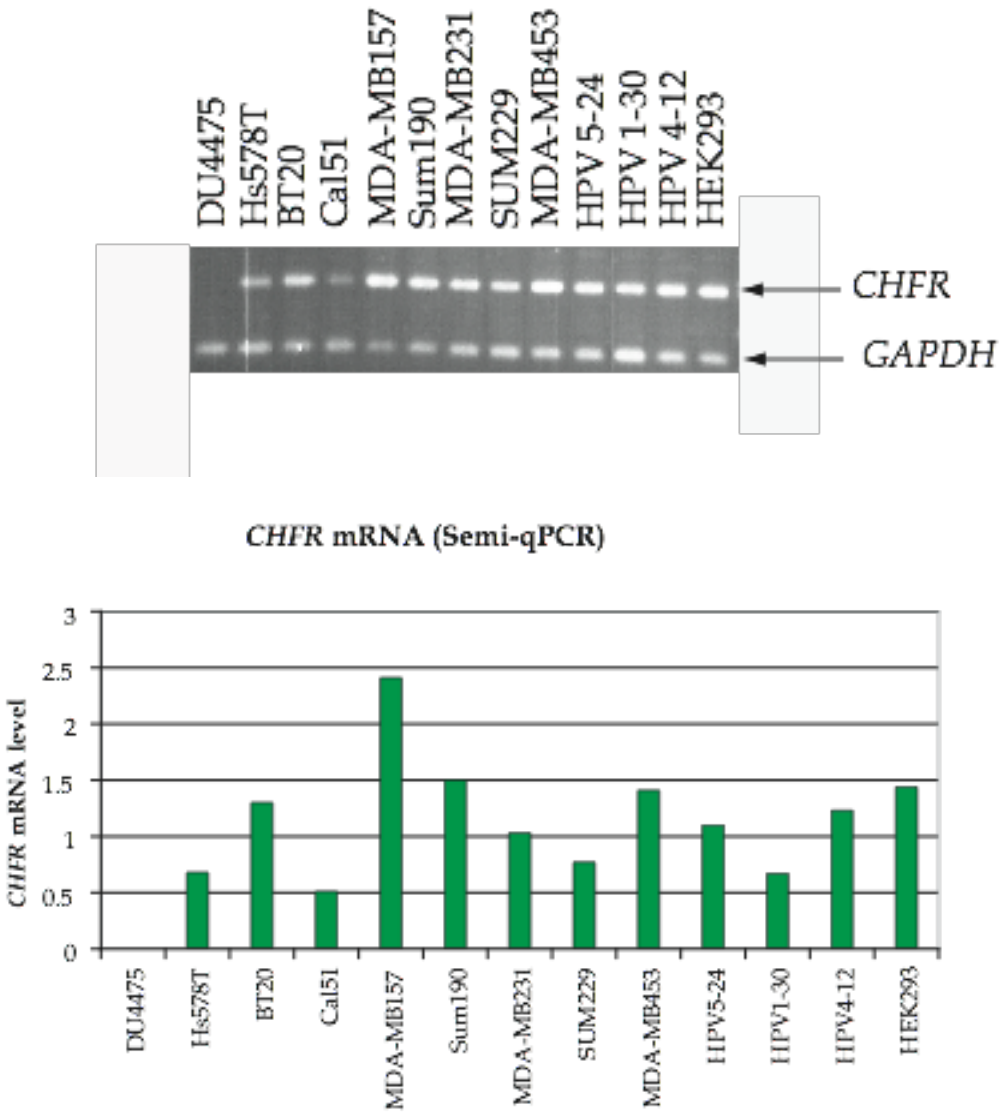

Figure 3.1. mRNA levels of *CHFR* in breast cancer cell lines, as measured by semi-quantitative PCR. Quantitation of PCR products relative to *GAPDH* is shown below the gel picture.

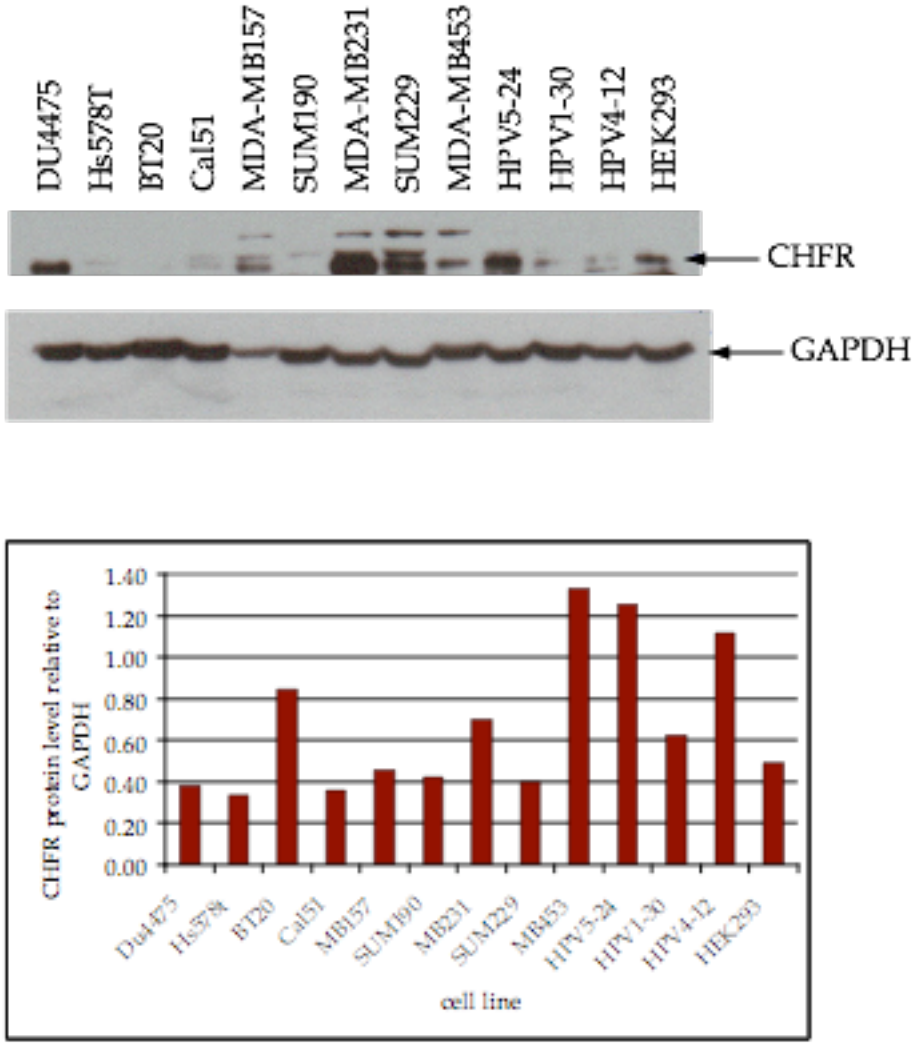

Figure 3.2. CHFR protein levels vary between breast cancer cell lines. BCC lines were analyzed for CHFR protein levels by Western blot. GAPDH protein levels were used as a loading control. Below, CHFR protein levels are quantitated relative to GAPDH and normalized to the average of the HPV lines.

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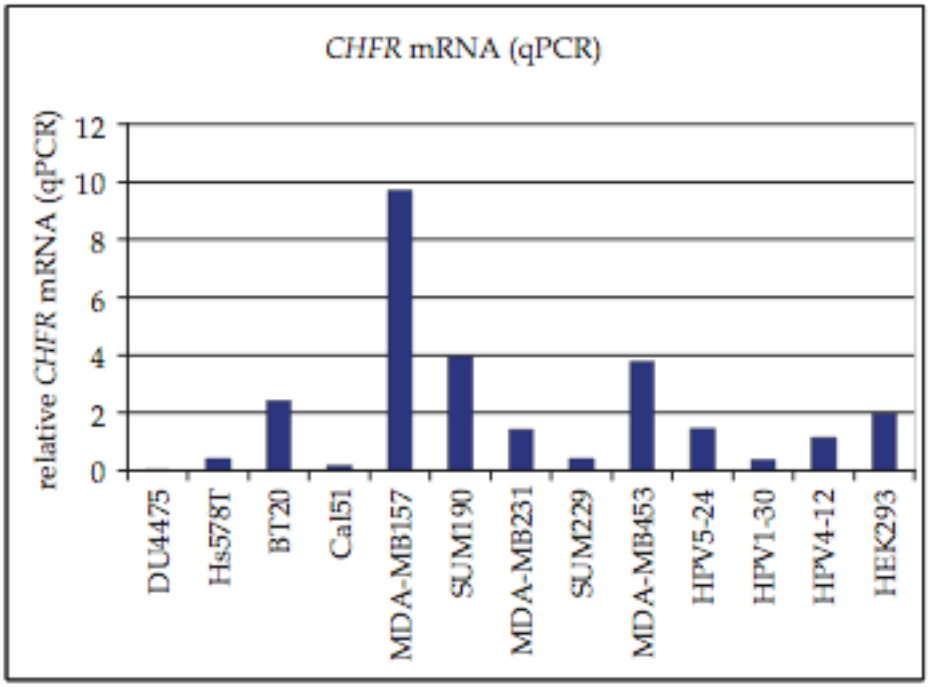

Figure 3.3. qPCR measurement of *CHFR* mRNA levels. *CHFR* levels were measured using qPCR and normalized to the average of the HPV lines.

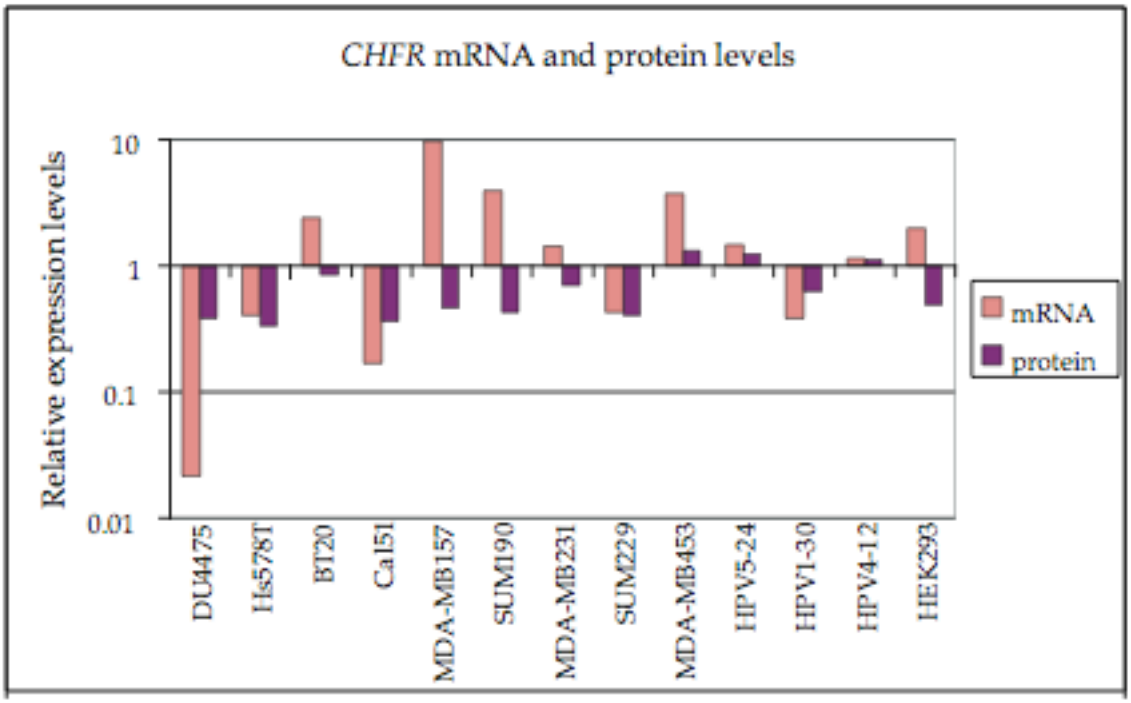

Figure 3.4. *CHFR* mRNA and protein levels do not correlate in BCC lines. Side-by-side comparison of mRNA levels measured by qPCR and protein levels. Values represent the relative expression of *CHFR* mRNA and protein normalized to GAPDH within each cell line. The value of '1' represents the average of the expression levels measured in HPV lines, to which each cell line was compared.

In cases in which translation is prevented, often seen when miRNA binds with imperfect base pairing [107], we expect to see low protein levels in spite of high mRNA levels, which we observed for multiple BCC lines. Targetscan (www.targetscan.org) search of human *CHFR* revealed several putative miRNA-binding sites in the 3'UTR of *CHFR* (Figure 3.5). Interestingly, three of the implicated miRNAs with high Targetscan scores, miR-26, miR-218 and miR-221/222, have previously been associated with cancer phenotypes (Table 3.1). Upregulation of miR-26, which encodes two isoforms (designated a and b), is associated with hypoxia and breast cancer, as well as other cancers [112, 113]. We chose to prioritize these microRNAs in our study due to the existing links between their expression and cancer phenotypes.

microRNA-26a and b have high expression in a subset of BCC lines expressing high CHFR mRNA levels and low CHFR protein levels

To understand the relationship between these microRNAs and CHFR protein expression, we first divided the BCC panel into three subsets based on *CHFR* mRNA and CHFR protein levels; high mRNA/low protein (high/low), high mRNA/high protein (high/high), and low mRNA/low protein (low/low). The designations of "high" and "low" expression indicate the levels relative to the average of three IHMEC lines. No lines were identified displaying low mRNA/high protein, possibly due to this normalization, and the IHMEC lines (HPV 1-30, HPV 5-24 and HPV 4-12) are categorized to the low/low and

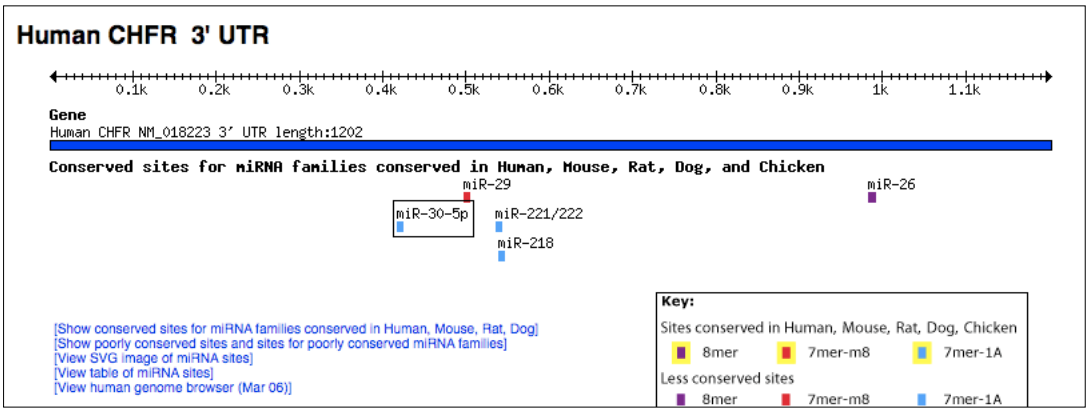

Figure 3.5. Putative miRNA binding sites on the *CHFR* 3'UTR. Targetscan results for *CHFR* indicate several putative microRNA binding sites in the 3'UTR of the *CHFR* gene.

miRNA	Targetscan score	Associated with:
hsa-miR-26	26a: 99 26b: 99	Colorectal Cancer [114] General cancers [112] Hepatocellular carcinoma [115] Hypoxia and breast cancer [112, 113] Nasopharyngeal carcinoma [116] Thyroid carcinoma [117] Tongue squamous cell carcinoma [118]
hsa-miR-218	57	ALL (leukemia) [119] Bladder cancer [120] Cervical carcinoma [121] Lung squamous cell carcinoma [122]
hsa-miR-221/222	221: 68 222: 66	Breast Cancer [123] Gastric Cancer [124] Glioma [125] Hepatocellular carcinoma [126, 127] Ovary and bladder cancer (221) [128] Papillary thyroid carcinoma [129, 130] Prostate cancer [131]

Table 3.1. Putative *CHFR*-targeting miRNAs are associated with cancers and cancer phenotypes.

high/high subsets. We examined the expression levels of miR-26a, miR-26b, miR-218, miR-221 and miR-222 in each of these subsets of cell lines using qPCR. Strikingly, each of the cell lines in the high/low subset expressed at least one form of miR-26 at high levels (Figure 3.6), while miR-218 and -221/222 were observed at both high and low levels within this subset (Figure 3.7). miRNA expression levels in the low/low and high/high subsets showed no obvious trends (Figures 3.8 and 3.9). This data suggest that miR-26 could be involved in preventing translation of *CHFR* mRNA, and could explain reduced CHFR expression in some BCC lines.

Inhibition of miR-26 has little effect on CHFR protein levels

To directly examine the effect of miR-26 modulation on CHFR protein levels, we employed a miR antisense inhibitor to reduce the levels of miR-26a and miR-26b in HEK293 cells. As illustrated in Figure 3.10, the miR-26 antisense inhibitor reduced the levels of miR-26a and miR-26b by approximately 90% and 70%, respectively. Cells that had been transfected with the miR-26 inhibitor were then analyzed for CHFR protein levels using western blot. Relative to the non-specific inhibitor, CHFR protein levels measured as increased by an average of 15% when miR-26 was inhibited (Figure 3.11), using ImageJ. This method of measuring band size and intensity is not as sensitive as required for this change to be considered significant. Upon more stringent testing, miR-26 inhibition could support the hypothesis that miR-26 is inhibiting CHFR protein translation in some cells.

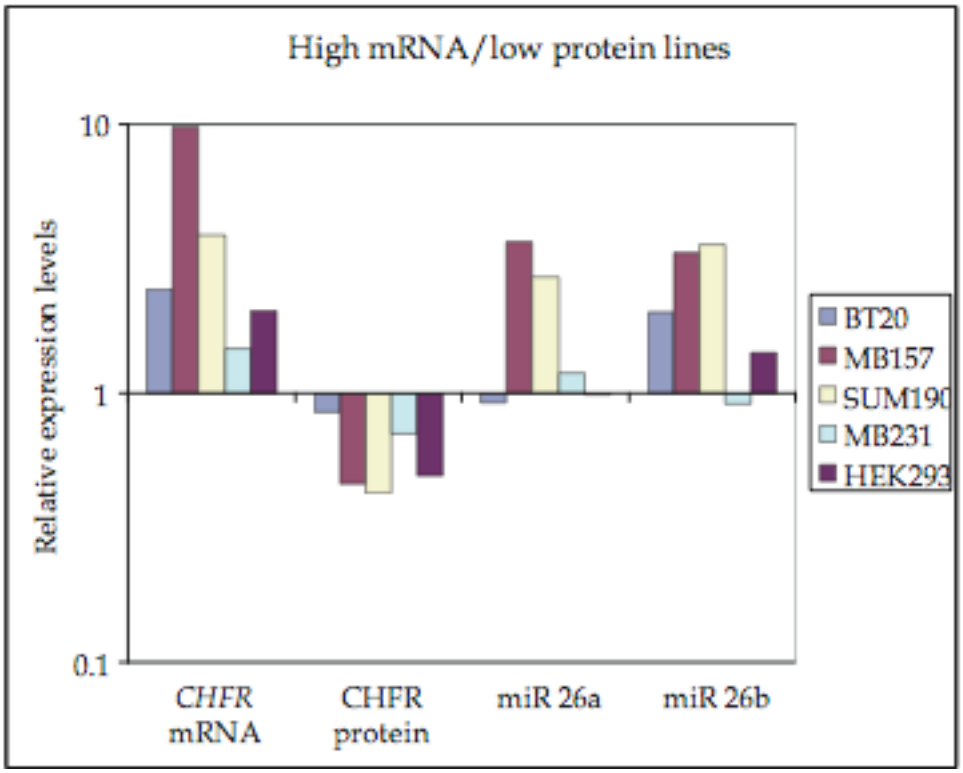

Figure 3.6. miR-26 is expressed at high levels in cells with high *CHFR* mRNA and low CHFR protein. Levels of miR-26a or -26b, or both are high in each cell line of the high/low set. Values represent the relative expression of *CHFR* mRNA and protein normalized to GAPDH within each cell line. The value of '1' represents the average of the expression levels measured in HPV lines, to which each cell line was compared.

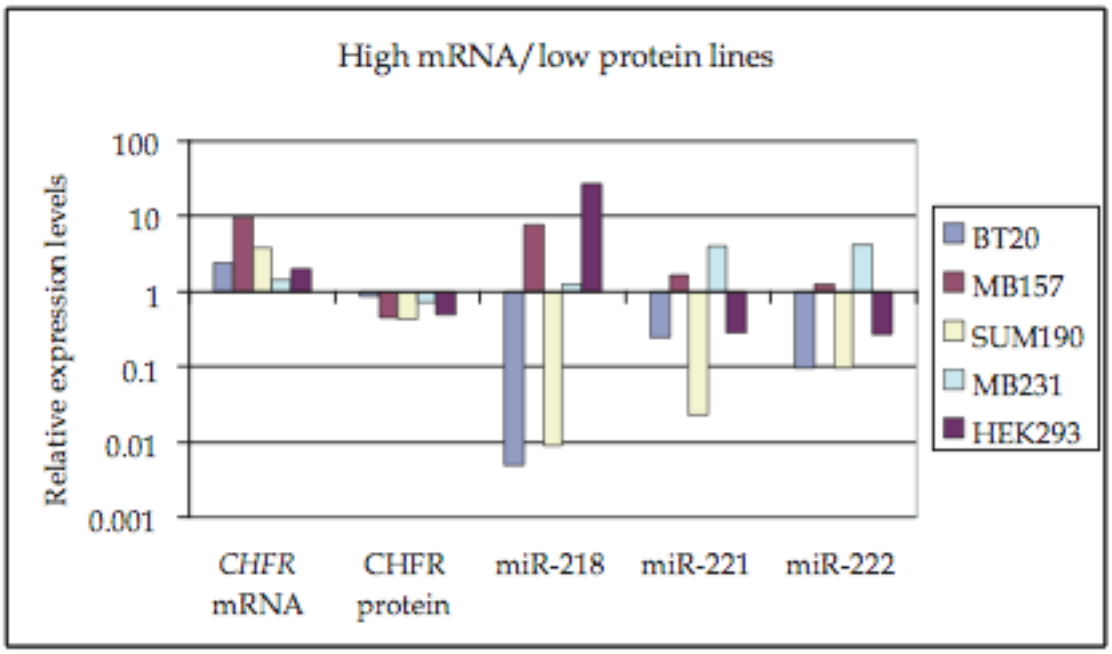

Figure 3.7. miR-218 and miR-221/222 expression in high mRNA/low protein lines. miR-218 and -221/222 do not show a trend in the high/low cell line set. Values represent the relative expression of *CHFR* mRNA and protein normalized to GAPDH within each cell line. The value of '1' represents the average of the expression levels measured in HPV lines, to which each cell line was compared.

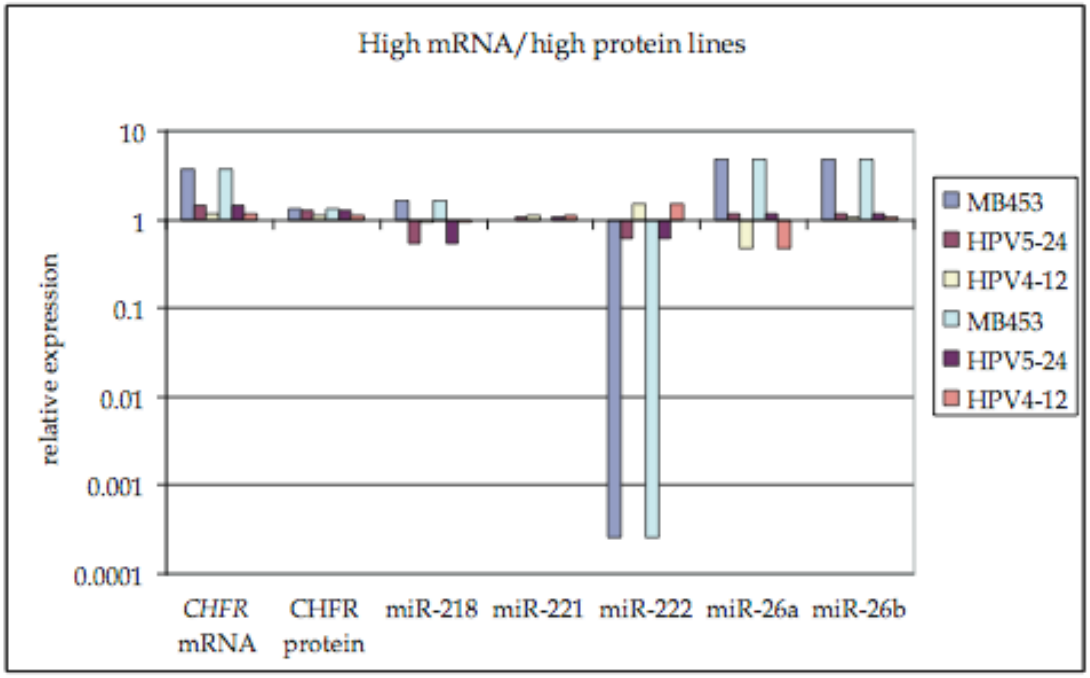

Figure 3.8. miRNA expression levels in the high mRNA/high protein cell lines. Values represent the relative expression of *CHFR* mRNA and protein normalized to GAPDH within each cell line. The value of '1' represents the average of the expression levels measured in HPV lines, to which each cell line was compared.

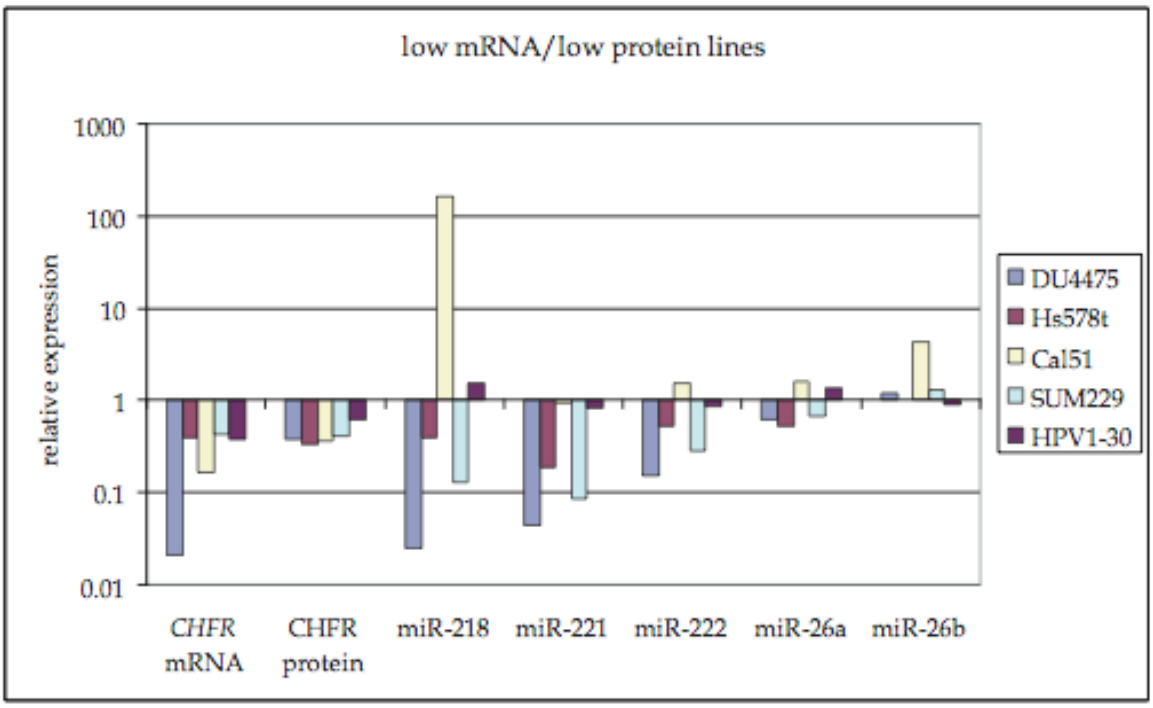

Figure 3.9. miRNA expression levels in the low mRNA/low protein cell lines. Values represent the relative expression of *CHFR* mRNA and protein normalized to GAPDH within each cell line. The value of '1' represents the average of the expression levels measured in HPV lines, to which each cell line was compared.

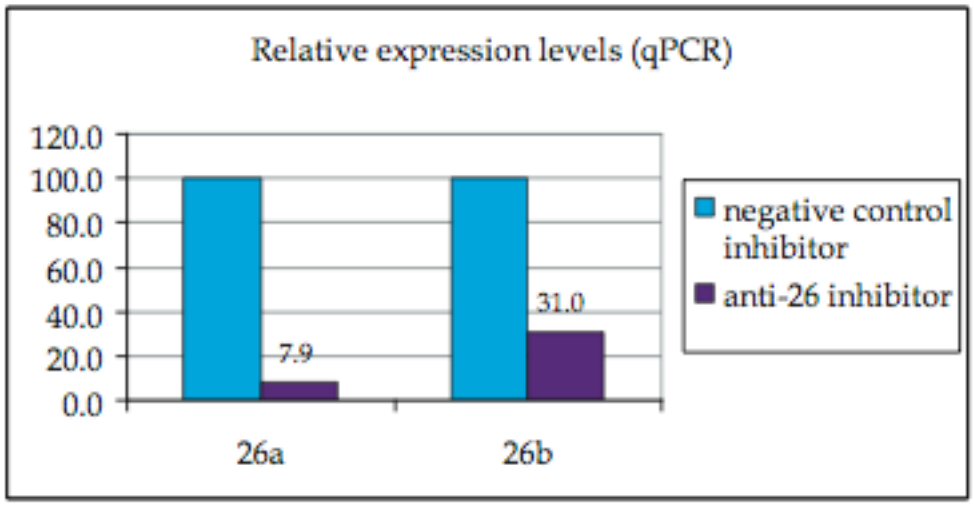

Figure 3.10. Inhibition of miR-26a and miR-26b by antisense inhibitors. Antisense miR-26 inhibitors were transfected into HEK293 cells. Reduction of miR-26a and miR-26b was measured by qPCR and the magnitude of knockdown relative to non-specific inhibitor is depicted in the histogram.

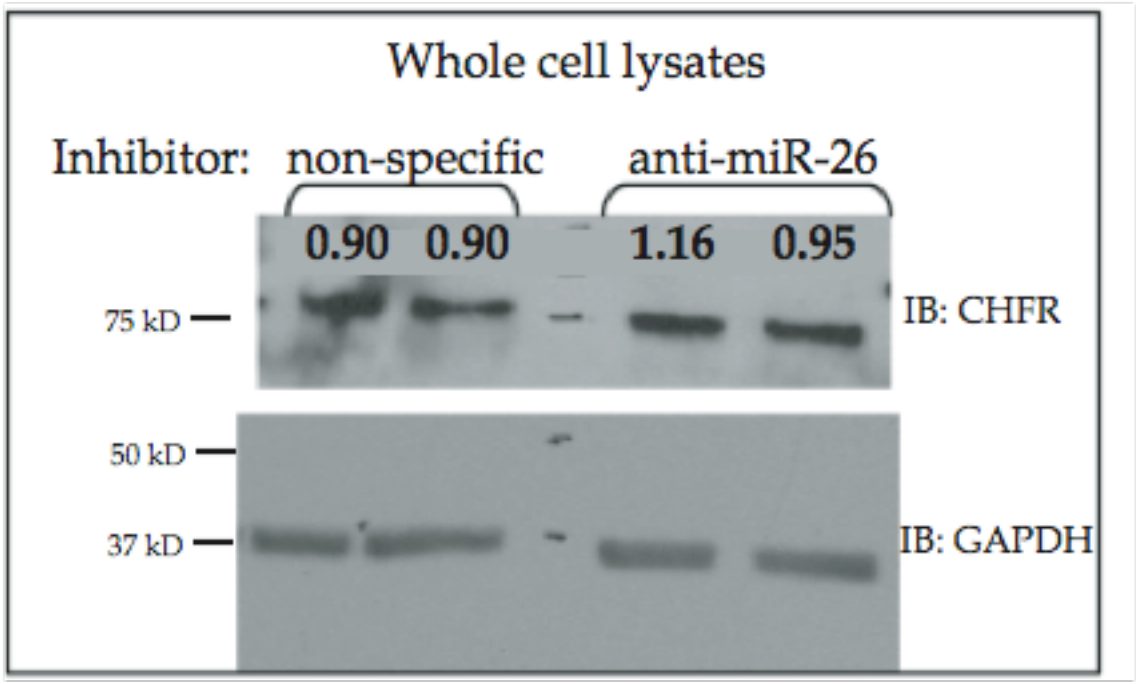

Figure 3.11. CHFR protein levels after miR-26 inhibition. Western blot was used to observe CHFR protein levels in HEK293 cells after transfection with miR-26 inhibitor, or a non-specific control inhibitor. Bands were quantitated using ImageJ, and normalized band measurements are indicated for each lane. On average, miR-26 inhibition increased the CHFR protein measurement by 15%.

Discussion

Regulation of transcripts by microRNAs is observed in a range of normal and disease-related processes including cancer [107]. Regulation of the cell cycle and cell proliferation are no exception [132, 133]. Expression profiles of miRNAs are currently studied in cancers to identify players involved in initiation and progression of tumors. MicroRNA expression has been investigated in breast cancers, and has identified several microRNAs of interest for understanding disease evolution as well as for use as prognostic biomarkers [113, 134]. Interestingly, several microRNAs have been identified that are modulated by estrogen receptor-α (ERα) expression in breast cancer cell lines, and some of those microRNAs appear to target ERα mRNA as well [135]. As estrogen receptor status has a major impact on efficacy of breast cancer treatment [136], an understanding of microRNA expression and regulation could have significant impact on clinical understanding of the disease.

Here, we show data that several breast cancer cell lines express high levels of *CHFR* mRNA, while maintaining low CHFR protein levels. One possible explanation for this finding is negative regulation at the level of mRNA translation, by microRNA binding. Using a panel of breast cancer cell lines, we found an association between high expression of miR-26 and a high/low relationship between *CHFR* mRNA and protein. We further investigated if miR-26 binding was responsible for inhibition of CHFR protein production using antisense miR-26 inhibitors. The results indicate that reduction of miR-26 in

HEK293 cells may result in a small increase in CHFR protein expression, lending support to the miR-26 inhibition model.

Several considerations must be made when interpreting these results. The miR-26 inhibition experiment was performed using only HEK293 cells, and a different outcome might be seen in other cell types. Additionally, the rapid growth rate of HEK293 cells limited the timeline of the experiment to 48 h post-transfection, as samples taken beyond 48 h no longer displayed miR-26 inhibition (data not shown). It is possible that this window of time is too short, and that if inhibition were continued a more dramatic effect could be seen. Alternatively, the small amount of miRNA remaining after inhibition could be sufficient to inhibit CHFR expression. We utilized IHMEC lines as a tool for normalizing the levels of miRNA expression measured in the breast cancer panel. It is entirely possible that these lines do not represent 'normal' levels of miRNA due to many factors such as immortalization or time in culture. Therefore the designations of 'high' or 'low' expression must be used cautiously.

Recent studies have identified miR-26 as a microRNA of interest in cancers. Sander *et al* demonstrated that repression of miR-26a by the oncogene c-MYC resulted in increased expression of Enhancer of Zeste Homolog 2 (EZH2), a protein that regulates gene expression and is a known oncogene [137]. A study of colorectal cancer cell lines identified miR-26b as a microRNA that was significantly downregulated in colorectal cancer cell lines, and overexpression of miR-26b reduced the growth of these cells [114]. Additionally, *CHFR* was

identified as a likely target of miR-26b in these cells, although the growth suppression phenotype seen in miR-26b overexpressing cells is opposite of what would be expected for an inhibitor of *CHFR* [114]. This most likely reflects the complexity of the regulation by microRNAs, and could be a result of miR-26 having different effects in different tissue types.

Several other explanations for loss of CHFR in breast cancer lines are possible. Two publications have reported evidence that *CHFR* promoter methylation is rare in breast cancers [4, 65], and the presence of high mRNA levels in some BCC lines supports this finding. However, each study utilized only one method of testing for promoter methylation, and methylation might have been observed in cell lines with low *CHFR* mRNA levels if multiple methods were utilized. It is possible that *CHFR* mRNA stability is altered by factors other than miRNA, such as altered binding of an unknown protein that targets *CHFR* mRNA for degradation. This could occur through mutation of binding sites on the *CHFR* mRNA, or altered activity of the binding protein. Possibly more likely is alteration of CHFR protein stability, through loss or mutation of USP7, which deubiquitinates and stabilizes CHFR protein [104], or through an increase in Stil activity, which increases ubiquitination and degradation of CHFR protein. To our knowledge, levels of these proteins in breast cancer lines have not been measured. Examination of the expression and mutation status of USP7 and Stil in BCC lines with low CHFR levels could lead to a new hypothesis for the cause of CHFR loss in breast cancer lines. It may turn

out that multiple mechanisms of CHFR loss are occurring independently in breast cancers, and that miRNA inhibition may only be a factor in a subset of BCC lines.

Clearly more experiments are needed to fully understand the role of microRNA inhibition in regulating CHFR expression. First steps should include overexpression of miR-26 in cells to examine if an increase in miR-26 will result in a decrease in CHFR protein levels. Expression constructs linking the *CHFR* 3'UTR to a fluorescent reporter can be used to measure the effect of miRNA modulation on protein expression, and miRNA targeting could be verified through mutation of the target sites. Finally, the impact of miR-26 expression modulation on cancer progression phenotypes in BCC lines would be extremely informative regarding the impact of miR-26 on breast cancer progression. Further study of this pathway could advance understanding of CHFR expression loss in breast cancers and open doors to new breast cancer treatments in the future.

Acknowledgements

We would like to thank Dr Elif Erson Bensan (Middle East Technical University) for critical discussion and advice. This project was inspired by observations made by Dr Lisa Privette Vinnedge during her work in the Petty lab.

CHAPTER 4

Conclusions

CHFR plays a role in the spindle assembly checkpoint through MAD2/CDC20

CHFR was originally identified as an antephase checkpoint protein important for cell cycle delay in response to microtubule stress before commitment to mitosis [1]. Privette *et al* reported that CHFR could bind to and appeared to regulate the function of MAD2, implicating CHFR in a second cell cycle checkpoint. The interaction between MAD2 and CDC20 has been established as a critical component of the spindle assembly checkpoint pathway important for maintaining genome stability [49, 51]. This interaction impairs the anaphase-promoting complex through CDC20, and is abolished when proper spindle attachment to kinetochores is achieved [51, 58, 98].

Our data show that loss of CHFR or the cysteine-rich domain of CHFR can disrupt the MAD2 spindle-assembly checkpoint pathway. We show that the cysteine-rich domain of CHFR, which includes the PBZ domain, was required for CHFR/MAD2 interaction. Furthermore, *CHFR*ΔCys expression in *Chfr*$^{-/-}$ MEF cells resulted in mislocalization of Mad2, impairing the interaction of Mad2 and Cdc20, and increasing the incidence of chromosome segregation defects.

In the absence of functional CHFR, MAD2 may not be efficiently transported to the kinetochores for activation (Figure 2.11), allowing the spindle assembly checkpoint to be bypassed. We were unable to show that the punctate localization of Mad2, which is lost in the *Chfr-/-* cells, is in fact at the kinetochore, however there is a clear mislocalization of Mad2 observed when Chfr is lost (Figure 2.4). This disruption could impair the production of active MAD2, resulting in reduced inhibition of the anaphase-promoting complex, effectively repressing the spindle-assembly checkpoint and promoting anaphase progression. The lack of phenotype observed in the CHFRΔFHA and CHFRΔRING expressing cells is of particular interest, because these two domains have been shown to be critical to CHFR function in the antephase checkpoint [1, 15]. This also suggests that the effect of CHFR on MAD2 activity is not dependent on ubiquitination, and must be occurring by some other mechanism.

The cysteine-rich domain appears to be a major protein-protein interaction domain for CHFR, important to the interactions of CHFR with several downstream targets including MAD2 [5, 20, 21]. The cysteine-rich domain also contains the PAR (poly(ADP-ribose))-binding zinc finger (PBZ) motif, which binds to PAR-bound proteins [22]. Interestingly, PAR-proteins accumulate on the mitotic machinery, and PAR addition to proteins is critical for spindle function [22, 23, 25]. This suggests that CHFR may depend on PAR-binding for some or all of its functions.

Significance

The data presented in Chapter 2 enhance our understanding of CHFR function by implicating CHFR in the spindle assembly checkpoint and indicate that the function of CHFR is not solely ubiquitination-related. CHFR interaction with MAD2 appears to be critical for MAD2 localization, and also MAD2 function in the spindle checkpoint. The interaction between CHFR and MAD2 represents another layer of complexity in the mitotic checkpoint function of CHFR, as well as MAD2.

Furthermore, the requirement of the cysteine-rich domain of CHFR but not the FHA or RING domains for MAD2 regulation indicates a more important role for the cysteine-rich domain than previously recognized. This domain has been understood to function in CHFR ubiquitination target recognition, however MAD2 does not appear to be ubiquitinated by CHFR. As the only domain found to affect MAD2-related function, the cysteine-rich domain, may have other properties important for MAD2 localization and activation.

Future work will be needed to determine the full function of the CHFR cysteine-rich domain. The presence of the PBZ domain points to PAR binding as a mechanism for CHFR target interactions. However, the cysteine-rich domain extends significantly beyond the PBZ domain, and it is well conserved between mouse and human [1, 22]. Conservation of this domain suggests that it may have an important function itself, and individual deletions of the PBZ domain or

the cysteine-rich domain outside of the PBZ domain will be needed to determine if this is the case.

Ahel *et al* have demonstrated that the PAR-binding function of CHFR is necessary for checkpoint activation, and inhibition of PARP also inhibited checkpoint activation [22]. While CHFR was shown to bind PAR [22], no PAR-modified substrates of CHFR have been identified, and identification of such substrates would enhance our understanding of CHFR function. Previous studies have concluded that the cysteine-rich domain of CHFR is not required for its growth inhibitory function [9]. Coupled with the data presented here indicating that the ubiquitin ligase function of CHFR is not required for regulation of MAD2, it seems that CHFR may have multiple functions that are dependent on distinct domains of the protein.

The mechanism of CHFR regulation of MAD2 is another question raised by this work. CHFR could be critical for MAD2 activation, and it would be interesting to examine if the levels of O-MAD2 and C-MAD2 are altered in cells lacking CHFR or expressing the ΔCys construct. Additionally, existing MAD2 mutants that favor the active conformation [97] could be used to test if expression of C-MAD2 can bypass the requirement for the CHFR cysteine-rich domain and attenuate the phenotypes shown in this work as well as tumor-related phenotypes seen in cancer cells lacking CHFR.

Loss of CHFR in cancer may occur by multiple pathways

CHFR protein levels are decreased in many cancers including breast cancers [2, 4]. *CHFR* promoter hypermethylation is observed in most cancer types, but has not been observed in breast cancers [2, 4]. In addition a small number of *CHFR* mutations have been identified, however these mutations are exceedingly rare [2].

In Chapter 3, evidence is presented that microRNA inhibition may be responsible for reduced CHFR levels in breast cancer cell lines. Investigation of mRNA and protein levels in a BCC panel indicates that the amount of mRNA does not correlate as expected to protein levels in several of the BCC lines. Additionally, cells in which high *CHFR* mRNA levels are accompanied by low CHFR protein levels display high expression of miR-26. This suggests that translation of CHFR protein may be inhibited by miR-26. Finally, preliminary evidence suggests that reduction of miR-26 in HEK293 cells could increase CHFR protein levels. These data indicate that microRNA pathways may be relevant to *CHFR* regulation, and could have special relevance to breast cancers.

microRNA inhibition of gene expression represents a significant increase in the complexity of the information carried by the genome [107]. MicroRNA expression has been investigated in breast cancers, and these studies have identified several microRNAs with potential to be disease biomarkers [113, 134]. This work is not the first to identify miR-26 as a microRNA of interest in cancer, lending support to the idea that it may be involved in *CHFR* regulation. miR-26b

was observed to be downregulated in colorectal cancer lines, and overexpression of miR-26b reduced the growth of these cells [114]. Additionally, *CHFR* was identified as a likely target of miR-26b in these cells [114]. However, we would expect that downregulation of *CHFR* by miR-26b would increase cancer-related phenotypes, while a decrease in growth was observed when miR-26b was overexpressed [114]. It is likely that the effect of miR-26 on cellular phenotypes is complex, involving multiple target genes, and different effects could be seen based on tissue-specific factors.

Significance

The work described in Chapter 3 forms the basis for further studies of microRNA regulation of *CHFR*. The association between apparent mRNA transcription levels and miR-26 expression implicates miR-26 in regulation of *CHFR* in breast cancer lines. This association is particularly interesting in the context of breast cancers in which the basis for reduced CHFR protein levels is unexplained, and could have implications for other cancer types as well. In addition, this association may be relevant to CHFR function in normal cells as well.

Further insight into the regulation of *CHFR* could inform both developmental biology and cancer biology fields. If miR-26 were found only to affect CHFR levels in cancers originating from the breast, it may indicate that tissue-specific factors were at play. miR-26 inhibitors could be explored as a

novel treatment option for patients displaying aberrantly high miR-26 expression in tumors. Further study of miRNA impact on *CHFR* could lead to a better understanding of CHFR regulation in normal cells as well as in breast cancer cells, and ultimately bring about more effective new treatments for cancer patients.

Future Directions

CHFR functions as a regulator of the spindle assembly checkpoint

The data shown here raise several new questions about CHFR function in the spindle checkpoint as well as the mechanisms regulating CHFR function itself. Further understanding of these properties could have significant impact on cancer treatment; so future work should include investigations into the properties of primary cancer samples and cell lines.

Many questions remain about the interaction between MAD2 and CHFR. While the interaction between the two proteins has been verified in this work, we do not know if this interaction is direct or occurs through a complex of proteins. Purified proteins must be used to determine if this interaction is indeed direct. If not, identification of the CHFR and MAD2 interacting protein could help to explain the mechanism by which CHFR impacts MAD2 localization and function.

Additionally, we have not identified the point in the MAD2 signaling pathway that the CHFR interaction is occurring. Based on the mislocalization of

Mad2 in *Chfr*$^{-/-}$ MEFs, and the reduction of the Mad2-Cdc20 interaction, we hypothesize that CHFR disrupts kinetochore localization and activation of Mad2 in mitosis. However, this mislocalization could be occurring after Mad2 activation, or it could be a result of Mad2 stabilization in the cell. Localization of CHFR relative to MAD2 could be examined using a very sensitive system such as FRET to determine if CHFR is bound to MAD2 at the kinetochores, or in the cytoplasm, or both. This will also lend insight into the mechanism by which CHFR regulates MAD2; does it simply affect MAD2 localization and therefore impact activation of MAD2, or is CHFR involved with MAD2 after activation as well?

We show evidence that MAD2 function is decreased in the absence of functional CHFR, and that the localization of MAD2 is also affected, suggesting that MAD2 fails to localize to the kinetochore to be activated. However, much more data is required to support this model. The conformation of MAD2 when bound to CHFR should be examined to identify where in the cycle of MAD2 CHFR is functioning. Furthermore, the conformation of MAD2 in the absence/presence of functional CHFR should be tested, to determine if C-MAD2 levels are in fact reduced when CHFR is absent or unable to bind.

The ability of MAD2 expression, or conformational mutants of MAD2, to compensate for a lack of CHFR could be explored with regard to the chromosome instability phenotype shown here. Mutants of MAD2 that favor one conformation over the other have been identified and could lend insight into

the specific impact of *CHFR* loss or mutation on MAD2 function, through rescue studies of phenotypes such as increased incidence of lagging chromosomes. Clearly the work here is merely the jumping off point for extensive inquiry into the role of CHFR in the MAD2-dependent spindle assembly checkpoint.

While the FHA and RING domain deletion constructs did not impact MAD2 localization, MAD2 interaction with CDC20, or the incidence of lagging chromosomes, it is possible that these domains still play a role in MAD2 function, for example as negative regulators of MAD2. The model for MAD2 deactivation involves $p31^{comet}$, which competes for binding sites on C-MAD2 to prevent conversion of O-MAD2 to C-MAD2 [100, 102]. This model implies that there is a mechanism to turn over C-MAD2 that has already been made. CHFR could be active in that process, by ubiquitination of C-MAD2, or other proteins that effect MAD2. Such an effect would not have been observed in our tests. Thus, MAD2 ubiquitination, and the effect of CHFR deletions on MAD2 stability should be closely examined.

Finally, the role of the PBZ domain of CHFR in the MAD2 spindle assembly checkpoint, as well as the early mitotic checkpoint, is a critical question in the understanding of CHFR function. PAR modifications are enriched at the mitotic spindle, and reduction of PAR has been shown to disrupt the mitotic spindle structure [25, 26]. ADP-ribose chains have been hypothesized to have a role in the mechanics of spindle movement as well [138]. While the existence of PAR modifications has been known for quite some time, how this modification

regulates cellular processes is still not understood, and few PAR-binding proteins have been identified. The importance of the PBZ domain of CHFR in its mitotic checkpoint functions suggests that PAR recognition could play a critical role in CHFR function. MAD2, as well as several other CHFR substrates, interacts with CHFR in a cysteine-rich domain-dependent fashion. The obvious next question is; does CHFR interact with MAD2 through the PAR-binding properties of its PBZ domain? Mutation of the PBZ domain of CHFR should be used to determine the importance of PBZ to MAD2 binding. In addition, poly(ADP-ribose)-specific antibody could be utilized to test purified MAD2 for PAR modification. It may also be that the PBZ domain is important not for CHFR/MAD2 interaction, but for proper localization of MAD2 after it is bound by CHFR. Therefore, additional tests of the effect of the PBZ-mutated CHFR on MAD2 localization and function (similar to those described in Chapter 2), would be informative. These studies are particularly interesting given PARP inhibitors are currently being tested as chemotherapeutic treatments for cancers [139, 140]. If PAR is indeed found to play a role in CHFR's spindle function, further tests of PARP inhibition on this function would be extremely valuable for chemotherapeutic development.

CHFR expression loss in breast cancer

Our data suggest that miR-26 could be a factor resulting in the loss of CHFR protein expression in some breast cancers. However, much more work is

required to determine if miR-26 indeed plays a role in CHFR expression regulation. The increase in CHFR protein levels we observed after miR-26 inhibition was minimal, and more sensitive assays, such as detection using fluorescent probes, should be used to validate this preliminary result. This should also be confirmed in multiple cell lines. Perhaps the more successful experiment will be the converse, overexpressing miR-26 in CHFR expressing cell lines with the hope of observing a reduction in CHFR protein levels. It may be easier to observe and measure a reduction in CHFR levels than to measure an increase above a level of expression within the cells. It is possible that the impact of miR-26 in HEK293 cells is minimal, making it a poor cell line for miR-26 inhibition experiments. In contrast, the overexpression experiment does not depend on *CHFR* inhibition by endogenous miR-26 occurring in the cell line tested. The easily-transfected HEK293 cell line could be utilized in this assay, as well as any other cell line in which CHFR protein is present in observable quantities.

Furthermore, direct study of the effect of miR-26 on the 3'UTR of *CHFR* should be performed using a construct containing a reporter gene followed by the *CHFR* 3'UTR. Using this tool, the effects of up and down-regulation of miR-26 could be quantitatively measured, and mutation of the putative miR-26 binding site can be performed to verify the role of miR-26. Clinical relevance should also be examined by measuring miR-26 levels in primary breast cancers

in which CHFR loss is observed, ideally in comparison to matched normal tissues.

The microRNA inhibition of *CHFR* also raises questions related to cancer treatment, specifically if miR-26 levels could be utilized as biomarkers for determining the proper course of treatments in cancers. Examination of the relationship between miR-26 levels and sensitivity to microtubule-targeting drugs could be performed in BCC lines, to determine if a correlation exists. *CHFR* loss by promoter methylation has been extensively examined as a marker for sensitivity to microtubule-targeting chemotherapeutics [89]. As we know, *CHFR* promoter methylation is not observed in many breast cancers with CHFR expression loss, so other tests are needed to determine CHFR status. Measurement of microRNA levels may be useful as a surrogate test for CHFR expression, especially in combination with tests of *CHFR* mRNA levels, to determine the most promising course of therapy for the patient.

It is also possible that miR-26 does not affect CHFR protein expression. As mentioned above, the change in CHFR levels after miR-26 inhibition was not significant. Furthermore, the subcategories of BCC lines we used were determined by comparing BCC lines to immortalized human mammary epithelial cell (IHMEC) lines, which may not be an appropriate normalization method. Considering that the IHMEC lines are immortalized, and have been passaged over time, the expression levels of CHFR, as well as of miR-26, may not

be a good representation of expression levels *in vivo*. Therefore, other explanations for the reduced expression of CHFR should be explored.

While we only observed a correlation between reduced *CHFR* translation and miR-26 expression, other microRNAs such as miR-218 or miR-221/222 could also be involved in *CHFR* regulation and should be examined as outlined for miR-26 above. In addition, more careful studies using multiple methods to test for *CHFR* promoter methylation should be performed. Promoter methylation could account for low *CHFR* mRNA expression, although some of our cell lines express *CHFR* mRNA at high levels suggesting that promoter methylation is not occurring in those cells.

Of particular interest for CHFR expression regulation are the proteins Stil and UBC7, which are known to affect CHFR ubiquitination and degradation. Changes in expression levels of these CHFR regulatory proteins should be explored as a mechanism responsible for CHFR loss in the BCC lines. These proteins are of particular interest because of their previously identified role in regulating CHFR protein levels. However, there may be other proteins also involved in CHFR protein stability, studies to determine the CHFR protein stability in BCC lines should also be performed.

CHFR as a tumor suppressor and cancer biomarker

CHFR has been identified as a tumor suppressor through studies of knockout mice, with support from studies showing CHFR loss in many cancer

types[2, 5]. To better understand how CHFR functions as a tumor suppressor, the impact of individual domain deletions on cancer-related phenotypes should be examined. The importance for each of the CHFR functional domains in the tumor suppressor function of CHFR is not known. While the FHA and RING domains have been identified as domains essential for the early mitotic checkpoint function of CHFR [1, 15], our data suggest that these domains are not essential for the spindle-assembly checkpoint function. Additionally, our work did not examine the impact of the ΔCys mutation on tumorigenic phenotypes, such as aneuploidy, motility, invasion, or growth rate. One question that is raised by this data is: is the cysteine-rich domain of CHFR actually required for tumor suppression in cells? It may be that the effects we observe on MAD2, and the phenotype of lagging chromosomes, ultimately do not lead to aneuploidy or other cancer-related phenotypes. To answer this question, CHFRΔCys could be expressed stably in *Chfr*$^{-/-}$ MEFs and the CHFR-lacking BCC line Hs578T, and examined for aneuploidy over time.

The FHA domain is known to function as a dominant negative, inhibiting the function of CHFR at the antephase checkpoint and resulting in a high mitotic index in a cancer cell line [1], however we did not see any effect of FHA deletion in our MAD2 studies. In addition, the RING domain of CHFR is required for the E3 ubiquitin ligase activity of CHFR but deletion of the RING domain did not affect MAD2 in our studies. This supports the hypothesis that the multiple functions of CHFR are dependent on discreet domains of the protein, and

suggests that each domain may have a different impact on tumor-related phenotypes. To that end, studies of the ability of deletions of each domain to rescue these cellular phenotypes in Hs578T and *Chfr*$^{-/-}$ cells can be performed. In Hs578T cells, addition of wild-type *CHFR* resulted in reduction of the mitotic index, growth rate, motility and invasiveness of cells [3]. The effectiveness of the CHFR deletion constructs in producing these effects could be examined to gain insight into the mechanisms of the tumor suppressive functions of CHFR, and would help to clarify the importance of each CHFR function to tumor suppression. In addition, cells stably expressing CHFR deletion mutants or wild type CHFR could be tested for tumorigenicity *in vivo* by injecting the cells into nude mice.

In addition, the role of the PBZ domain in CHFR function is particularly interesting, considering that PARP inhibitors are currently in clinical trials for the treatment of breast and ovarian cancers [139, 140]. These studies are targeted at cancers deficient in DNA repair pathways, but understanding the impact of PARP inhibitors on tumors that have lost or retained CHFR could lead to more effective, personalized treatment for each patient.

Finally the role of CHFR itself as a biomarker for prognosis or treatment efficacy in breast cancers should be more closely examined. Using patient samples and histories, correlations between CHFR protein expression and chemotherapeutic response (particularly to microtubule-targeting drugs), tumor

stage, metastases and recurrence can be measured for breast cancer, as is currently being done for other cancer types.

Summary

CHFR is clearly an important tumor suppressor involved in cancers of many types. The work presented here raises many questions about the role of CHFR in the spindle assembly checkpoint, and offers one possibility to explain the loss of CHFR in breast cancers. While several studies of the clinical relevance of CHFR loss have been performed, further study of the tumor suppressive functions of CHFR, as well as the mechanisms by which CHFR activity is regulated, is needed. Future work will aim to better understand the mechanism(s) by which CHFR prevents tumorigenesis, and to develop new and more effective cancer treatments.

REFERENCES

1. Scolnick, D.M., and Halazonetis, T.D. (2000). Chfr defines a mitotic stress checkpoint that delays entry into metaphase. Nature *406*, 430-435.
2. Privette, L.M., and Petty, E.M. (2008). CHFR: A Novel Mitotic Checkpoint Protein and Regulator of Tumorigenesis. Transl Oncol *1*, 57-64.
3. Privette, L.M., Gonzalez, M.E., Ding, L., Kleer, C.G., and Petty, E.M. (2007). Altered expression of the early mitotic checkpoint protein, CHFR, in breast cancers: implications for tumor suppression. Cancer Res *67*, 6064-6074.
4. Erson, A.E., and Petty, E.M. (2004). CHFR-associated early G2/M checkpoint defects in breast cancer cells. Mol Carcinog *39*, 26-33.
5. Yu, X., Minter-Dykhouse, K., Malureanu, L., Zhao, W.M., Zhang, D., Merkle, C.J., Ward, I.M., Saya, H., Fang, G., van Deursen, J., et al. (2005). Chfr is required for tumor suppression and Aurora A regulation. Nat Genet *37*, 401-406.
6. Brooks, L., 3rd, Heimsath, E.G., Jr., Loring, G.L., and Brenner, C. (2008). FHA-RING ubiquitin ligases in cell division cycle control. Cell Mol Life Sci *65*, 3458-3466.
7. Durocher, D., and Jackson, S.P. (2002). The FHA domain. FEBS Lett *513*, 58-66.
8. Hofmann, K., and Bucher, P. (1995). The FHA domain: a putative nuclear signalling domain found in protein kinases and transcription factors. Trends Biochem Sci *20*, 347-349.
9. Fukuda, T., Kondo, Y., and Nakagama, H. (2008). The anti-proliferative effects of the CHFR depend on the forkhead associated domain, but not E3 ligase activity mediated by ring finger domain. PLoS One *3*, e1776.
10. Toyota, M., Sasaki, Y., Satoh, A., Ogi, K., Kikuchi, T., Suzuki, H., Mita, H., Tanaka, N., Itoh, F., Issa, J.P., et al. (2003). Epigenetic inactivation of CHFR in human tumors. Proc Natl Acad Sci U S A *100*, 7818-7823.

11. Daniels, M.J., Marson, A., and Venkitaraman, A.R. (2004). PML bodies control the nuclear dynamics and function of the CHFR mitotic checkpoint protein. Nat Struct Mol Biol *11*, 1114-1121.

12. Stavridi, E.S., Huyen, Y., Loreto, I.R., Scolnick, D.M., Halazonetis, T.D., Pavletich, N.P., and Jeffrey, P.D. (2002). Crystal structure of the FHA domain of the Chfr mitotic checkpoint protein and its complex with tungstate. Structure *10*, 891-899.

13. Michael, S., Trave, G., Ramu, C., Chica, C., and Gibson, T.J. (2008). Discovery of candidate KEN-box motifs using cell cycle keyword enrichment combined with native disorder prediction and motif conservation. Bioinformatics *24*, 453-457.

14. Pfleger, C.M., and Kirschner, M.W. (2000). The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. Genes Dev *14*, 655-665.

15. Chaturvedi, P., Sudakin, V., Bobiak, M.L., Fisher, P.W., Mattern, M.R., Jablonski, S.A., Hurle, M.R., Zhu, Y., Yen, T.J., and Zhou, B.B. (2002). Chfr regulates a mitotic stress pathway through its RING-finger domain with ubiquitin ligase activity. Cancer Res *62*, 1797-1801.

16. Feine, O., Zur, A., Mahbubani, H., and Brandeis, M. (2007). Human Kid is degraded by the APC/C(Cdh1) but not by the APC/C(Cdc20). Cell Cycle *6*, 2516-2523.

17. Kang, D., Chen, J., Wong, J., and Fang, G. (2002). The checkpoint protein Chfr is a ligase that ubiquitinates Plk1 and inhibits Cdc2 at the G2 to M transition. J Cell Biol *156*, 249-259.

18. Bothos, J., Summers, M.K., Venere, M., Scolnick, D.M., and Halazonetis, T.D. (2003). The Chfr mitotic checkpoint protein functions with Ubc13-Mms2 to form Lys63-linked polyubiquitin chains. Oncogene *22*, 7101-7107.

19. Kim, J.M., Cho, E.N., Kwon, Y.E., Bae, S.J., Kim, M., and Seol, J.H. (2010). CHFR functions as a ubiquitin ligase for HLTF to regulate its stability and functions. Biochem Biophys Res Commun *395*, 515-520.

20. Maddika, S., Sy, S.M., and Chen, J. (2009). Functional interaction between Chfr and Kif22 controls genomic stability. J Biol Chem *284*, 12998-13003.

21. Oh, Y.M., Kwon, Y.E., Kim, J.M., Bae, S.J., Lee, B.K., Yoo, S.J., Chung, C.H., Deshaies, R.J., and Seol, J.H. (2009). Chfr is linked to tumour metastasis through the downregulation of HDAC1. Nat Cell Biol *11*, 295-302.

22. Ahel, I., Ahel, D., Matsusaka, T., Clark, A.J., Pines, J., Boulton, S.J., and West, S.C. (2008). Poly(ADP-ribose)-binding zinc finger motifs in DNA repair/checkpoint proteins. Nature *451*, 81-85.

23. Oberoi, J., Richards, M.W., Crumpler, S., Brown, N., Blagg, J., and Bayliss, R. (2010). Structural basis of poly(ADP-ribose) recognition by the multizinc binding domain of checkpoint with forkhead-associated and RING Domains (CHFR). J Biol Chem *285*, 39348-39358.

24. Hassa, P.O., and Hottiger, M.O. (2008). The diverse biological roles of mammalian PARPS, a small but powerful family of poly-ADP-ribose polymerases. Front Biosci *13*, 3046-3082.

25. Chang, P., Jacobson, M.K., and Mitchison, T.J. (2004). Poly(ADP-ribose) is required for spindle assembly and structure. Nature *432*, 645-649.

26. Chang, P., Coughlin, M., and Mitchison, T.J. (2005). Tankyrase-1 polymerization of poly(ADP-ribose) is required for spindle structure and function. Nat Cell Biol *7*, 1133-1139.

27. Loring, G.L., Christensen, K.C., Gerber, S.A., and Brenner, C. (2008). Yeast Chfr homologs retard cell cycle at G1 and G2/M via Ubc4 and Ubc13/Mms2-dependent ubiquitination. Cell Cycle *7*, 96-105.

28. Fraschini, R., Bilotta, D., Lucchini, G., and Piatti, S. (2004). Functional characterization of Dma1 and Dma2, the budding yeast homologues of Schizosaccharomyces pombe Dma1 and human Chfr. Mol Biol Cell *15*, 3796-3810.

29. Privette, L.M., Weier, J.F., Nguyen, H.N., Yu, X., and Petty, E.M. (2008). Loss of CHFR in human mammary epithelial cells causes genomic instability by disrupting the mitotic spindle assembly checkpoint. Neoplasia *10*, 643-652.

30. Kashima, L., Toyota, M., Mita, H., Suzuki, H., Idogawa, M., Ogi, K., Sasaki, Y., and Tokino, T. (2009). CHFR, a potential tumor suppressor, downregulates interleukin-8 through the inhibition of NF-kappaB. Oncogene *28*, 2643-2653.

31. Matsusaka, T., and Pines, J. (2004). Chfr acts with the p38 stress kinases to block entry to mitosis in mammalian cells. J Cell Biol *166*, 507-516.

32. Chin, C.F., and Yeong, F.M. (2010). Safeguarding entry into mitosis: the antephase checkpoint. Mol Cell Biol *30*, 22-32.

33. Toyoshima-Morimoto, F., Taniguchi, E., Shinya, N., Iwamatsu, A., and Nishida, E. (2001). Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase. Nature *410*, 215-220.

34. Summers, M.K., Bothos, J., and Halazonetis, T.D. (2005). The CHFR mitotic checkpoint protein delays cell cycle progression by excluding Cyclin B1 from the nucleus. Oncogene *24*, 2589-2598.

35. Eckerdt, F., and Strebhardt, K. (2006). Polo-like kinase 1: target and regulator of anaphase-promoting complex/cyclosome-dependent proteolysis. Cancer Res *66*, 6895-6898.

36. Smits, V.A., Klompmaker, R., Arnaud, L., Rijksen, G., Nigg, E.A., and Medema, R.H. (2000). Polo-like kinase-1 is a target of the DNA damage checkpoint. Nat Cell Biol *2*, 672-676.

37. Castiel, A., Danieli, M.M., David, A., Moshkovitz, S., Aplan, P.D., Kirsch, I.R., Brandeis, M., Kramer, A., and Izraeli, S. (2011). The Stil protein regulates centrosome integrity and mitosis through suppression of Chfr. J Cell Sci *124*, 532-539.

38. Cooper, G.M. (2000). The cell : a molecular approach, 2nd Edition, (Washington, D.C.; Sunderland, Mass.: ASM Press; Sinauer Associates).

39. Hirota, T., Kunitoku, N., Sasayama, T., Marumoto, T., Zhang, D., Nitta, M., Hatakeyama, K., and Saya, H. (2003). Aurora-A and an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells. Cell *114*, 585-598.

40. Vader, G., and Lens, S.M. (2008). The Aurora kinase family in cell division and cancer. Biochim Biophys Acta *1786*, 60-72.

41. Hannak, E., Kirkham, M., Hyman, A.A., and Oegema, K. (2001). Aurora-A kinase is required for centrosome maturation in Caenorhabditis elegans. J Cell Biol *155*, 1109-1116.

42. Dutertre, S., Descamps, S., and Prigent, C. (2002). On the role of aurora-A in centrosome function. Oncogene *21*, 6175-6183.

43. Zhou, H., Kuang, J., Zhong, L., Kuo, W.L., Gray, J.W., Sahin, A., Brinkley, B.R., and Sen, S. (1998). Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat Genet *20*, 189-193.

44. Cazales, M., Schmitt, E., Montembault, E., Dozier, C., Prigent, C., and Ducommun, B. (2005). CDC25B phosphorylation by Aurora-A occurs at the G2/M transition and is inhibited by DNA damage. Cell Cycle *4*, 1233-1238.

45. Macurek, L., Lindqvist, A., Lim, D., Lampson, M.A., Klompmaker, R., Freire, R., Clouin, C., Taylor, S.S., Yaffe, M.B., and Medema, R.H. (2008). Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery. Nature *455*, 119-123.

46. Seki, A., Coppinger, J.A., Jang, C.Y., Yates, J.R., and Fang, G. (2008). Bora and the kinase Aurora a cooperatively activate the kinase Plk1 and control mitotic entry. Science *320*, 1655-1658.

47. Anand, S., Penrhyn-Lowe, S., and Venkitaraman, A.R. (2003). AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. Cancer Cell *3*, 51-62.

48. Musacchio, A., and Salmon, E.D. (2007). The spindle-assembly checkpoint in space and time. Nat Rev Mol Cell Biol *8*, 379-393.

49. Fang, G., Yu, H., and Kirschner, M.W. (1998). The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. Genes Dev *12*, 1871-1883.

50. Wassmann, K., and Benezra, R. (1998). Mad2 transiently associates with an APC/p55Cdc complex during mitosis. Proc Natl Acad Sci U S A *95*, 11193-11198.

51. Yu, H. (2002). Regulation of APC-Cdc20 by the spindle checkpoint. Curr Opin Cell Biol *14*, 706-714.

52. Burgess, A., Labbe, J.C., Vigneron, S., Bonneaud, N., Strub, J.M., Van Dorsselaer, A., Lorca, T., and Castro, A. (2008). Chfr interacts and colocalizes with TCTP to the mitotic spindle. Oncogene *27*, 5554-5566.

53. Gachet, Y., Tournier, S., Lee, M., Lazaris-Karatzas, A., Poulton, T., and Bommer, U.A. (1999). The growth-related, translationally controlled protein P23 has properties of a tubulin binding protein and associates transiently with microtubules during the cell cycle. J Cell Sci *112 (Pt 8)*, 1257-1271.

54. Yarm, F.R. (2002). Plk phosphorylation regulates the microtubule-stabilizing protein TCTP. Mol Cell Biol *22*, 6209-6221.

55. Webster, D.R., and Borisy, G.G. (1989). Microtubules are acetylated in domains that turn over slowly. J Cell Sci *92 (Pt 1)*, 57-65.

56. Levesque, A.A., and Compton, D.A. (2001). The chromokinesin Kid is necessary for chromosome arm orientation and oscillation, but not congression, on mitotic spindles. J Cell Biol *154*, 1135-1146.

57. Ding, H., Descheemaeker, K., Marynen, P., Nelles, L., Carvalho, T., Carmo-Fonseca, M., Collen, D., and Belayew, A. (1996). Characterization of a helicase-like transcription factor involved in the expression of the human plasminogen activator inhibitor-1 gene. DNA Cell Biol *15*, 429-442.

58. Suijkerbuijk, S.J., and Kops, G.J. (2008). Preventing aneuploidy: the contribution of mitotic checkpoint proteins. Biochim Biophys Acta *1786*, 24-31.

59. Bischoff, J.R., Anderson, L., Zhu, Y., Mossie, K., Ng, L., Souza, B., Schryver, B., Flanagan, P., Clairvoyant, F., Ginther, C., et al. (1998). A

homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. EMBO J *17*, 3052-3065.

60. Takai, N., Hamanaka, R., Yoshimatsu, J., and Miyakawa, I. (2005). Polo-like kinases (Plks) and cancer. Oncogene *24*, 287-291.

61. Park, S.Y., Jun, J.A., Jeong, K.J., Heo, H.J., Sohn, J.S., Lee, H.Y., Park, C.G., and Kang, J. (2011). Histone deacetylases 1, 6 and 8 are critical for invasion in breast cancer. Oncol Rep *25*, 1677-1681.

62. Chen, W., Li, Z., Bai, L., and Lin, Y. (2011). NF-kappaB in lung cancer, a carcinogenesis mediator and a prevention and therapy target. Front Biosci *16*, 1172-1185.

63. Mariatos, G., Bothos, J., Zacharatos, P., Summers, M.K., Scolnick, D.M., Kittas, C., Halazonetis, T.D., and Gorgoulis, V.G. (2003). Inactivating mutations targeting the chfr mitotic checkpoint gene in human lung cancer. Cancer Res *63*, 7185-7189.

64. Kang, H.C., Kim, I.J., Jang, S.G., Hong, S.H., Hwang, J.A., Shin, H.R., and Park, J.G. (2008). Coding region polymorphisms in the CHFR mitotic stress checkpoint gene are associated with colorectal cancer risk. Cancer Lett *260*, 170-179.

65. Tokunaga, E., Oki, E., Nishida, K., Koga, T., Yoshida, R., Ikeda, K., Kojima, A., Egashira, A., Morita, M., Kakeji, Y., et al. (2006). Aberrant hypermethylation of the promoter region of the CHFR gene is rare in primary breast cancer. Breast Cancer Res Treat *97*, 199-203.

66. Ogi, K., Toyota, M., Mita, H., Satoh, A., Kashima, L., Sasaki, Y., Suzuki, H., Akino, K., Nishikawa, N., Noguchi, M., et al. (2005). Small interfering RNA-induced CHFR silencing sensitizes oral squamous cell cancer cells to microtubule inhibitors. Cancer Biol Ther *4*, 773-780.

67. Sui, J.L., An, J., Sun, J.F., Chen, Y., Wu, D.C., and Zhou, P.K. (2004). Spindle checkpoint and apoptotic response in alpha-particle transformed human bronchial epithelial cells. Radiat Environ Biophys *43*, 257-263.

68. Sakai, M., Hibi, K., Kanazumi, N., Nomoto, S., Inoue, S., Takeda, S., and Nakao, A. (2005). Aberrant methylation of the CHFR gene in advanced hepatocellular carcinoma. Hepatogastroenterology *52*, 1854-1857.

69. Chen, K., Sawhney, R., Khan, M., Benninger, M.S., Hou, Z., Sethi, S., Stephen, J.K., and Worsham, M.J. (2007). Methylation of multiple genes as diagnostic and therapeutic markers in primary head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg *133*, 1131-1138.

70. Morioka, Y., Hibi, K., Sakai, M., Koike, M., Fujiwara, M., Kodera, Y., Ito, K., and Nakao, A. (2006). Aberrant methylation of the CHFR gene in digestive tract cancer. Anticancer Res *26*, 1791-1795.

71. Takeshita, M., Koga, T., Takayama, K., Yano, T., Maehara, Y., Nakanishi, Y., and Sueishi, K. (2010). Alternative efficacy-predicting markers for paclitaxel instead of CHFR in non-small cell lung cancer. Cancer Biol Ther *10*.

72. Yoshida, K., Hamai, Y., Suzuki, T., Sanada, Y., Oue, N., and Yasui, W. (2006). DNA methylation of CHFR is not a predictor of the response to docetaxel and paclitaxel in advanced and recurrent gastric cancer. Anticancer Res *26*, 49-54.

73. Gao, Y.J., Xin, Y., Zhang, J.J., and Zhou, J. (2008). Mechanism and pathobiologic implications of CHFR promoter methylation in gastric carcinoma. World J Gastroenterol *14*, 5000-5007.

74. Hu, S.L., Huang, D.B., Sun, Y.B., Wu, L., Xu, W.P., Yin, S., Chen, J., Jiang, X.D., and Shen, G. (2011). Pathobiologic implications of methylation and expression status of Runx3 and CHFR genes in gastric cancer. Med Oncol *28*, 447-454.

75. Koga, Y., Kitajima, Y., Miyoshi, A., Sato, K., Sato, S., and Miyazaki, K. (2006). The significance of aberrant CHFR methylation for clinical response to microtubule inhibitors in gastric cancer. J Gastroenterol *41*, 133-139.

76. Satoh, A., Toyota, M., Itoh, F., Sasaki, Y., Suzuki, H., Ogi, K., Kikuchi, T., Mita, H., Yamashita, T., Kojima, T., et al. (2003). Epigenetic inactivation of CHFR and sensitivity to microtubule inhibitors in gastric cancer. Cancer Res *63*, 8606-8613.

77. Oki, E., Zhao, Y., Yoshida, R., Masuda, T., Ando, K., Sugiyama, M., Tokunaga, E., Morita, M., Kakeji, Y., and Maehara, Y. (2009). Checkpoint with forkhead-associated and ring finger promoter hypermethylation correlates with microsatellite instability in gastric cancer. World J Gastroenterol *15*, 2520-2525.

78. Takeshita, M., Koga, T., Takayama, K., Kouso, H., Nishimura-Ikeda, Y., Yoshino, I., Maehara, Y., Nakanishi, Y., and Sueishi, K. (2008). CHFR expression is preferentially impaired in smoking-related squamous cell carcinoma of the lung, and the diminished expression significantly harms outcomes. Int J Cancer *123*, 1623-1630.

79. Koga, T., Takeshita, M., Yano, T., Maehara, Y., and Sueishi, K. (2011). CHFR hypermethylation and EGFR mutation are mutually exclusive and exhibit contrastive clinical backgrounds and outcomes in non-small cell lung cancer. Int J Cancer.

80. Kobayashi, C., Oda, Y., Takahira, T., Izumi, T., Kawaguchi, K., Yamamoto, H., Tamiya, S., Yamada, T., Iwamoto, Y., and Tsuneyoshi, M. (2006). Aberrant expression of CHFR in malignant peripheral nerve sheath tumors. Mod Pathol *19*, 524-532.

81. Brandes, J.C., van Engeland, M., Wouters, K.A., Weijenberg, M.P., and Herman, J.G. (2005). CHFR promoter hypermethylation in colon cancer correlates with the microsatellite instability phenotype. Carcinogenesis *26*, 1152-1156.

82. Tanaka, M., Chang, P., Li, Y., Li, D., Overman, M., Maru, D.M., Sethi, S., Phillips, J.K., Bland, G., Abbruzzese, J.L., et al. (2011). Association of CHFR Promoter Methylation with Disease Recurrence in Locally Advanced Colon Cancer. Clin Cancer Res.

83. Yanokura, M., Banno, K., Kawaguchi, M., Hirao, N., Hirasawa, A., Susumu, N., Tsukazaki, K., and Aoki, D. (2007). Relationship of aberrant DNA hypermethylation of CHFR with sensitivity to taxanes in endometrial cancer. Oncol Rep *17*, 41-48.

84. Banno, K., Yanokura, M., Kawaguchi, M., Kuwabara, Y., Akiyoshi, J., Kobayashi, Y., Iwata, T., Hirasawa, A., Fujii, T., Susumu, N., et al. (2007). Epigenetic inactivation of the CHFR gene in cervical cancer contributes to sensitivity to taxanes. Int J Oncol *31*, 713-720.

85. Cheng, Z.D., Hu, S.L., Sun, Y.B., Xu, W.P., Shen, G., and Kong, X.Y. (2010). Promoter methylation of CHFR gene in gastric carcinoma tissues detected using two methods. Chin J Cancer *29*, 163-166.

86. Inoue, T., Hiratsuka, M., Osaki, M., Yamada, H., Kishimoto, I., Yamaguchi, S., Nakano, S., Katoh, M., Ito, H., and Oshimura, M. (2007). SIRT2, a tubulin deacetylase, acts to block the entry to chromosome condensation in response to mitotic stress. Oncogene *26*, 945-957.

87. Michel, L.S., Liberal, V., Chatterjee, A., Kirchwegger, R., Pasche, B., Gerald, W., Dobles, M., Sorger, P.K., Murty, V.V., and Benezra, R. (2001). MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. Nature *409*, 355-359.

88. Sotillo, R., Hernando, E., Diaz-Rodriguez, E., Teruya-Feldstein, J., Cordon-Cardo, C., Lowe, S.W., and Benezra, R. (2007). Mad2 overexpression promotes aneuploidy and tumorigenesis in mice. Cancer Cell *11*, 9-23.

89. Keller, J.A., Erson-Bensan, A.E., and Petty, E.M. (2010). Connections between CHFR, the cell cycle, and chemosensitivity: Are they critical in cancer? Cancer Biol Ther *10*, 942-944.

90. Fang, G. (2002). Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex. Mol Biol Cell *13*, 755-766.

91. Perez de Castro, I., de Carcer, G., and Malumbres, M. (2007). A census of mitotic cancer genes: new insights into tumor cell biology and cancer therapy. Carcinogenesis *28*, 899-912.

92. Kato, T., Daigo, Y., Aragaki, M., Ishikawa, K., Sato, M., Kondo, S., and Kaji, M. (2011). Overexpression of MAD2 predicts clinical outcome in primary lung cancer patients. Lung Cancer.

93. Luo, X., Tang, Z., Xia, G., Wassmann, K., Matsumoto, T., Rizo, J., and Yu, H. (2004). The Mad2 spindle checkpoint protein has two distinct natively folded states. Nat Struct Mol Biol *11*, 338-345.

94. Luo, X., Fang, G., Coldiron, M., Lin, Y., Yu, H., Kirschner, M.W., and Wagner, G. (2000). Structure of the Mad2 spindle assembly checkpoint protein and its interaction with Cdc20. Nat Struct Biol *7*, 224-229.

95. Skinner, J.J., Wood, S., Shorter, J., Englander, S.W., and Black, B.E. (2008). The Mad2 partial unfolding model: regulating mitosis through Mad2 conformational switching. J Cell Biol *183*, 761-768.

96. De Antoni, A., Pearson, C.G., Cimini, D., Canman, J.C., Sala, V., Nezi, L., Mapelli, M., Sironi, L., Faretta, M., Salmon, E.D., et al. (2005). The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint. Curr Biol *15*, 214-225.

97. Mapelli, M., Massimiliano, L., Santaguida, S., and Musacchio, A. (2007). The Mad2 conformational dimer: structure and implications for the spindle assembly checkpoint. Cell *131*, 730-743.

98. Ma, H.T., and Poon, R.Y. (2011). Orderly Inactivation of the Key Checkpoint Protein Mitotic Arrest Deficient 2 (MAD2) during Mitotic Progression. J Biol Chem *286*, 13052-13059.

99. Wassmann, K., Liberal, V., and Benezra, R. (2003). Mad2 phosphorylation regulates its association with Mad1 and the APC/C. EMBO J *22*, 797-806.

100. Yang, M., Li, B., Tomchick, D.R., Machius, M., Rizo, J., Yu, H., and Luo, X. (2007). p31comet blocks Mad2 activation through structural mimicry. Cell *131*, 744-755.

101. Peters, J.M. (2008). Checkpoint control: the journey continues. Curr Biol *18*, R170-172.

102. Xia, G., Luo, X., Habu, T., Rizo, J., Matsumoto, T., and Yu, H. (2004). Conformation-specific binding of p31(comet) antagonizes the function of Mad2 in the spindle checkpoint. EMBO J *23*, 3133-3143.

103. Yu, L., Guo, W.C., Zhao, S.H., Tang, J., and Chen, J.L. (2010). Mitotic arrest defective protein 2 expression abnormality and its clinicopathologic significance in human osteosarcoma. APMIS *118*, 222-229.

104. Oh, Y.M., Yoo, S.J., and Seol, J.H. (2007). Deubiquitination of Chfr, a checkpoint protein, by USP7/HAUSP regulates its stability and activity. Biochem Biophys Res Commun *357*, 615-619.

105. Erez, A., Perelman, M., Hewitt, S.M., Cojacaru, G., Goldberg, I., Shahar, I., Yaron, P., Muler, I., Campaner, S., Amariglio, N., et al. (2004). Sil overexpression in lung cancer characterizes tumors with increased mitotic activity. Oncogene *23*, 5371-5377.

106. Moelans, C.B., Verschuur-Maes, A.H., and van Diest, P.J. (2011). Frequent promoter hypermethylation of BRCA2, CDH13, MSH6, PAX5, PAX6 and WT1 in ductal carcinoma in situ and invasive breast cancer. J Pathol.

107. Erson, A.E., and Petty, E.M. (2008). MicroRNAs in development and disease. Clin Genet *74*, 296-306.

108. Calin, G.A., Sevignani, C., Dumitru, C.D., Hyslop, T., Noch, E., Yendamuri, S., Shimizu, M., Rattan, S., Bullrich, F., Negrini, M., et al. (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A *101*, 2999-3004.

109. Selcuklu, S.D., Yakicier, M.C., and Erson, A.E. (2009). An investigation of microRNAs mapping to breast cancer related genomic gain and loss regions. Cancer Genet Cytogenet *189*, 15-23.

110. Silveri, L., Tilly, G., Vilotte, J.L., and Le Provost, F. (2006). MicroRNA involvement in mammary gland development and breast cancer. Reprod Nutr Dev *46*, 549-556.

111. Tavazoie, S.F., Alarcon, C., Oskarsson, T., Padua, D., Wang, Q., Bos, P.D., Gerald, W.L., and Massague, J. (2008). Endogenous human microRNAs that suppress breast cancer metastasis. Nature *451*, 147-152.

112. Kulshreshtha, R., Ferracin, M., Wojcik, S.E., Garzon, R., Alder, H., Agosto-Perez, F.J., Davuluri, R., Liu, C.G., Croce, C.M., Negrini, M., et al. (2007). A microRNA signature of hypoxia. Mol Cell Biol *27*, 1859-1867.

113. Iorio, M.V., Ferracin, M., Liu, C.G., Veronese, A., Spizzo, R., Sabbioni, S., Magri, E., Pedriali, M., Fabbri, M., Campiglio, M., et al. (2005). MicroRNA gene expression deregulation in human breast cancer. Cancer Res *65*, 7065-7070.

114. Ma, Y.L., Zhang, P., Wang, F., Moyer, M.P., Yang, J.J., Liu, Z.H., Peng, J.Y., Chen, H.Q., Zhou, Y.K., Liu, W.J., et al. (2010). Human embryonic stem cells and metastatic colorectal cancer cells shared the common endogenous human microRNA-26b. J Cell Mol Med.

115. Ji, J., Shi, J., Budhu, A., Yu, Z., Forgues, M., Roessler, S., Ambs, S., Chen, Y., Meltzer, P.S., Croce, C.M., et al. (2009). MicroRNA expression, survival, and response to interferon in liver cancer. N Engl J Med *361*, 1437-1447.

116. Lu, J., He, M.L., Wang, L., Chen, Y., Liu, X., Dong, Q., Chen, Y.C., Peng, Y., Yao, K.T., Kung, H.F., et al. (2011). MiR-26a inhibits cell growth and

tumorigenesis of nasopharyngeal carcinoma through repression of EZH2. Cancer Res *71*, 225-233.

117. Visone, R., Pallante, P., Vecchione, A., Cirombella, R., Ferracin, M., Ferraro, A., Volinia, S., Coluzzi, S., Leone, V., Borbone, E., et al. (2007). Specific microRNAs are downregulated in human thyroid anaplastic carcinomas. Oncogene *26*, 7590-7595.

118. Wong, T.S., Liu, X.B., Wong, B.Y., Ng, R.W., Yuen, A.P., and Wei, W.I. (2008). Mature miR-184 as Potential Oncogenic microRNA of Squamous Cell Carcinoma of Tongue. Clin Cancer Res *14*, 2588-2592.

119. Zanette, D.L., Rivadavia, F., Molfetta, G.A., Barbuzano, F.G., Proto-Siqueira, R., Silva-Jr, W.A., Falcao, R.P., and Zago, M.A. (2007). miRNA expression profiles in chronic lymphocytic and acute lymphocytic leukemia. Braz J Med Biol Res *40*, 1435-1440.

120. Tatarano, S., Chiyomaru, T., Kawakami, K., Enokida, H., Yoshino, H., Hidaka, H., Yamasaki, T., Kawahara, K., Nishiyama, K., Seki, N., et al. (2011). miR-218 on the genomic loss region of chromosome 4p15.31 functions as a tumor suppressor in bladder cancer. Int J Oncol *39*, 13-21.

121. Martinez, I., Gardiner, A.S., Board, K.F., Monzon, F.A., Edwards, R.P., and Khan, S.A. (2008). Human papillomavirus type 16 reduces the expression of microRNA-218 in cervical carcinoma cells. Oncogene *27*, 2575-2582.

122. Davidson, M.R., Larsen, J.E., Yang, I.A., Hayward, N.K., Clarke, B.E., Duhig, E.E., Passmore, L.H., Bowman, R.V., and Fong, K.M. (2010). MicroRNA-218 is deleted and downregulated in lung squamous cell carcinoma. PLoS One *5*, e12560.

123. Miller, T.E., Ghoshal, K., Ramaswamy, B., Roy, S., Datta, J., Shapiro, C.L., Jacob, S., and Majumder, S. (2008). MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1. J Biol Chem *283*, 29897-29903.

124. Chun-Zhi, Z., Lei, H., An-Ling, Z., Yan-Chao, F., Xiao, Y., Guang-Xiu, W., Zhi-Fan, J., Pei-Yu, P., Qing-Yu, Z., and Chun-Sheng, K. (2010). MicroRNA-221 and microRNA-222 regulate gastric carcinoma cell proliferation and radioresistance by targeting PTEN. BMC Cancer *10*, 367.

125. Zhang, J., Han, L., Ge, Y., Zhou, X., Zhang, A., Zhang, C., Zhong, Y., You, Y., Pu, P., and Kang, C. (2010). miR-221/222 promote malignant progression of glioma through activation of the Akt pathway. Int J Oncol *36*, 913-920.

126. Fornari, F., Gramantieri, L., Ferracin, M., Veronese, A., Sabbioni, S., Calin, G.A., Grazi, G.L., Giovannini, C., Croce, C.M., Bolondi, L., et al. (2008). MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. Oncogene *27*, 5651-5661.

127. Pineau, P., Volinia, S., McJunkin, K., Marchio, A., Battiston, C., Terris, B., Mazzaferro, V., Lowe, S.W., Croce, C.M., and Dejean, A. (2010). miR-221 overexpression contributes to liver tumorigenesis. Proc Natl Acad Sci U S A *107*, 264-269.

128. Gottardo, F., Liu, C.G., Ferracin, M., Calin, G.A., Fassan, M., Bassi, P., Sevignani, C., Byrne, D., Negrini, M., Pagano, F., et al. (2007). Micro-RNA profiling in kidney and bladder cancers. Urol Oncol *25*, 387-392.

129. Chen, Y.T., Kitabayashi, N., Zhou, X.K., Fahey, T.J., 3rd, and Scognamiglio, T. (2008). MicroRNA analysis as a potential diagnostic tool for papillary thyroid carcinoma. Mod Pathol *21*, 1139-1146.

130. Visone, R., Russo, L., Pallante, P., De Martino, I., Ferraro, A., Leone, V., Borbone, E., Petrocca, F., Alder, H., Croce, C.M., et al. (2007). MicroRNAs (miR)-221 and miR-222, both overexpressed in human thyroid papillary carcinomas, regulate p27Kip1 protein levels and cell cycle. Endocr Relat Cancer *14*, 791-798.

131. Galardi, S., Mercatelli, N., Giorda, E., Massalini, S., Frajese, G.V., Ciafre, S.A., and Farace, M.G. (2007). miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. J Biol Chem *282*, 23716-23724.

132. Bueno, M.J., Perez de Castro, I., and Malumbres, M. (2008). Control of cell proliferation pathways by microRNAs. Cell Cycle *7*, 3143-3148.

133. Bueno, M.J., and Malumbres, M. (2011). MicroRNAs and the cell cycle. Biochim Biophys Acta *1812*, 592-601.

134. Iorio, M.V., Casalini, P., Tagliabue, E., Menard, S., and Croce, C.M. (2008). MicroRNA profiling as a tool to understand prognosis, therapy response and resistance in breast cancer. Eur J Cancer *44*, 2753-2759.

135. Castellano, L., Giamas, G., Jacob, J., Coombes, R.C., Lucchesi, W., Thiruchelvam, P., Barton, G., Jiao, L.R., Wait, R., Waxman, J., et al. (2009). The estrogen receptor-alpha-induced microRNA signature regulates itself and its transcriptional response. Proc Natl Acad Sci U S A *106*, 15732-15737.

136. Ellis, P.A., Saccani-Jotti, G., Clarke, R., Johnston, S.R., Anderson, E., Howell, A., A'Hern, R., Salter, J., Detre, S., Nicholson, R., et al. (1997). Induction of apoptosis by tamoxifen and ICI 182780 in primary breast cancer. Int J Cancer *72*, 608-613.

137. Sander, S., Bullinger, L., Klapproth, K., Fiedler, K., Kestler, H.A., Barth, T.F., Moller, P., Stilgenbauer, S., Pollack, J.R., and Wirth, T. (2008). MYC stimulates EZH2 expression by repression of its negative regulator miR-26a. Blood *112*, 4202-4212.

138. Compton, D.A. (2005). Mitosis: PARty time in the spindle. Curr Biol *15*, R178-179.

139. Mukhopadhyay, A., Curtin, N., Plummer, R., and Edmondson, R.J. (2011). PARP inhibitors and epithelial ovarian cancer: an approach to targeted chemotherapy and personalised medicine. BJOG *118*, 429-432.

140. Kruse, V., Rottey, S., De Backer, O., Van Belle, S., Cocquyt, V., and Denys, H. (2011). PARP inhibitors in oncology: a new synthetic lethal approach to cancer therapy. Acta Clin Belg *66*, 2-9.