CHCHD2 Is Coamplified with EGFR in NSCLC and Regulates Mitochondrial Function and Cell Migration

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Abstract

Coiled-coil-helix-coiled-coil-helix domain-containing 2, a mitochondrial protein, encoded by CHCHD2 is located at chromosome 7p11.2 and proximal to the EGFR gene. Here, bioinformatic analyses revealed that CHCHD2 is consistently coamplified with EGFR in non–small cell lung carcinoma (NSCLC). In addition, CHCHD2 and EGFR protein expression levels were positively correlated and upregulated relative to normal lung in NSCLC tumor-derived xenografts. Knockdown of CHCHD2 expression in NSCLC cells attenuated cell proliferation, migration, and mitochondrial respiration. CHCHD2 protein–protein interactions were assessed by the complementary approaches of affinity purification mass spectrometry and in vivo proximity ligation. The CHCHD2 interactome includes the apparent hub proteins C1QBP (a mitochondrial protein) and YBX1 (an oncogenic transcription factor), and an overlapping set of hub-associated proteins implicated in cell regulation.

Implications: CHCHD2 influences mitochondrial and nuclear functions and contributes to the cancer phenotype associated with 7p11.2 amplification in NSCLC. Mol Cancer Res; 13(7); 1119–29. ©2015 AACR.

Introduction

Lung cancer is the most common cause of cancer-related mortality in the world (1). Eighty-five percent of lung cancers are non–small cell lung carcinoma (NSCLC), a heterogeneous group composed of two main subtypes, squamous cell carcinoma (SCC) and adenocarcinoma (ADC; ref. 2). NSCLC are characterized by somatic genetic alterations that recur according to histology, and which include oncogenic driver mutations that in some instances are associated with genomic amplification or deletion (3–6). For example, in NSCLC, recurrent amplification is observed in the 7p11 region encoding the drug target EGFR (5, 6, 7). Indeed, EGFR gene copy number is a significant molecular predictor of a differential survival benefit from treatment of NSCLC with the EGFR inhibitor erlotinib (8). Despite advances in the identification and pharmacologic modulation of cancer drivers such as the EGFR, and improved responses to combination therapies, NSCLC outcomes remain poor. Contributing to this, tumor heterogeneity and genetic complexity are significant factors that confound NSCLC tumor classification and treatment (2–4, 9).

Regions of DNA amplification can be up to 1 Mb in size, and therefore often encompass neighboring genes in addition to the suspected driver gene (10–12). It has been suggested that genes coamplified within a given amplicon could be functionally relevant (12) and that coamplified genes may cooperate to promote tumor progression (2, 10). Indeed, in a series of NSCLC-derived cell lines, several genes mapping proximal to EGFR, including CHCHD2, have been reported as coamplified and with elevated mRNA expression (13). Similarly, a recent integrated omic analysis of NSCLC indicated consistent upregulation of proteins genetically linked to EGFR within the recurrent 7p11.2 amplicon, including the chaperonin subunit CCT6A, and CHCHD2 (14).

However, the genes frequently coamplified with EGFR, including CHCHD2, and their protein products have not been systematically investigated for roles in NSCLC.

According to The Human Protein Atlas, the CHCHD2 gene product is a widely expressed 16.7-kDa mitochondrion-localized protein that is upregulated in lung cancer (15). CHCHD2 consists of an N-terminal mitochondrion localization sequence and a strongly conserved C-terminal CHCH domain, which is characterized by twin CX6C motifs in a CXnCX6CnC configuration. Twin CX6C proteins, such as CHCHD2, have been proposed to function as scaffolding proteins in the mitochondrion, and have been identified as part of respiratory chain complexes.
cytochrome c oxidase (COX) assembly factors, or involved in the maintenance of mitochondrion structure and function (16–18). Depletion of CHCHD1 proteins decreases cellular oxygen consumption and ATP production (16, 18, 19). Recently, CHCHD2 was predicted through computational expression screening, and then experimentally validated, as a regulator of oxidative phosphorylation (oxphos; ref. 20). It was further demonstrated that CHCHD2 could bind to the promoter of COX subunit 4 isoform 2 (COX4I2) and interact with two other transcription factors (RBPs, CXCC5C) in regulating COX4I2 gene expression under the influence of oxygen concentration (21). The CHCHD2 cDNA was found to promote cell migration and alter cell adhesion when ectopically overexpressed in NIH3T3 fibroblasts (22). Dysregulated metabolism and altered migration/invasion are pivotal factors for tumor initiation and progression (23). Therefore, the EGFR-linked CHCHD2 gene product is implicated as a factor contributing to the cancer phenotype in NSCLC.

In this study, the role of CHCHD2 in NSCLC was addressed by analysis of primary NSCLC-derived xenografts, and tumor-derived cell lines. An integrative approach including measurement and modulation of CHCHD2 protein expression, affinity purification-mass spectrometry (AP-MS), in vivo proximity ligation, and computational methods was used to gain insight into the interactions and function of CHCHD2. The results suggest that CHCHD2 functions in the regulation of NSCLC cell growth, migration, and mitochondrial function via complex protein–protein interaction networks.

Materials and Methods

Cells, constructs, and reagents

The established NSCLC cell line HCC827 and the xenograft derived lung primary cell line LPC43 (Supplementary Table S1) were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin (Invitrogen). HEK293 cells were grown in DMEM with 10% FBS, and penicillin/streptomycin. All cells were cultured at 37°C in 5% CO2-humidi ed incubator.

The plasmid containing human CHCHD2 full-length cDNA was obtained from the SPARC BioCentre (Hospital for Sick Children, Toronto, ON, Canada). To generate a C-terminal Flag-tagged construct, CHCHD2 cDNA was amplified by PCR using specific primers with a linker encoding the Flag tag (Supplementary Table S1). The PCR product was subcloned into a pcDNA3.1-Myc/His expression vector (Invitrogen). All expression vectors were sequenced to confirm their authenticity.

Antibodies used were rabbit polyclonal anti-CHCHD2 (HPA027407; Sigma-Aldrich), mouse monoclonal anti-FLAG M2 (Sigma-Aldrich), rabbit polyclonal anti-EGFR (SC-03; Santa Cruz Biotechnology), rabbit monoclonal anti-CIQB (6502S; Cell Signaling Technology) and rabbit monoclonal anti-YB1 (9744; Cell Signaling Technology). All other reagents were purchased from Sigma-Aldrich unless otherwise specified.

Generation of CHCHD2 knockdown and rescue stable cell lines

Stable knockdown at the protein level was achieved by using shRNAi: pGIPIZ-GFP-human CHCHD2-shRNAmir (Clone ID: V21HL5_97752) and pGIPIZ-GFP-scrambled shRNAmir lentiviral vectors were sourced from Open Biosystems. Lentiviral particles, prepared by SPARC BioCentre, were used to infect target cells (HCC827, LPC43) following standard protocols. The stably transduced CHCHD2 knockdown (KD) and nonsilencing control (NSC) cell lines were selected by growth in medium containing 2 μg/mL puromycin. The rescue/overexpression cell lines (RES/OE) were established by ectopic expression of an shRNA-resistant human CHCHD2 cDNA in the knockdown lines: CHCHD2 full-length cDNA, resistant to pGIPIZ-CHCHD2 shRNAmir by introduction of a silent mutation, was subcloned into pLX303 (from Dr. Sergio Grinstein, Hospital for Sick Children), and used to prepare virus particles. Transduced, blasticidin-resistant CHCHD2-KD cells (derived from HCC827 and LPC43) were selected and verified for CHCHD2 expression.

Affinity purification and Western blotting

For the isolation of immunoprecipitates (IPs) containing CHCHD2 protein complexes, HEK293 cells were transiently transfected with Flag-tagged CHCHD2. Cells were lysed in NP-40 buffer [50 mmol/L Tris–HCl, pH 7.5, 150 mmol/L sodium chloride, 1% (v/v) Nonidet P-40, 100 mmol/L NaF, 1 mmol/L sodium orthovanadate, and protease inhibitors; ref. 24], clarified, and subjected to immunopurification using immobilized anti-FLAG M2 agarose beads (Sigma-Aldrich).

For coimmunoprecipitation (co-IP), immune complexes were eluted from beads by using 2X Laemmli sample buffer, followed by Western blotting. Western blotting of total cell protein extracts (15 μg protein/sample) or IP eluates involved SDS–PAGE followed by electrophoretic transfer to nitrocellulose membranes (Amersham Bioscience), blocking, and probing with primary and secondary antibodies as previously described (24). For IP followed by liquid chromatography-tandem mass spectrometry (LC/MS–MS), isolated immune complexes were dissociated by using 0.15% trifluoroacetic acid (TFA), neutralized, and then immediately processed for downstream proteomic analysis (25).

Immunofluorescence staining and confocal microscopy

LPC43 and Hela cells were grown on coverslips for 24 hours. For transient CHCHD2-flag expression, Hela cells were transfected by using Lipofectamine reagent (Life Technologies), and then incubated for 24 hours. For CHCHD2 imaging, cells were grown on coverslips submerged in a 24-well plate until subconfluent. The mitochondria of live cells were stained with Mitotracker Red CMXRos (red color) for 30 minutes (37°C, 5% CO2). After paraformaldehyde fixation, cells were permeabilized with 0.2% Triton X-100, incubated with rabbit antibody to CHCHD2 (Sigma-Aldrich; HPA027407) and then detected with Alexa Fluor 647–conjugated secondary antibodies (far-red). Confocal microscopy was performed by using a Zeiss LSM 510 META laser scanning microscope equipped with a ×64 objective. Green fluorescence was an indication of lentivirus infection. Counterstaining of DNA with 4′,6-diamidino-2-phenylindole (DAPI) was used to localize cell nuclei (blue). Additional details on experimental protocols were published previously (24, 26).

Liquid chromatography-tandem mass spectrometry analysis

MS sample preparation was performed essentially as described previously (27). Tryptic peptides were concentrated and purified on homemade C18 columns or C18 StageTips (Thermo Fisher Scientific) before LC/MS-MS. For MS analysis, peptides were separated by reverse-phase chromatography using a nanoflow UPLC system (Thermo Fisher Scientific) with a 240-minute linear gradient. The UPLC system was coupled to an Orbitrap Elite MS...
instrument (Thermo Fisher Scientific), and peptides were fragmented by collision-induced dissociation (CID).

MS data processing and analysis

Raw MS files acquired from the Orbitrap-Elite were processed by MaxQuant software (version 1.3.0.5) according to the standard workflow (28). MS/MS spectra were searched against the UniProt human proteome (release 2013_03_06) containing 87,656 entries (including common contaminants) by using the Andromeda search engine (29). For statistical evaluation of the data, a false discovery rate of 0.01 was set for peptide and protein identification. MS data related to NSCLC primary tumors were accessed from ProteomeXchange (http://www.proteomexchange.org) with the dataset identifier PXD000853, and analyzed by using MaxQuant as described in Li and colleagues (14). Protein LFQ (label-free quantification) intensity obtained from MaxQuant was chosen as the quantitative value representing protein abundance, and used for calculation of protein differential expression. For quantitative proteomics, protein LFQ intensities were exported and statistical analysis was performed by using the R software package. For quantitative mass spectrometric analysis of anti-Flag IPs, protein LFQ intensities across different samples were first normalized according to the intensities of the bait protein CHCHD2 in each sample. Normalized LFQ intensities were then used for determination of specific protein–protein interactions by using Perseus software tools available in the MaxQuant environment.

Identification of CHCHD2 interactions by proximity-dependent biotinylation (BiodI) and MS

To identify proximate and interacting proteins in living cells, the BiodI method (30) was used. By this approach, proteins in the vicinity of the FlagBirAR118G–CHCHD2 fusion protein were covalently modified by biotin in vivo, followed by MS identification of biotinylated proteins isolated by streptavidin-based affinity capture in vitro. In brief, full-length CHCHD2 coding sequence was subcloned into the pcDNA5/FRT/TO-FlagBirA’ expression vector. HEK293T-Rex cells stably expressing FlagBirA’–CHCHD2 or CHCHD2-BirA’Flag were generated by using the Flp-In system (Invitrogen). Cells were incubated for 24 hours in complete medium supplemented with 1 μg/mL tetracycline (Sigma-Aldrich) and 50 μmol/L biotin (Bioshop), and then lysed in RIPA buffer (50 mmol/L Tris–HCl pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, protease inhibitors). Clarified cell lysates were subject to streptavidin affinity purification followed by trypsin digestion and MS analysis, essentially as described previously (31). Detailed information is included in Supplementary Methods.

CHCHD2 quantification by selected reaction monitoring-MS

Selected reaction monitoring (SRM) was performed as described (27) with slight modifications. SRM peak intensities were calculated by using Skyline software (32), and signal intensity of each peptide was the average of two technical replicates. Total intensities from three CHCHD2 peptides (Supplementary Table S2) were summed and multiplied by a correction factor, which was determined by normalization of the sum of MYH9 and ACTG total peak intensities across the different samples.

Cell proliferation and migration assays

Real-time cell proliferation and migration activities were monitored by using the xCELLigence system (AECa Biosciences) according to the manufacturer’s instructions. For growth curves, cells were seeded in triplicate into 96-well E-plates, and the cell index, reflecting electrical impedance was measured every 15 minutes throughout the experiment. For migration, cells were serum-starved for 16 hours, trypsinized, resuspended in serum-free media and then seeded in triplicate on the top chamber of CIM-16 plates. Serum-containing medium was added to the lower chamber as a migratory stimulant. Cells were allowed to settle for 30 minutes before measurements were taken. The cell index was collected at 5-minute intervals. Proliferation and migration rates were determined from the slope of cell index curve.

Oxygen consumption and extracellular acidification measurement

To measure mitochondria function in intact cells, a Seahorse Bioscience XF96 Extracellular Flux Analyzer was used. Brieﬂy, cells were seeded in XF96-well microplates (25,000/well) with RPMI-1640 complete medium, and incubated for 16 hours. The pH of medium was adjusted to 7.4, and ﬁnal concentrations of glucose and glutamine were 11 mmol/L and 2 mmol/L, respectively. Cells were subsequently washed and restored with bicarbonate-free medium and incubated in a CO2-free incubator for 2 hours. Oxygen consumption rate and extracellular acidification rate were measured under basal conditions, in the presence of the ATP-synthase inhibitor oligomycin (1 μmol/L) and the mitochondri- on uncoupling agent, FCCP (500 nmol/L). Data were normalized according to the number of seeded cells.

Statistical analysis and bioinformatics

The signiﬁcance of difference between groups was determined by the Student t test using R software or by one-way ANOVA followed by Bonferroni post hoc test using GraphPad Prism 3.0 (GraphPad Software Inc.), unless stated otherwise.

Gene ontology (GO) enrichment analysis was performed by using DAVID bioinformatics resources (33). A P value less than 0.05 was considered statistically signiﬁcant. Protein interaction network analysis was based on literature investigations and the Biological General Repository for Interaction Datasets (BioGRID; ref. 34). Cytoscape version 3.0 (35) was used for visualization of protein interaction networks.

Results

Correlated expression of EGFR and CHCHD2 in NSCLC

As EGFR overexpression in NSCLC is often a consequence of gene amplification (36), we examined whether the EGFR-proximal gene CHCHD2 is coamplified. Data on somatic copy number variation (SCNV) in lung adenocarcinoma (LADC) and lung squamous cell carcinoma (LSCC) were retrieved from The Cancer Genome Atlas (TCGA) data portal (www.cancergenome.nih.gov) and through cBioPro- tal (37). This indicated that CHCHD2 is indeed frequently coampliﬁed along with EGFR in both subtypes, and with a strong tendency toward cooccurrence (odds ratio >10; Fig. 1A). As shown in Fig. 1A, it is apparent that EGFR as expected, but not CHCHD2, is in some instances mutated in addition to being ampliﬁed. We recently reported that EGFR and CHCHD2 protein levels are upregulated in primary NSCLC (14). Figure 1B shows that the levels of EGFR and CHCHD2 are consistently elevated relative to normal lung in a set of 11 NSCLC patient-derived xenograft (PDX) tumors (listed in Supplementary Table S1), and show a strong correlation in their expression levels, with a
Proteins remained moderately correlated in their expression level (Fig. 1D). This suggests that the correlated expression of the two gene products that occurs at the protein level, at least in NSCLC contexts, extends beyond their genetic linkage to the protein level, but, as shown by the HEK293 experiment (Fig. 1D), does not appear to reflect increased synthesis or stability of CHCHD2 as a product of elevated EGFR expression. Western blot analysis of four NSCLC cell lines, including LPC43 and LPC72, which were generated from NSCLC PDX sample A6 (see Supplementary Table S1), and the established cell lines RVH6849 and HCC827, showed that EGFR and CHCHD2 were coexpressed in vitro (Supplementary Fig. S1).

Modulation of CHCHD2 expression alters NSCLC cell proliferation and migration

The coamplification and elevated protein expression of EGFR and CHCHD2 in NSCLC led us to consider whether CHCHD2 expression contributes to key aspects of the cancer phenotype, including cell proliferation and migration. The NSCLC cell lines HCC827 and PDX A6-derived LPC43 were used to generate cell lines stably expressing (i) NSC shRNA, (ii) shRNA directed against human CHCHD2 (knockdown), and (iii) CHCHD2 knockdown cells "rescued" with ectopic overexpression of an shRNA-resistant human CHCHD2 cDNA (RES/OE). CHCHD2 protein in LPC43-derived cells, was mainly localized in mitochondria, as determined by colocalization with MitoTracker, whereas CHCHD2 staining was minimal on knockdown cells (Fig. 2). Consistent with the cell imaging results, quantification of CHCHD2 by SRM-MS analysis of three different CHCHD2 peptide ions confirmed that protein expression was greatly reduced in knockdown and increased in RES/OE lines relative to NSC cells (Fig. 3A).

The impact of CHCHD2 depletion and overexpression on NSCLC cell proliferation and migration were considered. Cell proliferation kinetics were measured in real-time by monitoring the electrical impedance of the substrate surface, which is affected by the area of cell contact, and hence cell number (Fig. 3B). In order to measure cell migration, a Transwell migration assay based on cell-surface impedance was used (Fig. 3C). In HCC827 knockdown cells, the level of CHCHD2 was 7% of the level measured in NSC, and in RES/OE the amount of CHCHD2 was approximately 2.4-fold greater than NSC. In LPC43 the amount of CHCHD2 in knockdown was 12% of that seen in NSC, and in RES/OE expressed 6.7-fold more than RES (Fig. 3A).

In HCC827 knockdown cells, migration was significantly decreased in CHCHD2 knockdown compared with NSC and/or RES/OE. In LPC43, CHCHD2 knockdown significantly reduced the rate of proliferation, but proliferation remained impaired in the RES/OE cells. In HCC827 and LPC43, migration was significantly decreased in CHCHD2 knockdown cells. This effect was not recued by CHCHD2 overexpression in LPC43 cells compared with knockdown. In LPC43, CHCHD2 knockdown significantly reduced the rate of proliferation, but proliferation remained impaired in the RES/OE cells. In HCC827 and LPC43, migration was significantly decreased in CHCHD2 knockdown cells. This effect was not recued by CHCHD2 overexpression in LPC43 cells compared with knockdown. In LPC43, CHCHD2 knockdown significantly reduced the rate of proliferation, but proliferation remained impaired in the RES/OE cells. In HCC827 and LPC43, migration was significantly decreased in CHCHD2 knockdown cells. This effect was not recued by CHCHD2 overexpression in LPC43 cells compared with knockdown. In LPC43, CHCHD2 knockdown significantly reduced the rate of proliferation, but proliferation remained impaired in the RES/OE cells.
of oxphos, along with FCCP-stimulated maximal oxygen consumption, and mitochondrial reserve capacity. In both cell types (HCC827 and LPC43) basal and maximal OCR and mitochondrial reserve capacity were significantly decreased in knockdown cells compared with NSC controls (Fig. 4A and B). In both of the RES/OE cell types, these parameters showed a general trend intermediate between control NSC and knockdown values, but were not significantly different from each. This suggests that in terms of mitochondrial function, overexpression of CHCHD2 in RES/OE cells did not achieve a true "rescue" effect. Analysis of additional RES clones with CHCHD2 expression closer to endogenous levels might address this possibility. Basal extracellular acidification rate (ECAR), a marker for glycolysis was not significantly changed as a function of CHCHD2 in the two cell lines (Supplementary Fig. S2).

The effect of CHCHD2 expression on NSCLC proteomes

To elucidate the molecular changes that contribute to the phenotypes observed, MS-based quantitative proteome analyses were performed to identify proteins differentially expressed as a function of CHCHD2 expression in the LPC43- and HCC827-based, NSC, knockdown, and RES/OE cell lines. Three independent analyses of each cell line were completed and in aggregate a total of 4,308 nonredundant proteins were identified and quantified. An overlap of 70% to 75% (at the protein level) was observed between the independent replicate experiments (Supplementary Fig. S3). After pooling the respective NSC, knockdown, and RES/OE datasets derived from LPC43 and HCC827, a majority of proteins (3,249; 75%) was shared by the NSC, knockdown, and RES/OE groups (Fig. 5A). Statistical analysis (paired t test) identified 205 proteins as significantly differentially expressed (P < 0.05) between knockdown and NSC cells (Supplementary Table S3). Go analysis revealed that the differentially expressed proteins are implicated in diverse biologic processes (Fig. 5B), and distributed across various cellular compartments, primarily mitochondrion, nuclear lumen, ribonucleoprotein complex, and cytoskeleton (Supplementary Fig. S3).

In order to more stringently identify candidates whose expression levels changed upon CHCHD2 depletion, the list of differentially expressed proteins was filtered to include only proteins showing ≥2-fold expression difference between NSC and knockdown, but not between NSC and RES/OE. This produced a smaller set of 13 proteins, excluding CHCHD2, regarded as CHCHD2 regulated proteins (Table 1). Ten proteins showed parallel expression changes with CHCHD2 expression: downregulation in CHCHD2 knockdown cells, and recovered expression in CHCHD2 RES/OE cells (Table 1). This set includes two protein kinases: the tyrosine-specific anaplastic lymphoma kinase (ALK), and the MAP2K4-encoded dual specificity enzyme known as mitogen-activated protein kinase kinase 4 (MKK4). Substrates activated by MKK4 include the MAP kinases JNK1 and -2 and the stress-activated MAP kinase p38 (25). In contrast, the expression levels of three proteins, including ATP-dependent Clp protease (CLPP), WD repeat-containing protein 20 (WDR20) and Agrin (AGRN), were increased upon CHCHD2 knockdown (Table 1).

Proteomic analysis of the CHCHD2 protein interactome

As a putative scaffolding protein and transcription factor, CHCHD2 is expected to function through protein–protein interactions. To test this, CHCHD2-associated proteins were characterized by affinity purification mass spectrometry (AM-MS). C-terminal Flag-tagged CHCHD2 constructs were ectopically expressed in transiently transfected cells, which were verified by immunoblot and immunostaining analysis (Supplementary Fig. S4). Anti-Flag IPs were prepared from cells expressing epitope-tagged CHCHD2 and control cells not expressing ectopic CHCHD2. The criterion for a specific CHCHD2-interacting protein was differential recovery relative to the negative control (fold enrichment ≥ 32, and P ≤ 0.01; Fig. 6A; Supplementary Fig. S4). By this criterion, 58 proteins were identified as binding directly or indirectly to CHCHD2 (Supplementary Table S4).

To further validate the CHCHD2-interacting proteins, a recently developed proximity-dependent in vivo biotinylation method termed BioID (30) was applied in combination with MS to identify proteins that interact or come into close proximity with CHCHD2 within cells. Following the BioID methodology, CHCHD2 was expressed in transfected cells as a fusion protein including the
promiscuous biotin protein ligase domain BirA’ fused to the carboxyl end of CHCHD2. A total of 65 proteins that became biotinylated in vivo due to their proximity with CHCHD2-BirA’ passed stringent criteria applied by using SAINT software (Fig. 6A and Supplementary Table S5; ref. 39). Seven proteins were identified by both the AP-MS and proximity ligation methods, suggesting they are central, or at least high-confidence CHCHD2-associated proteins (Fig. 6B). Among the remaining CHCHD2-interacting proteins that were identified by only one of the methods (i.e., AP-MS or BioID), interrogation of BioGRID (34) verified eight as having interactions with one or more of the seven high-confidence CHCHD2-associated proteins. These proteins were organized and visualized by using Cytoscape tools (Fig. 6B; ref. 35). This revealed two highly connected protein subclusters that were centered on proteins C1QBP and YBX1, suggesting that they may function as hubs within a CHCHD2 network. The protein CAND1 was common to both the C1QBP and YBX1 complexes. It is reportedly a transcription factor, and also an F-box protein exchange factor, influencing the association of diverse F-box proteins in SCF ubiquitin ligase complexes (40). In addition to C1QBP and YBX1, which were previously reported as CHCHD2-interacting proteins (22, 41–43), five other CHCHD2-interacting candidates were identified by both AP-MS and proximity ligation including the uncharacterized protein coiled-coil domain containing 71-like (CCDC71L); cytidine monophosphate N-acetylneuraminic acid synthetase (CMAS), the inner nuclear membrane protein LBR; the putative RNA helicase DHXS7; and the nuclear chaperone THOC4 (encoded by ALYREF). Two previously reported CHCHD2-interacting proteins RPL24 and polyubiquitin-C (UBC; ref. 41) also were detected by AP-MS, but did not pass the selection criteria. Further support for the interaction of CHCHD2 with YBX1 and C1QBP was obtained by demonstration of associations between ectopic CHCHD2 and endogenous YBX1 and C1QBP by Western blot analysis of CHCHD2-Flag IPs recovered from transfected HEK293 cells (Fig. 6C).

**Discussion**

Recurrent cancer-associated amplifications and deletions are logically thought to be driven by changes in expression of encoded...
oncogenes and tumor suppressors, respectively. Integrated analysis of NSCLC proteomes and somatic gene copy number variation suggests that genomes are organized such that coamplified genes are functionally linked to the cancer phenotype (14). Coamplification of EGFR and CHCHD2 has been reported in NSCLC (13) and other cancer types, including glioma (12). However, not all amplified genes in tumors are overexpressed (12). Our analysis of TCGA datasets clearly indicates that EGFR and CHCHD2 are indeed coamplified in both the ADC and SCC subtypes of NSCLC. Consistent with this trend, we found that CHCHD2 and EGFR protein expression levels are elevated relative to normal lung and are positively correlated with each other in primary NSCLC xenografts. However, in HEK293 cells, CHCHD2 protein expression was not increased as a product of elevated ectopic EGFR expression. This negates a model, at least in the HEK293 system, wherein CHCHD2 protein expression is itself elevated as a product of EGFR protein expression. Although we do not yet understand the mechanisms regulating EGFR or CHCHD2 protein levels, the observations that both proteins appear consistently upregulated in NSCLC (Fig. 1B; ref. 14) provided a rationale for experiments to address whether CHCHD2 contributes to the transformed cell phenotype.

Cancer metabolism is known to affect cell migration (44). CHCHD2 was implicated as a regulator of oxphos (20), and

Figure 4.
Dependence of mitochondrial respiration on CHCHD2 protein expression. Control (NSC), knockdown, and rescue/overexpression (RESOE) cells were seeded in specialized microplates and cultured for 16 hours. Cells were then switched to bicarbonate-free medium and mitochondrial function was assessed using sequential injection of oligomycin and FCCP. Shown are representative OCR curves and summarized quantification of basal OCR, maximal OCR, and reserve capacity of (A) HCC827 and (B) LPC43. Results are mean ± SEM from three independent experiments.

**P < 0.01; *P < 0.05.
Ectopic CHCHD2 promoted NIH3T3 cell migration (22). Consistent with these observations, we found that cell migration and proliferation rates in the NSCLC cell lines HCC827 and LPC43 were stimulated by CHCHD2 expression. Furthermore, knockdown of CHCHD2 significantly attenuated mitochondrial respiratory activity, typified by reduced basal and maximum OCR, and diminished mitochondrial reserve capacity. In breast cancer cells, loss of reserve capacity is linked to apoptosis (45), and maintenance of reserve capacity positively correlates with rapid proliferation (46). Therefore, we speculate that the impaired NSCLC cell proliferation associated with CHCHD2 knockdown was, at least in part, due to the measured reduction in mitochondrial reserve capacity. Consistent with this, our comprehensive analysis of NSCLC cell proteomes revealed proteins that changed in abundance as a function of CHCHD2 knockdown and rescue, and including significant enrichment for biologic processes involved in cell proliferation and migration (i.e., GO categories cell proliferation, cell cycle, and cell projection morphogenesis). For example, Agrin protein expression increased 2-fold upon CHCHD2 knockdown and this effect was reversed by CHCHD2 rescue. Overexpression of Agrin increased substrate adhesion in COS7 cells (47). We therefore speculate that elevated Agrin may have stimulated cell adhesion, and consequently hindered cell migration, in response to CHCHD2 knockdown. These results are consistent with observations of increased cell motility associated with EGFR amplification (48), and suggest that coamplification of CHCHD2 may contribute to this cancer-associated phenomenon.

The receptor tyrosine kinase ALK is activated as a consequence of somatic chromosomal rearrangements in a subset (3%–7%) of ADC-subtype NSCLC (49). Knockdown of CHCHD2 was accompanied by reduced expression of ALK; however, to the best of our knowledge, wild-type ALK, which is known to function in brain development and neural
function, has not been implicated as an oncogenic driver. Interestingly, recent observations of concomitant EGFR mutation and ALK gene rearrangement has suggested that coordinated activation of the signaling networks of EGFR and ALK may be important in lung ADC (50). Although not tested in this study, our results suggest that coamplification of CHCHD2 and EGFR may be linked to ALK.

In addition to Agrin, the expression of other proteins was affected by CHCHD2 protein levels (Table 1). WDR20 displayed increased expression upon CHCHD2 depletion and dropped back

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<td>Q5UM73</td>
<td>ALK receptor tyrosine kinase</td>
<td>0.014</td>
<td>-1.024</td>
</tr>
<tr>
<td>AGRN</td>
<td>O00468</td>
<td>Agrin</td>
<td>0.001</td>
<td>1.016</td>
</tr>
<tr>
<td>WDR20</td>
<td>E7EUY8</td>
<td>WD repeat-containing protein 20</td>
<td>0.047</td>
<td>1.529</td>
</tr>
<tr>
<td>CLPP</td>
<td>Q16740</td>
<td>Putative ATP-dependent Clp protease proteolytic subunit, mitochondrial</td>
<td>0.027</td>
<td>1.610</td>
</tr>
</tbody>
</table>

Table 1. Proteins differentially expressed between CHCHD2 knockdown and NSC

Figure 6.
Analysis of CHCHD2 interactome by MS approach. A, schematic depiction of the experimental plan for the identification of CHCHD2 associated proteins. In one arm, CHCHD2-associated proteins were identified by AP-MS analysis of proteins recovered by anti-Flag immunoprecipitation of ectopically expressed CHCHD2-Flag. In the other arm, the BioID method was used to characterize by MS proteins that became covalently modified by ubiquitin in cells expressing a fusion protein of CHCHD2 linked to the promiscuous ubiquitin ligase BirA*. Seven proteins in addition to the CHCHD2 bait proteins were identified by both approaches. B, network analysis of the CHCHD2 interactome. Proteins and their interactions are shown as nodes and edges. Interacting proteins were identified by AP-MS (light purple), BioID (orange), or both methods (pink), as indicated. Solid black lines indicate interactions also present in the BioGRID database search (http://thebiogrid.org). The dashed boxes encompass two potential CHCHD2-associated complexes. C, Western blot validation of CHCHD2 interacting proteins. Lysates and anti-Flag IPs from transfected HEK293 cells expressing ectopic CHCHD2-Flag and control cells not expressing CHCHD2-Flag were analyzed by SDS-PAGE and immuno-blotting. Expression of YBX1 and C1QBP was confirmed by analysis of lysates (lanes 1 and 2). Both proteins were recovered by co-IP with CHCHD2-Flag (lane 4), but not detected in anti-Flag IPs from control cells (lane 3).
toward control levels after CHCHD2 was reexpressed. WDR20 interacts with and stimulates the activity of ubiquitin-specific protease USP12 and also interacts with USP46 (51). USP12 regulates Notch signaling (52) and USP46 is a tumor suppressor (53). However, this study did not address mechanisms through which CHCHD2 might affect protein production, modifications, or stability. The proteins that changed in abundance in cells depleted of CHCHD2 relate to several different cellular functions and subcellular localizations other than mitochondrion.

The CHCHD2 interactome was defined by the complementary approaches of AM-MS and proximity ligation. This analysis revealed two highly connected subnetworks centered on putative hub proteins C1QBP and YBX1. The mitochondrial protein C1QBP is upregulated in a variety of neoplasms, including lung cancer (43, 54), and has promotion activity in cancer cell lines (43, 54). Moreover, C1QBP has been shown to promote cancer cell proliferation and resistance to cell death (54). YBX1 is an oncogenic transcription factor with roles in cell proliferation, invasion, and metastasis as well as energy metabolism (55). It was suggested that nuclear translocation of YBX1 could induce upregulation of EGFR expression and a more aggressive NSCLC phenotype (56). A recent report further revealed YBX1 as a convergent hub for lysophosphatidic acid and EGF signaling, and activation of YBX1 contributed to ovarian cancer cell invasion (57). Considering the multifaceted roles of C1QBP and YBX1, together with our observation that they present as “hubs” within the CHCHD2 interactome, we propose that CHCHD2 functions, at least in part, through interactions with C1QBP and YBX1. Our nonquantitative indirect immunofluorescence microscopic analysis most obviously depicted a mitochondrial localization of CHCHD2, which may reflect its major subcellular localization under the conditions of our analysis, but may also mean that nonmitochondrial CHCHD2 was below our level of detection. The changes in protein levels in response to CHCHD2 knockdown (Table 1), could be downstream effects stemming from the loss of CHCHD2 function in mitochondria, and/or a consequence of changes in gene expression related to the noted transcription factor activity of CHCHD2 (21), and possibly involving its interactions with YBX1. In conclusion, our analyses indicate that CHCHD2 gene copy number and protein levels are linked with EGFR in NSCLC. CHCHD2 participates in mitochondrial and extra-mitochondrial protein–protein interactions and is an effecter of cell proliferation, migration, and respiration. Therefore, along with EGFR, it should be considered as a driver of 7p11.2 amplification and the cancer phenotype.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Study supervision: M.S. Tsao, M.F. Moran

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References
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