Proteomics Analysis of the Interactome of N-myc Downstream Regulated Gene 1 and Its Interactions with the Androgen Response Program in Prostate Cancer Cells*

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NDRG1 is known to play important roles in both androgen-induced cell differentiation and inhibition of prostate cancer metastasis. However, the proteins associated with NDRG1 function are not fully enumerated. Using coimmunoprecipitation and mass spectrometry analysis, we identified 58 proteins that interact with NDRG1 in prostate cancer cells. These proteins include nuclear proteins, adhesion molecules, endoplasmic reticulum (ER) chaperons, proteasome subunits, and signaling proteins. Integration of our data with protein-protein interaction data from the Human Proteome Reference Database allowed us to build a comprehensive interactome map of NDRG1. This interactome map consists of several modules such as a nuclear module and a cell membrane module; these modules explain the reported versatile functions of NDRG1. We also determined that serine 330 and threonine 366 of NDRG1 were phosphorylated and demonstrated that the phosphorylation of NDRG1 was prominently mediated by protein kinase A (PKA). Further, we showed that NDRG1 directly binds to β-catenin and E-cadherin. However, the phosphorylation of NDRG1 did not interrupt the binding of NDRG1 to E-cadherin and β-catenin. Finally, we showed that the inhibition of NDRG1 expression by RNA interference decreased the ER inducible chaperon GRP94 expression, directly proving that NDRG1 is involved in the ER stress response. Intriguingly, we observed that many members of the NDRG1 interactome are androgen-regulated and that the NDRG1 interactome links to the androgen response network through common interactions with β-catenin and heat shock protein 90. Therefore we overlaid the transcriptomic expression changes in the NDRG1 interactome in response to androgen treatment and built a dual dynamic picture of the NDRG1 interactome in response to androgen. This interactome map provides the first road map for understanding the functions of NDRG1 in cells and its roles in human diseases, such as prostate cancer, which can progress from androgen-dependent curable stages to androgen-independent incurable stages. Molecular & Cellular Proteomics 6:575–588, 2007.
expression of NDRG1 can be induced by a variety of stimulus including ER stress-inducing agents such as β-mercaptopo-
ethanol and tunicamycin (16). We and others have shown that
NDRG1 expression was induced by androgen (17, 18). Phos-
phatase and tensin homolog (PTEN) (7) and p53 (10), two
important tumor suppressor genes, also up-regulate the ex-
pression of NDRG1. Other inducers include metal ions (19–
22), DNA damage agents such as camptothecin (23), intracel-
lar calcium concentrations (24), and hypoxic condition (25).
Hypoxia and its mimetics (e.g. nickel and cobalt) are probably
the most important inducers of the NDRG1 gene (26). Nickel
and cobalt induce NDRG1 expression by creating hypoxia-
like conditions in cells (20). Like other hypoxia responsive
genes, the induction of NDRG1 is prominently mediated by
the transcription factor HIF-1 and HIF-1α (20, 27). HIF-1α induces
NDRG1 through a phosphatidylinositol 3-kinase/Akt-depend-
ent pathway (28), but HIF-1-independent pathways may also
be involved (29). These results imply that multiple proteins and
pathways contribute to the regulation of NDRG1.

Although we know a lot about the genomic actions that
regulate NDRG1 expression, we know little about the non-
genomic regulation of NDRG1 at the protein level, which
includes the proteins that interact with NDRG1 (the NDRG1
interactome) and execute its versatile functions in both normal
and abnormal physiological conditions. With the advance of
mass spectrometry technology, we sought to comprehensively
profile the NDRG1 interactome by immunoprecipitation
coupled with high throughput tandem mass spectrometric
analysis. Because NDRG1 is an androgen-regulated gene, we
conducted the experiments in androgen-treated prostate cancer
cells LNCaP to gain additional information on the relation-
ship between the androgen response network and the
NDRG1 interactome.

We identified 58 novel NDRG1-interacting proteins by im-
munoprecipitation (IP)-LC/MS/MS and demonstrated that
NDRG1 directly binds to β-catenin and E-cadherin. Intrigu-
ingly, the NDRG1 interactome links to the androgen network
through interactions with β-catenin and heat shock protein
90. The identified NDRG1 interacting proteins also correlate
well with the functions of NDRG1 including NDRG1 phospho-
rylation, the regulation of ER chaperon expression, and pro-
teasome activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic androgen methyltrienolone (R1881) was pur-
chased from PerkinElmer Life Sciences. R1881 (10 μM) stock solution
was prepared in ethanol and stored at −20 °C. Kinase inhibitors
including H-89, GF109203X, KN-62, and 5,6-dichloro-1-β-d-ribo-
furanosylbenzimidazole (DRB) were purchased from Sigma-Aldrich.
Stock solutions (10 mM) were prepared in DMSO and stored at
−20 °C. Proteasome substrate III Z-LLL AMC was purchased from
Calbiochem and was dissolved in DMSO, stored at −20 °C. Protein
A-Sepharose was purchased from Invitrogen. IgY microbeads and
anti-NDRG1 polyclonal IgY and rabbit anti-IgY antibodies were pur-
chased from GenWay Biotech (San Diego, CA). Monoclonal antibody
against E-cadherin and β-catenin was ordered from BD Transduction
Laboratories, monoclonal antibody against proliferating cell nuclear
antigen (PCNA) was from Millipore Corporation, and antibody against
amino acid residue KDEL in the protein GRP78 and TRA1 was from
Abcam Inc. (Cambridge, MA). Trypsin and ECL reagent were pur-
chased from Amersham Biosciences. NDRG1 siRNA smart pool and
Dharmacon 3 reagent were purchased from Dharmacon (Lafayette,
CO).

**Cell Culture and Treatments**—Human prostate cancer cell line LN-
CaP was obtained from American Type Culture Collection (ATCC,
Rockville, MD) and maintained in phenol red-free RPMI 1640 medium
supplemented with 10% fetal bovine serum (FBS), 100 units/ml pen-
icillin, and 100 μg/ml streptomycin at 37 °C under 5% CO2.
For androgen deprivation, LNCaP cells were cultured in phenol red-
free RPMI 1640 medium with 10% dextran-coated charcoal-stripped FBS
for 60 h prior to treatment with 10 nM R1881 or siRNA transfection. All
treatments were done in phenol red-free RPMI 1640 medium with
10% dextran-coated charcoal-stripped FBS. For treatment of cells
with protein kinase inhibitors, cells were pretreated for 30 min with 10
μM H-89 (protein kinase A inhibitor), 2 μM GF109203X (protein kinase
C (PKC) inhibitor), 10 μM KN-62 (calcium-calmodulin kinase II inhib-
itor) (Sigma), 10 μM DRB (casein kinase-II inhibitor), or vehicle control
(DMSO) in medium.

**Immunoblotting**—Cells were washed twice with PBS and lysed at 4
°C in a lysis buffer (50 mM Hepes, pH 7.4, 4 mM EDTA, 2 mM EGTA,
10 mM sodium fluoride, 2 mM sodium pyrophosphate, 1% Triton
X-100, 2 μM PMSF, 1× protease inhibitor mixture, 1 mM Na3VO4).
Lysates were centrifuged at 14,000 rpm for 20 min. The protein
concentration in the lysates was determined using the Bio-Rad pro-
tein assay kit. Cell lysates were subjected to SDS-PAGE electropho-
resis and transferred to a PVDF membrane (Hybond-P, Amer-
sham Biosciences). The membrane was blocked with 4% nonfat milk
in TBS (25 mM Tris, pH 7.4, 125 mM NaCl) for 1 h at room temperature
followed by incubation with primary antibodies against NDRG, PCNA,
and KDEL. The membranes were washed 3 times with TBS and then
incubated with horseradish peroxidase conjugated anti-rabbit IgY or
anti-mouse IgG for 1 h. The immunoblot was then washed five times
with TBS and developed using ECL. Experiments were repeated at
least three times.

**Immunoprecipitation**—Cell lysates were incubated with 10 μl of
IgY microbeads or 30 μl of protein A-Sepharose for 1 h at 4 °C to
eliminate nonspecific absorption of proteins to the beads. After brief
centrifugation, the supernatants were incubated at 1 h at 4 °C with
IgY-microbeads or protein A-Sepharose that had been incubated with
anti-NDRG1, anti-E-cadherin, anti-β-catenin, anti-Ku70, or anti-
CANX antibodies overnight at 4 °C. Beads were then washed twice
with buffer B (20 mM Tris-Cl, pH 7.4, 0.5 mM NaCl) and twice with
buffer C (20 mM Tris-Cl, pH 7.4, 0.5 mM DTT). After washing, the
beads were boiled with SDS sample buffer and analyzed by SDS-
PAGE. After SDS-PAGE electrophoresis, proteins were transferred
to PVDF membrane and followed by Western blotting with anti-NDRG1,
anti-E-cadherin, anti-β-catenin, anti-Ku70, or anti-CANX antibodies.
For MS analysis, proteins were detected by silver staining and fol-
lowed by in-gel trypsin digestion.

**In-gel Trypsin Digestion**—For mass spectrometry analysis, the gels
were silver stained. Proteins of interest were cut from gels. Each
cuted gel was placed in an Eppendorf tube, cut into smaller (less
than 1 mm in each dimension) pieces, and incubated with 100 mM
ammonium bicarbonate for 30 min. The solution was discarded. The gel
pieces were then incubated in 100 mM ammonium bicarbonate con-
taining 45 mM DTT at 60 °C for 30 min. After cooling to room tem-
perature, the DTT solution was replaced with 100 mM iodoacetamide
in 100 mM ammonium bicarbonate for 30 min at room temperature
in the dark. The gel pieces were washed in 50% acetonitrile, 100 mM
ammonium bicarbonate for 1 h, dehydrated in 100% acetonitrile, and
dried. The gel pieces were swollen in a digestion buffer containing 25
mM ammonium bicarbonate and 250 ng of trypsin. Following enzy-
matic digestion overnight at 37 °C, the peptides were extracted with
50 μl of 5% acetonitrile for 15 min at 37 °C followed by addition of 100
μl of 100% acetonitrile for another 15 min at 37 °C. The peptides were then dried and rehydrated in 1% formic acid.

**Mass Spectrometry Analysis**—Proteins were visualized on SDS-PAGE by silver stain. Bands of interest were excised and subjected to trypsin digestion. Peptides were analyzed by microcapillary high pressure liquid chromatography nanoelectrospray tandem mass spectrometry on a LTQ mass spectrometer (Thermo Electron).

The sample was loaded automatically onto a 2-cm-long 5 μm 200A Magic C18 (Michrom Bioresources) precolumn using a FAMOS autosampler ( Dionex). The sample was washed for 15 min with a solution of 5% acetonitrile, 0.1% formic acid. A gradient was then delivered from 10 to 35% acetonitrile over 30 min. This eluted the peptides off of the precolumn onto a 10-cm-long 5 μm 100A Magic C18 analytical column and then finally into the LTQ. Both the wash and the gradient were delivered using an Agilent 1100 series binary pump, and the gradient was followed by a cleaning and equilibration step. Thermo LTQ mass spectrometer parameters were as follows: Ion Max™ source with sweep cone, ESI probe, capillary temperature 250 °C, sheath gas flow 0, auxiliary gas flow 0. Sweep gas flow 0, positive polarity; source voltage 2 kV, capillary voltage 16 V, tube lens (V) 55.00.

LC/MS spectra were acquired using 4 scans events. Data-dependent acquisition was set to require a minimal signal of 1000. We used the SEQUEST database to match peptide tandem mass spectra to sequences in the human International Protein Index (IPI) database (17) (30). The SEQUEST parameter file is shown in supplemental Doc 1. We used the Peptide Prophet and Protein Prophet programs to measure the quality of peptide and protein identification (31, 32). To assess the MS spectra quality, we applied a filter with a Protein Prophet probability >0.9 (31) and then performed visual inspection of the spectra.

**siRNA Transfection**—Dharmafect was used for transfection of siRNAs into LNCaP cells according to protocols provided by Dharmacon. Expression of NDRG1 was monitored by Western blotting.

**Proteasome Function Assays**—Proteasome function was measured as described previously (33), with some minor modifications. To measure 26S proteasome activity, 100-μg proteins of cell lysates were diluted with buffer I (50 mM Tris (pH 7.4), 2 mM DTT, 5 mM MgCl2, 2 mM MgCl2, 2 mM ATP) to a final volume of 200 μl in triplicate. The fluorogenic proteasome substrate Suc-LLVY-AMC (chymotrypsin-like, Calbiochem) was dissolved in DMSO and added to a final concentration of 80 μM in 1% DMSO. Proteolytic activity was measured as the release of the fluorescent group 7-amido-4-methylcoumarin in a fluorescence plate reader (BioTex FLx800™, Winooski, VT) at 380/460 nm.

**RESULTS**

**Interactome of NDRG1: NDRG1-interacting Proteins Identified by Immunoprecipitation and LC/MS/MS**—To explore proteins that interact with NDRG1, we performed a proteomics analysis of the NDRG1 containing complex by IP coupled with LC/MS/MS. Because androgen dramatically increases NDRG1 protein expression (see Fig. 2A), we used LNCaP cells treated with 10 nM R1881 for 24 h for the IP experiment. The IP complex was eluted, separated on SDS-PAGE, and subjected to in-gel trypsin digestion. The tryptic digests were analyzed through microcapillary liquid chromatography MS/MS followed by protein database searching of the acquired spectra. To control for nonspecific IP, IgY preimmune complex was also analyzed. The experiments were performed in three replicates, and each replicate was run through MS/MS three times. Fifty-eight proteins were identified in the NDRG1 complex after 1) filtering proteins that had a Protein Prophet probability >0.9 (31) and passed visual inspection to assess the MS spectra quality, 2) removing proteins that were not found in the replicate experiments, and 3) subtracting common proteins that were found in the IgY preimmune complex. One known NDRG1 interacting partner heat shock protein 70 (34) was excluded from the list because it was also identified from the IgY control IP/MS analysis.

The identified proteins can be classified into several functional categories such as ER chaperons, vesicle-mediated protein trafficking, DNA repair and transcription, cell adhesion and cytoskeleton organization, signal transduction, RNA processing, protein translation, and metabolism (Table 1). Most of the proteins identified in the NDRG1 complex correlate well with the reported functions of NDRG1 such as differentiation, metastasis, and ER stress responses. All the protein interactions identified here were novel.

Since this study was performed under androgen-stimulated conditions, many proteins that were identified from the NDRG1 complex are also known androgen-regulated proteins such as heat shock protein 90 α (HSPA), β-catenin (CTNNB1), calnexin (CANX), SEC23, 26S protease regulatory subunit 7 (PSMC2), and 26S protease regulatory subunit 6A (PSMC3). Based on the identified NDRG1-interacting proteins, we retrieved protein-protein interactions from the Human Protein Reference Database (HPRD, www.hprd.org) and built a map of the interactome of NDRG1 (Fig. 1). Interestingly, the NDRG1 interactome can link to the androgen receptor interactome (www.hprd.org) through CTNNB1 and HSPCA.

To understand the androgen regulation of the proteins in the NDRG1 interactome map, the androgen-responsive expression levels of each gene were also indicated on the map (colored circles, Fig. 1). Expression levels were measured using massively parallel signature sequencing (MPSS) analysis (35, 36) comparing of LNCaP cells that had been treated with R1881 against untreated cells. Only those genes with a Z-test p value < 0.05 were indicated, and the red-colored nodes indicate androgen up-regulated (p value < 0.05) genes, and green-colored nodes indicate androgen down-regulated (p < 0.05) genes.

**Confirmation of the Interactome of NDRG1: E-cadherin and β-Catenin Interact with NDRG1**—Our proteomics analysis revealed many interesting protein interactions for NDRG1. To confirm our IP-LC/MS/MS analysis results, we selected two interesting protein candidates and confirmed their binding to NDRG1 by reciprocal coimmunoprecipitation and Western blot based on the availability and quality of the available antibodies.

First we chose β-catenin and its binding partner E-cadherin. Interestingly, E-cadherin was also identified in the NDRG1 IP complex. However, it was only identified in two of three replicate experiments and therefore not included in Ta-

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2 X. Yan, L. Hood, and B. Lin, unpublished results.
| Gene symbol | Total no. of peptides | No. of independent experiments | IPI RefSeq accession no. | Protein description | Amino acid coverage % |
|-------------|----------------------|--------------------------------|--------------------------|---------------------|----------------------|
| NDRG1       | 23                   | 3                              | IPI00022078 NP_006087    | NDRG1               | 28.2                 |
| Chaperon protein |                     |                                 |                          |                     |                      |
| HSPCA       | 57                   | 3                              | IPI00382470 NP_005339    | Heat shock protein HSP 90-α | 44.7                 |
| HSPA5 (GRP78)| 79                   | 3                              | IPI00003362 NP_005338    | Heat shock 70 kDa protein 5 | 42                   |
| VCP         | 19                   | 3                              | IPI000222774 NP_009057   | Transitional endoplasmic reticulum ATPase | 23.2                 |
| CANX        | 18                   | 3                              | IPI00020984 NP_001737    | Calnexin            | 21.5                 |
| Ubiquitin-dependent |              |                                 |                          |                     |                      |
| protein catabolism |                |                                 |                          |                     |                      |
| PSMC2       | 17                   | 3                              | IPI00021435 NP_002794    | 26S protease regulatory subunit 7 | 25.5                 |
| PSM2        | 32                   | 3                              | IPI00012268 NP_002799    | 26S proteasome non-ATPase regulatory subunit 2 | 37.1                 |
| DNA repair proteins |                |                                 |                          |                     |                      |
| XRCC6 (Ku70)| 32                   | 3                              | IPI00465430 NP_001460    | ATP-dependent DNA helicase II | 48.8                 |
| RUVBL2      | 8                    | 3                              | IPI00009104 NP_006657    | RuvB-like-2         | 21.9                 |
| Transcriptional factor |            |                                 |                          |                     |                      |
| ILF3 (NAT90) | 16                   | 3                              | IPI00219330 NP_006350    | Interleukin enhancer-binding factor 3 | 19.5                 |
| SEC23A      | 19                   | 3                              | IPI0017375 NP_006355     | Protein transport protein Sec23A | 23                   |
| COPB2       | 54                   | 3                              | IPI00220219 NP_004757    | Coatomer β subunit  | 36.7                 |
| CLTC        | 86                   | 3                              | IPI00204067 NP_004850    | Similar to clathrin heavy chain | 27.2                 |
| AP2M1       | 5                    | 3                              | IPI0022256 NP_004059     | AP-2 complex subunit-μ1 | 12.5                 |
| AP1M2       | 28                   | 3                              | IPI00002552 NP_005489    | Splice isoform 1 of AP-1 complex subunit-μ-2 | 31                   |
| Cell adhesion and cytoskeleton organization |        |                                 |                          |                     |                      |
| CTNNB1      | 39                   | 3                              | IPI00017292 NP_001895    | Splice isoform 1 of β-catenin | 38.7                 |
| ACTB        | 50                   | 3                              | IPI00021440 NP_001605    | Actin               | 60.5                 |
| Kif5B       | 12                   | 3                              | IPI00012837 NP_004512    | Kinesin heavy chain | 13.6                 |
| Signal transduction |                |                                 |                          |                     |                      |
| PPP2R2A     | 17                   | 3                              | IPI00332511 NP_002708    | Serine/threonine protein phosphatase 2A | 31.3                 |
| TLE3        | 3                    | 3                              | IPI00219368 NP_005069    | Splice isoform 1 of transducin-like enhancer protein 3 | 3.4                   |
| Metabolic enzymes, mitochondria precursors, and carrier HSD17B4 | |                                 |                          |                     |                      |
| CNPD2       | 31                   | 3                              | IPI00177728 NP_006070    | Cytosolic nonspecific dipeptidase | 33.5                 |
| DARS        | 38                   | 3                              | IPI00216951 NP_001340    | Aspartyl-tRNA synthetase | 48.1                 |
| DLST        | 14                   | 3                              | IPI00421018 NP_001924    | Dihydrolipoate-residue succinyl-transferase | 13.9                 |
| ACSL3       | 16                   | 3                              | IPI00401055 NP_004448    | acyl-CoA synthetase long chain family member 3 | 16.7                 |
| FASN        | 33                   | 3                              | IPI00645907 NP_004095    | Fatty acid synthase | 11.5                 |
| MAOA        | 26                   | 3                              | IPI00008483 NP_000231    | Aminoxidase (flavin containing) A | 25.2                 |
| LDHA        | 14                   | 3                              | IPI00217966 NP_005557    | Lactate dehydrogenase A | 22.6                 |
| PKM2        | 41                   | 3                              | IPI00479186 NP_002645    | Pyruvate kinase 3 isoform 1 | 40.9                 |
| Protein translation regulators EEF2 |            |                                 |                          |                     |                      |
| EEF1G       | 38                   | 3                              | IPI00000875 NP_001395    | Similar to elongation factor 1-gamma | 37.2                 |
| EIF2S3      | 15                   | 3                              | IPI00297982 NP_001406    | Eukaryotic translation initiation factor 2 subunit 3 | 33.1                 |
Table I—continued

| Gene symbol | Total no. of peptides | No. of independent experiments | IPI | RefSeq accession no. | Protein description | Amino acid coverage |
|-------------|-----------------------|--------------------------------|-----|----------------------|---------------------|-------------------|
| EIF3S6 | 11 | 3 | IPI00013068 | NP_001559 | Eukaryotic translation initiation factor 3 subunit 6 | 24.7 |
| PABPC1 | 52 | 3 | IPI0008524 | NP_002559 | Splice isoform 1 of polyadenylate-binding protein 1 | 42.9 |

Ribosomal proteins

| Gene symbol | Total no. of peptides | No. of independent experiments | IPI | RefSeq accession no. | Protein description | Amino acid coverage |
|-------------|-----------------------|--------------------------------|-----|----------------------|---------------------|-------------------|
| RPS3 | 35 | 3 | IPI0011253 | NP_000996 | 40S ribosomal protein S3 | 57.2 |
| RPS6 | 17 | 3 | IPI0021840 | NP_001001 | 40S ribosomal protein S6 | 21.3 |
| RPL24 | 7 | 3 | IPI00306332 | NP_000977 | 60S ribosomal protein L24 | 13.4 |
| RPL3 | 31 | 3 | IPI0050021 | NP_000958 | 60S ribosomal protein L3 | 34.3 |
| RPS16 | 15 | 3 | IPI00221092 | NP_001011 | 40S ribosomal protein S16 | 24.8 |
| RPS8 | 13 | 3 | IPI00216587 | NP_001003 | 40S ribosomal protein S8 | 31.9 |
| RPS20 | 6 | 3 | IPI00012493 | NP_001014 | 40S ribosomal protein S20 | 25.2 |
| RPS9 | 18 | 3 | IPI0021088 | NP_001004 | 40S ribosomal protein S9 | 30.6 |
| RPL4 | 79 | 3 | IPI0003918 | NP_000959 | 60S ribosomal protein L4 | 39.2 |
| RPS26 | 9 | 3 | IPI00655650 | NP_001020 | 40S ribosomal protein S26 | 21.1 |

RNA processing

| Gene symbol | Total no. of peptides | No. of independent experiments | IPI | RefSeq accession no. | Protein description | Amino acid coverage |
|-------------|-----------------------|--------------------------------|-----|----------------------|---------------------|-------------------|
| NCL | 21 | 3 | IPI00444262 | NP_005372 | Nucleolin | 25.2 |
| HNRPF | 25 | 3 | IPI00003881 | NP_004957 | Heterogeneous nuclear ribonucleoprotein F | 20.5 |
| HNRPU | 22 | 3 | IPI00644079 | NP_114032 | Heterogeneous nuclear ribonucleoprotein U | 18.5 |
| HNRPH1 | 25 | 3 | IPI0013881 | NP_005511 | Heterogeneous nuclear ribonucleoprotein H1 | 20.5 |
| DDX1 | 22 | 3 | IPI00293655 | NP_004930 | ATP-dependent helicase DDX1 | 29.5 |
| DDX5 | 6 | 3 | IPI0017617 | NP_004837 | Probable RNA-dependent helicase p68 | 17.6 |
| UPF1 | 21 | 3 | IPI0034049 | NP_002902 | Regulator of nonsense transcripts 1 | 19.2 |
| EWSR1 | 11 | 3 | IPI0009841 | NP_005234 | EWS-B of RNA-binding protein EWS | 15.5 |

N-myc Downstream Regulated Gene 1 Interactome

Table I—continued

| Gene symbol | Total no. of peptides | No. of independent experiments | IPI | RefSeq accession no. | Protein description | Amino acid coverage |
|-------------|-----------------------|--------------------------------|-----|----------------------|---------------------|-------------------|
| EIF3S6 | 11 | 3 | IPI00013068 | NP_001559 | Eukaryotic translation initiation factor 3 subunit 6 | 24.7 |
| PABPC1 | 52 | 3 | IPI0008524 | NP_002559 | Splice isoform 1 of polyadenylate-binding protein 1 | 42.9 |

Ribosomal proteins

| Gene symbol | Total no. of peptides | No. of independent experiments | IPI | RefSeq accession no. | Protein description | Amino acid coverage |
|-------------|-----------------------|--------------------------------|-----|----------------------|---------------------|-------------------|
| RPS3 | 35 | 3 | IPI0011253 | NP_000996 | 40S ribosomal protein S3 | 57.2 |
| RPS6 | 17 | 3 | IPI0021840 | NP_001001 | 40S ribosomal protein S6 | 21.3 |
| RPL24 | 7 | 3 | IPI00306332 | NP_000977 | 60S ribosomal protein L24 | 13.4 |
| RPL3 | 31 | 3 | IPI0050021 | NP_000958 | 60S ribosomal protein L3 | 34.3 |
| RPS16 | 15 | 3 | IPI00221092 | NP_001011 | 40S ribosomal protein S16 | 24.8 |
| RPS8 | 13 | 3 | IPI00216587 | NP_001003 | 40S ribosomal protein S8 | 31.9 |
| RPS20 | 6 | 3 | IPI00012493 | NP_001014 | 40S ribosomal protein S20 | 25.2 |
| RPS9 | 18 | 3 | IPI0021088 | NP_001004 | 40S ribosomal protein S9 | 30.6 |
| RPL4 | 79 | 3 | IPI0003918 | NP_000959 | 60S ribosomal protein L4 | 39.2 |
| RPS26 | 9 | 3 | IPI00655650 | NP_001020 | 40S ribosomal protein S26 | 21.1 |

RNA processing

| Gene symbol | Total no. of peptides | No. of independent experiments | IPI | RefSeq accession no. | Protein description | Amino acid coverage |
|-------------|-----------------------|--------------------------------|-----|----------------------|---------------------|-------------------|
| NCL | 21 | 3 | IPI00444262 | NP_005372 | Nucleolin | 25.2 |
| HNRPF | 25 | 3 | IPI00003881 | NP_004957 | Heterogeneous nuclear ribonucleoprotein F | 20.5 |
| HNRPU | 22 | 3 | IPI00644079 | NP_114032 | Heterogeneous nuclear ribonucleoprotein U | 18.5 |
| HNRPH1 | 25 | 3 | IPI0013881 | NP_005511 | Heterogeneous nuclear ribonucleoprotein H1 | 20.5 |
| DDX1 | 22 | 3 | IPI00293655 | NP_004930 | ATP-dependent helicase DDX1 | 29.5 |
| DDX5 | 6 | 3 | IPI0017617 | NP_004837 | Probable RNA-dependent helicase p68 | 17.6 |
| UPF1 | 21 | 3 | IPI0034049 | NP_002902 | Regulator of nonsense transcripts 1 | 19.2 |
| EWSR1 | 11 | 3 | IPI0009841 | NP_005234 | EWS-B of RNA-binding protein EWS | 15.5 |
performed using anti-Ku70 or anti-CANX antibodies. However, this is likely because of the fact that most of the proteins pulled down by these two antibodies are the free forms, and only a small portion of these proteins were actually bound to NDRG1. The amount of bounded forms (to NDRG1) pulled down by anti-Ku70 or anti-CANX antibodies was too low to give a signal above background.

Non-genomic Function of the Interactome of NDRG1: NDRG1 Is Phosphorylated in LNCaP Cells, and the Phosphorylation Is Mediated by PKA—As shown in Fig. 2A, NDRG1 was phosphorylated in LNCaP cells (upper band, phosphorylated form), and the phosphorylation was dramatically increased after R1881 treatment.

To identify the phosphorylation site, we performed a SEQUEST database search with serine, threonine, and tyrosine phosphorylation as optional search parameters. We were able to identify three hits for the serine phosphorylated peptide R.TAS167GSSVTSLDGTR.S with Peptide Prophet scores /H11022 0.95 (the dot in the peptide sequence indicates trypsin cleavage sites) and a single peptide hit for the threonine phosphorylated peptide R.SHT181SEGAHLDITPNSGAAGNS-AGPK.S. with a Peptide Prophet score of 0.97. Fig. 3 showed both the singly (y') and doubly charged (y''+) y-ions of the phosphorylated (bottom panel) and unphosphorylated (upper panel) tryptic peptide R.TASGSSVTSLDGTR.S. We observed y12 ions for both the unphosphorylated form (m/z of
Our data suggested that both phospho-amino acid residue 366 of the NDRG1 protein sequence (RefSeq ID: NP_006087) corresponds to the doubly charged y12 ions for both phosphorylated and unphosphorylated forms of NDRG1 co-exist in cells. Other peptides with no phosphorylation sites were also identified and listed in Table II. We identified seven peptides with Peptide Prophet scores greater than 0.9. The total residues covered by these seven peptides are 82. However, among many putative phosphorylated amino acid residues in these 82 amino acid residues, only the two sites (serine 330 and threonine 366) were phosphorylated under our assessment conditions.

We utilized several kinase inhibitors to study which kinase mediates the phosphorylation of NDRG1 in LNCaP cells. LNCaP cells were pretreated with kinase inhibitors including the calmodulin kinase inhibitor KN-62, the cascein kinase II inhibitor DRB, the PKA inhibitor H89, and the PKC kinase inhibitor GF109230F for 30 min and then incubated with 10 nM R1881 for 24 h. Subsequently, cell lysates were prepared and analyzed for NDRG1 phosphorylation by Western blotting. As shown in Fig. 4, the phosphorylation of NDRG1 is reduced by all inhibitors that we tested. However, complete loss of phosphorylation was only observed in H89 (a PKA inhibitor)-treated cell lysates, suggesting that the phosphorylation of NDRG1 is prominently mediated by PKA. Our results were similar to the observation of Sugiki et al. (38) that NDRG1 undergoes phosphorylation in mast cells and that the phosphorylation depended on calmodulin kinase-II and PKA but not PKC. Interestingly, we also identified PPP2R2A (serine/threonine protein phosphatase 2A) as an NDRG1 interacting protein (Table I). This protein could potentially remove the phosphate in the NDRG1 protein added by PKA.

Non-genomic Function of the Interactome of NDRG1: NDRG1 Is Implicated in the ER Stress Response and Proteasome Activity—NDRG1 was postulated to play a role in ER stress response (39), and some NDRG1 interacting proteins that we identified were also involved in the ER stress response (Table I). To investigate whether changes in NDRG1 protein levels alter the protein levels of ER stress response proteins, siRNA interference was applied to knock-down the level of NDRG1 in LNCaP cells followed by measurement of expression levels of two ER stress inducible chaperon proteins GRP78 (HSPA5, heat shock 70 kDa protein 5) and GRP94 (HSP90B1, heat shock protein 90kDa β, member 1). LNCaP cells were transfected with siRNA against NDRG1 and treated with or without 10 nM R1881. Cell lysates were collected at 72 h after treatments, and two ER stress markers (GRP78 and GRP94) were analyzed by Western blotting. PCNA was used as a loading control because its expression is unaffected by either R1881 or NDRG1 siRNA. As shown in Fig. 5A, in the absence of R1881 the level of NDRG1 was dramatically reduced in siRNA-transfected cells as compared with mock-transfected cells, and the level of GRP94 but not GRP78 was decreased under the NDRG1 knock-down condition (lane 2 versus lane 1), suggesting that NDRG1 may modulate ER inducible gene expression. Despite the fact that the level of NDRG1 was decreased by siRNA, it remained unphosphorylated and unphosphorylated forms of NDRG1 co-exist in cells. Other peptides with no phosphorylation sites were also identified and listed in Table II. We identified seven peptides with Peptide Prophet scores greater than 0.9. The total residues covered by these seven peptides are 82. However, among many putative phosphorylated amino acid residues in these 82 amino acid residues, only the two sites (serine 330 and threonine 366) were phosphorylated under our assessment conditions.

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FIG. 3. Close up view of the spectra of the phosphorylated and unphosphorylated peptide TASGSSVTSLDGTR of NDRG1. Only the zoomed spectra in the $m/z$ range between 1000 and 1200 were shown to show the singly charged $y_{12}^+$ ions clearly. Both the singly ($y^+$) and doubly charged ($y^{2+}$) $y$-ions of the phosphorylated (bottom panel) and unphosphorylated (upper panel) tryptic peptide R.TASGSSVTSLDGTR.S were identified. The $y_{12}$ ions for both the unphosphorylated form ($m/z$ 1167.2181, upper panel) and the phosphorylated form ($m/z$ 1247.2181, bottom panel) of the peptide are shown. The spectra for the doubly charged $y_{12}$ ions for both phosphorylated and unphosphorylated forms ($m/z$ 584.1130 and 624.1130) are not shown; only the identification was indicated in the table below the close up view of the spectra panels.
inducible in the presence of R1881 (lane 4 versus lane 2). The level of GRP94 was also inducible by R1881 (lanes 3 and 4 versus lanes 1 and 2). Unlike GRP94, the level of GRP78 was not altered regardless of the level of NDRG1.

ER stress response can activate ER-associated protein degradation, which is executed by proteasomes in the cytosol to relieve the overwhelmed stress in the ER (40). Because many of the proteasome subunits were identified in NDRG1 complex (Table I), it would be interesting to investigate whether NDRG1 plays a role in the regulation of proteasome activity. The proteasome possesses multiple peptidase activities including chymotrypsin-like activity, postglutamyl peptidase activity, and trypsin-like activity (41). We utilized a fluorogenic peptide substrate to measure the chymotrypsin-like activity of the proteasome under the NDRG1 suppressed condition. LNCaP cells were transfected with siRNA against NDRG1 and treated with or without 10 nM R1881. Cell lysates were collected 72 h after incubation.

Fig. 5. Measurement of the effects of NDRG1 on ER stress responses and proteasome activity. LNCaP cells were transfected with an siRNA against NDRG1 and were treated with or without 10 nM R1881. Cell lysates were collected 72 h after incubation. A, proteins were subjected to SDS-PAGE and immunoblotted with anti-NDRG1, anti-KDEL (which recognizes both GRP78 and -94), and PCNA. B, cell lysates were subjected to proteasome activity assays by incubating with a fluorogenic substrate Suc-LLVY-AMC for 30 min. Release of the fluorescent group 7-amido-4-methylcoumarin from the proteasome substrate Suc-LLVY-AMC was monitored in a fluorescence plate (excitation/emission, 380/460 nm). The proteasome activities were represented as the fluorescence intensity (n = 3). These results suggested that NDRG1 plays a negative regulatory role on proteasome activity.

**DISCUSSION**

The role(s) of NDRG1 in tumor progression, invasion, and metastasis remains somewhat enigmatic and controversial. To understand the protein network by which NDRG1 executes its biological functions, we performed a comprehensive analysis of the interactome of NDRG1 in the androgen responsive LNCaP cell line using co-IP and tandem MS (Table I). We adopted a very stringent criterion to filter out those proteins that were not identified in three independent biological repli-

| Peptide sequences                                      | No. of peptide hits | Peptide Prophet scores   |
|--------------------------------------------------------|---------------------|--------------------------|
| R.SHTSEGAHLDITPNAGNSAGPK.S                            | 1                   | 1                        |
| K.MACCGGPGQISQPAGK.L                                   | 5                   | 0.9979–9999              |
| K.SIGMGTQAGAY.I                                        | 2                   | 0.9886–0.9886            |
| R.EMQDVLAEVPLKVE.G                                     | 3                   | 0.9538–1                 |
| R.SHT181SEGAHLDITPNAGNSAGPK.S                         | 1                   | 0.9694                   |
| R.TAS167GSSVSLDGTR.S                                    | 3                   | 0.9477–0.9694            |
| R.TASGSSVSLDGTR.S                                      | 4                   | 0.9996–1                 |

**TABLE II**

NDRG1 peptides identified from our MS/MS analysis

The dots in the peptide sequences indicate trypsin cleavage sites.
NDRG1-boundments suggested that cells contain significant amount of or the anti-CANX antibodies and Western blot was performed when the IP experiments were performed using the anti-Ku70 anti-CANX antibodies, but we were not able to detect NDRG1 when the IP was performed by the anti-NDRG1 antibody and perplexed because we were able to detect Ku70 and CANX NDRG1 using reciprocal IP experiments. Initially we were to confirm the interaction of LC/MS/MS and tried to confirm their interaction using reciprocal co-IP. Among 58 proteins, only 16 of them have antibody available. We have tried all 16 antibodies. We were able to confirm the interaction of β-catenin, E-cadherin, and NDRG1 using reciprocal IP experiments. Initially we were perplexed because we were able to detect Ku70 and CANX when the IP was performed by the anti-NDRG1 antibody and the Western blot was performed using the anti-Ku70 or the anti-CANX antibodies, but we were not able to detect NDRG1 when the IP experiments were performed using the anti-Ku70 or the anti-CANX antibodies and Western blot was performed using the anti-NDRG1 antibody (Fig. 2). Additional experiments suggested that cells contain significant amount of NDRG1-bound β-catenin and E-cadherin. However, the level of NDRG1-bound Ku70 and CANX is extremely low compared with the level of Ku70 and CANX in total cell lysates, suggesting that most of the Ku70 and CANX exist as free forms or the interactions are transient, i.e. only exist when they are needed. When the amount of NDRG1-bound Ku70 and CANX proteins are much lower than that of the unbound forms, it is not surprising that an IP experiment can be directional, i.e. depending on which antibody is used in the IP and which is used in the Western blot analysis after IP. Alternatively, difference in the quality of antibodies for IP experiments can also play a role. As shown in Fig. 2E (top panel, lane 7), CANX antibody did not work well in IP. Many other factors can contribute to the success of an co-IP experiment including 1) the quality and efficiency of antibody to pull down the target protein, 2) the level of the bounded form to free forms for a specific interacting protein, 3) whether the epitope of an antibody is also involved in protein interaction and therefore is mutually exclusive (i.e. if bound to antibody, it may prevent it from binding to the interacting protein), 4) whether the protein-protein interaction is direct or indirect, lasting or transient etc. In addition, reciprocal IP experiments are tedious and not amenable for high throughput analysis. Therefore, alternative strategies such as fluorescence resonance energy transfer may be needed to confirm interacting proteins identified by large scale proteomics studies.

As part of our proteomics analysis, we also determined that two positions, serine residue 330 and threonine residue 366 of the NDRG1 protein (RefSeq ID: NP_006087) were phosphorylated under our assessment conditions, whereas the other putative residues among the 82 total amino acid residues we found in MS/MS analysis were not phosphorylated (Table II). Integration of our IP/MS/MS data with HPRD data suggested that NDRG1 forms a complex with β-catenin and androgen receptor, two key control proteins in cells (Fig. 2). It is well known that androgen mediates its action by binding to an androgen receptor. Upon androgen binding, the androgen receptor translocates from the cytosol to the nucleus and transactivates downstream genes (44). To respond to androgen quickly, the androgen receptor recruits different co-regulators such as heat shock proteins (Hsp), co-chaperones, and tetratricopeptide repeat containing proteins to obtain appropriate conformation changes upon androgen binding (45). Although we did not identify the androgen receptor itself in the NDRG1 complex, we identified three androgen receptor co-regulators: HSPCA, CTNNB1, and XRCC6 protein. HSPCA is known to bind to AR when it is in a ligand-unbound state (46). The HSPCA-AR complex then binds androgen (46). The binding is important for the stability and activation of AR as it has been demonstrated that AR is transformed into a DNA binding competent status with the assistance of HSPCA; AR then initiates nuclear translocation, recruitment of cofactors, and transactivation of target genes (47). CTNNB1 is a co-activator of the androgen receptor and a component of the Wnt signaling pathway (48). CTNNB1 can promote androgen signaling by binding to the liganded AR, which leads to the transactivation of androgen-regulated genes (49, 50). XRCC6 and Ku80 were recently demonstrated to bind to AR and act as co-activator of AR (51). Ku80 was excluded from the 58 interacting proteins (Table I) because it was only identified in two of three replicate experiments.

We also overlaid the expression changes in response to androgen for the proteins in the NDRG1 interactome (Fig. 1). The integrated interactome map provides an overview of a dynamic (dual) mode of the NDRG1 interactome. It is a first step toward a better understanding of the interactions between the androgen response program and the NDRG1 interactome as well as the effects and roles of these interactions in prostate cancer progression.

We showed that cell adhesion molecules including E-cadherin and β-catenin interact with NDRG1. The interaction of NDRG1 with E-cadherin or β-catenin is not affected by androgen stimulation (Fig. 2, B–D) or by the phosphorylation status of NDRG1 (data not shown). E-cadherin, β-catenin, and α-catenin are known to form a network in the adhesion junction (52). E-cadherin is a transmembrane protein having an extracellular domain that mediates homotypic cell-to-cell adhesion and a cytoplasmic tail that links to β-catenin and α-catenin, which in turn provides anchorage to the actin cytoskeleton. The loss of E-cadherin or disruption of cadherin-catenin interaction is known to increase the potential of metastasis in various tumors including prostate cancer (37). It was postulated that NDRG1 is functionally linked to the formation of the E-cadherin-β-catenin complex (53). Our study provides di-
Our proposed model of the NDRG1 interactome indicating several modules of NDRG1 interactome. In the cytosol, NDRG1 can form complex with TLE3 and PPP2R2A, two protein kinases. NDRG1 can form complex with ER chaperons, suggesting that either NDRG1 is a target to be folded into correct conformation by chaperons or acts as a chaperon itself. The complex of NDRG1 and AR co-regulators including CTNNB1, HSPCA, and XRCC6 could be in the cytosol or in the nuclear because these AR co-regulators can be distributed to both compartments.

We also identified ER stress response proteins in the NDRG1 complex including HSPA5, CANX, and transitional endoplasmic reticulum ATPase (VCP) in NDRG1 complex (Table I). NDRG1 is a known component of the ER stress response because it is sensitive to the redox status of the cells and the intracellular calcium concentration (16, 39). We showed that the expression of HSPA5 was not affected by NDRG1 knock-down, but the ER inducible chaperon protein TRA1 was decreased after knocking down NDRG1 in LNCaP cells (Fig. 5A), indicating that NDRG1 is involved in the induction of ER inducible chaperons. CANX is another chaperone that is structurally different from molecular chaperones of the Hsp60, Hsp70, and Hsp90 families (54) and is involved in the retention of incorrectly or incompletely folded proteins (54).

VCP, the 97-kDa valosin containing protein is another chaperone that was shown to be associated with Hsp90 (55). We showed that NDRG1 interacted with these three chaperons, suggesting that NDRG1 might be a chaperon protein itself or the target of these chaperons (Fig. 6). Further experimentation is necessary to sort this out.

The proteasome is an essential component of the ATP-dependent proteolytic pathway that is responsible for the degradation of most cellular proteins (41). 26S proteasome is a multimeric protein complex that is composed of a 20S core particle and a 19S regulatory particle (41, 56). The 20S core particle possesses the catalytic activities that hydrolyze proteins into small pieces, whereas the 19S complex controls the entry of a substrate into the 20S core particle (41, 56). We identified several 19S subunits from the NDRG1 complex. We further showed that knocking down NDRG1 under androgen-starved conditions (no R1881) increases proteasomal activity (Fig. 5B). Androgen stimulation itself also increased proteasomal activity in native LNCaP cells that were not subject to NDRG1 knock-down (Fig 5B, columns a and c). However, the combination of androgen stimulation and NRDG1 knock-down (Fig 5B, column d) did not further increase proteasome activity (i.e. no additive effects). It is possible that androgen induction of NDRG1 expression counteracted the effect of NDRG1 knock-down and therefore could not have additive effects in increasing proteasome activity.

Proteasome activity is critical for androgen receptor transcriptional activity, as it was demonstrated that the inhibition of proteasome function attenuated androgen-induced AR nuclear translocation, whereas overexpression of PSMA7, a catalytic subunit of the 20S core particle, enhanced AR transactivation (57). We showed that androgen up-regulated PSMA7 but down-regulated several 19S subunits and proteasomal activators (Fig. 1). Our interactome map provides physical basis for the interaction between proteasome activity and the AR.

NDRG1 is localized in the cytoplasm (11). However, in response to p53 and DNA damage, NDRG1 can redistribute to the nucleus (58). The role of NDRG1 in the nucleus is unknown. We identified a transcriptional factor NFAT90 (ILF3) in the NDRG1 complex. This transcriptional factor in turn interacts with other transcriptional factors such as TP53, ILF2, FUS (fusion in t (12, 16) in malignant liposarcoma) (Fig. 1). These data suggest that NDRG1 may modulate the expression of genes that are controlled by these transcriptional factors.

We also identified several nucleoproteins such as XRCC6 and RuvB like-2 (RUVBL2) in the NDRG1 complex. XRCC6 is a regulatory subunit of a nuclear serine/threonine kinase DNA-dependent protein kinase that is involved in non-homologous end joining recombination (59, 60). RUVBL2 participates in DNA repair by driving the branch migration of the Holliday junction (61). DNA damage agents such as camptothecin can induce NDRG1 expression, and the induction seems to be associated with drug resistance (62). Our data suggest that the drug resistance may be caused by increased DNA repair ability induced by NDRG1 and its interactome.

Furthermore, some of the NDRG1 interacting proteins are also involved in the regulation of cell differentiation and tumor progression. Transducin-like enhancer protein 3 (TLE3) is a member of the Notch signaling pathway (63). This pathway controls the prostate epithelial cell differentiation and also is involved in prostate cancer progression. 17β-Hydroxysteroid dehydrogenase 4 (HSD17B4) catalyzes branched chain fatty acid β-oxidation in the peroxisome and works in the downstream from α-methylacyl-CoA racemase (AMACR). Both enzymes have been found to be up-regulated in human prostate cancer, and the selective up-regulation of peroxisomal branched chain fatty acid β-oxidation may be involved in the progression of prostate cancer (64). Further investigation of the interaction between NDRG1 and these proteins
may uncover the mechanisms by which NDRG1 induces differentiation.

Protein-protein interactions play critical roles in the biologic function of proteins. A proteome-wide approach such as IP/MS/MS was heralded as the method of choice for building a global protein interactome map (65). However, the limitation of the IP/MS/MS approach is that it cannot distinguish between direct and indirect interacting partners of a protein because both can be immunoprecipitated and therefore identified. The interacting proteins of NDRG1 we identified will contain both direct and indirect interacting proteins, and further experimentation is necessary to distinguish them. Furthermore, because NDRG1 is a protein with multiple possible cellular localizations (10, 66) and we only performed IP from the total cell lysates (not from different cellular compartments), our interactome map will be a combination of interactomes of all possible combinations. Nonetheless, we were able to identify different interactome modules at different cellular localization by the specific localization of the NDRG1 interacting proteins. For example, NDRG1-E-cadherin-β-catenin complex is likely to form at cellular junctions because E-cadherin is a cellular membrane transmembrane protein. The NDRG1-ILF3-XRCC6 complex is likely to form in the nucleus because ILF3 and XRCC6 are DNA binding proteins. We propose a model in which NDRG1 is associated with its interacting proteins at different cellular compartments as shown in Fig. 6. As we discussed earlier, NDRG1 is associated with chaperon proteins HSPA5, CANX, and VCP in ER. While in the cytosol, NDRG1 can bind to proteins like TLE3 and participate in signal transduction. The complex of NDRG1 and AR co-regulators including CTNNB1, HSPCA, and XRCC6 could be in the cytosol or in the nucleus because these AR co-regulators can be distributed to both compartments (67–69). The androgen receptor can promote CTNNB1 nuclear translocation (70). It is possible that NDRG1, which binds to CTNNB1, also plays a role in the AR-mediated transport of CTNNB1 to the nucleus. Multiple localization and interaction with multiple proteins may explain the multiple roles of NDRG1 in response to a variety of stimuli.

In summary, we have built a comprehensive interactome map of NDRG1, a versatile and a multiple functional molecule in the cell. This interactome map consists of several modules, which correspond to the reported functions of NDRG1. We also provide evidence suggesting that the NDRG1 interactome interacts closely with the androgen response program and that the expression of many genes in the interactome is affected by androgen. This interactome map provides the first road map for understanding the pleiotropic functions of NDRG1 at cellular level and its roles in human diseases.

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