Global Gene Expression Analysis Reveals a Link between NDRG1 and Vesicle Transport

Hanne A. Askautrud1,2,*, Elisabet Gjernes1,2,*, Gjermund Gunnes3, Marit Sletten1,2, Douglas T. Ross4, Anne Lise Børresen-Dale5, Nina Iversen1,2, Michael A. Tranulis3, Eirik Frengen1,2

1 Department of Medical Genetics, University of Oslo, Oslo, Norway, 2 Department of Medical Genetics, Oslo University Hospital, Oslo, Norway, 3 Department of Basic Sciences and Aquatic Medicine, Norwegian School of Veterinary Science, Oslo, Norway, 4 Clarient Diagnostic Services, Aliso Viejo, California, United States of America, 5 Department of Genetics, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet, Oslo, Norway

Abstract

N-myc downstream-regulated gene 1 (NDRG1) is induced by cellular stress such as hypoxia and DNA damage, and in humans, germ line mutations cause Charcot-Marie-Tooth disease. However, the cellular roles of NDRG1 are not fully understood. Previously, NDRG1 was shown to mediate doxorubicin resistance under hypoxia, suggesting a role for NDRG1 in cell survival under these conditions. We found decreased apoptosis in doxorubicin-treated cells expressing NDRG1 shRNAs under normoxia, demonstrating a requirement for NDRG1 in apoptosis in breast epithelial cells under normal oxygen pressure. Also, different cellular stress regimens, such as hypoxia and doxorubicin treatment, induced NDRG1 through different stress signalling pathways. We further compared expression profiles in human breast epithelial cells ectopically over-expressing NDRG1 with cells expressing NDRG1 shRNAs in order to identify biological pathways where NDRG1 is involved. The results suggest that NDRG1 may have roles connected to vesicle transport.

Introduction

N-myc downstream-regulated gene 1 (NDRG1) is also known as Drg-1 (differentiation-related gene 1) [1], Cap43 (p43 protein induced by free intracellular Ca2+) [2], and RTP (reducing agents and tunicamycin-responsive protein) [3], named by its perceived functions in different in vitro assays. The NDRG gene family contains NDRG1, -2, -3 and -4, which have 57–65% amino acid sequence identity [4]. The tissue-distribution of NDRG1 mRNA is widespread [4,5], and the NDRG1 amino acid sequence is >80% identical in a variety of species, ranging from amphibians and fish to mammals [6]. However, the physiological functions of NDRG1 remain elusive. NDRG1 encodes a protein that belongs to the α/β hydrolase superfamily, but lacks an apparent hydrolytic catalytic site [7]. NDRG1 has been shown to exist as a multi-phosphorylated protein, lacks an ER-targeting signal-sequence and has no apparent transmembrane domain [3,8]. The protein has primarily been detected in the cytoplasm, but is also seen associated with the plasma membrane, nucleus and the inner mitochondrial membrane, depending on the cell type assessed [5].

NDRG1 missense mutations cause hereditary motor and sensory neuropathy-Lom (HMSN(L)), an autosomal recessive form of Charcot-Marie-Tooth disease (CMT) also called CMT4D [9]. Patients with CMT4D exhibit early-onset peripheral neuropathy, which progresses in adulthood to severe disability characterized by muscle weakness, sensory loss, and neural deafness [9,10]. A tumor-promoting role has also been reported for NDRG1 [11,12], and it has been implicated in metastasis suppression [13–15]. Further, NDRG1 has been shown to have a role in myelin sheath maintenance [9,16], exocytosis in mast cells [8,17], differentiation [1,13,18], and NDRG1 expression is induced by homocysteine [3], androgen [19], as well as in response to various forms of cellular stress such as hypoxia [5,20,21], heavy metals [20] or DNA damage [22,23].

In the present study, we induced DNA damage by doxorubicin treatment of epithelial cell lines expressing NDRG1 at relatively high (SUM102, ME16C2 and HCT116) or low (ZR-75-1 and MCF-7) levels. We found that doxorubicin-induced apoptosis was inhibited in epithelial breast cells expressing shRNAs targeting NDRG1, demonstrating that NDRG1 is required for apoptosis in these cells under normoxic conditions. We also present results showing that NDRG1 is not a regulator of hypoxia-induced gene expression in the epithelial cells studied, and that hypoxia and doxorubicin treatment initiate different NDRG1 responses. Global gene expression analysis of cell lines with manipulated NDRG1 expression demonstrates that NDRG1 influences the expression of genes related to vesicular structures and transport. The roles of NDRG1 in cellular responses to stress, such as hypoxia and...
apoptosis may converge by NDRG1 exerting its functions at the level of endomembrane structures.

Results
NDRG1 does not Regulate Hypoxia-induced Gene Expression
We first studied the two luminal breast cancer cell lines, ZR-75-1 and MCF-7, and demonstrated increased NDRG1 expression when these cells were grown at 1% O2 for 24 hours (Figure 1A). Then we grew both cell lines under hypoxic conditions for 24 or 48 hours and compared the global gene expression profile with cells grown at normoxia. Five microarrays were used per cell line (Figure 1C). Sixteen out of 18 genes in a robust hypoxia signature previously described by Chi et al. [24] showed increased expression on the microarrays (Figure 1C), documenting the hypoxia response in the cells analyzed.

We further investigated whether ectopic NDRG1 expression would result in similar gene expression changes as those detected in cells grown under hypoxic conditions. To facilitate ectopic expression, a construct carrying NDRG1 cDNA (see Figure S1) was transduced into ZR-75-1 in parallel experiments resulting in six cell populations, ZR-75-1/NDRG1 A–F, all showing increased NDRG1 expression (Figure 1B). Global gene expression profiling was performed using the six ZR-75-1 cell populations ectopically expressing NDRG1 grown in 20% O2 (9 microarrays) (see experimental procedures for details). A one class significance analysis of microarrays (SAM) was performed to identify genes with similar expression patterns on these 9 microarrays and the 5 microarrays from ZR-75-1 cells grown under hypoxia. Only genes represented on more than 60% of the arrays were included. The SAM analysis identified only 6 genes in addition to NDRG1: AGMAT, FTL, KLF12, LMNB1, SChHP1, and STAT3 (FDR = 0), documenting a limited overlap in the global gene expression response when NDRG1 is induced by hypoxia compared to ectopic NDRG1 over-expression. Furthermore, the only gene in the hypoxia signature showing increased expression in the cells ectopically expressing NDRG1 was NDRG1 itself (Figure 1D). Thus, ectopic over-expression of NDRG1 does not trigger a gene expression profile resembling the hypoxic response, showing that NDRG1 is not itself a regulator of hypoxia-induced gene expression in the cells analyzed.

DNA Damage and Hypoxic Growth Induce different NDRG1 Responses in the Cell
NDRG1 has previously been shown to have a role in TP53-mediated apoptosis in colon and pancreatic cell lines [25,26]. We detected increased TP53 levels and a distinct increase in NDRG1 levels in a panel of doxorubicin treated cells (Figure 2A). Furthermore, we detected a significantly reduced fraction of apoptotic cells when NDRG1 shRNAs [27] were expressed in the immortalized breast cell line SUM102 (Figure 2B). This shows that NDRG1 is necessary for doxorubicin-induced apoptosis in SUM102 under normoxic conditions.

We then treated SUM102 cells with doxorubicin and in parallel grew cells in hypoxic conditions, and used immunofluorescence to detect NDRG1 (Figure 3A). Cells grown at 1% O2 show increased NDRG1 levels in the cytoplasm, whereas doxorubicin treatment resulted in increased NDRG1 levels with a coarsely granulated cytoplasmic staining pattern (Figure 3A). Staining with markers for ER (calnexin) and early endosomes (EEA1) did not show co localization with NDRG1 in SUM102 under the conditions tested (Figure 3A,B). Furthermore, we did not observe any obvious morphological differences in the SUM102 cells with or without NDRG1 shRNA expression under the conditions tested (results not shown). In summary, our results indicate that hypoxia and doxorubicin treatments initiate different NDRG1 responses in SUM102 cells.
Global Gene Expression Analysis Indicate that NDRG1 Function is Connected to Endomembrane Structures and Vesicular Transport

To investigate the global gene expression changes caused by manipulated NDRG1 expression, we focused on the breast epithelial cell lines SUM102 and ME16C2, because both have high endogenous NDRG1 expression (Figure 4). Two NDRG1 shRNA constructs, NDRG1si4 and NDRG1si7 [27], were transduced into these cell lines in two parallel experiments resulting in a total of eight cell populations with reduced NDRG1 expression: SUM102/NDRG1si4A and 4B, SUM102/NDRG1si7A and 7B, ME16C2/NDRG1si4A and 4B, and ME16C2/NDRG1si7A and 7B (Figure 4). Global gene expression profiling was performed and a two class SAM analysis was used to identify genes showing different expression in SUM102 and ME16C2 with NDRG1 knock-down (16 microarrays) compared to ZR-75-1 ectopically over-expressing NDRG1 (9 microarrays). The SAM delta value was adjusted to obtain the largest gene list that gave a false discovery rate of less than 5% (FDR = 3.5%) (table S1). The remaining probes represented control oligos, duplicated oligos and multiple oligos targeting the same gene. Clustering of the resulting 83 genes identified across the different cell populations are visualized in Figure 5.

Functional information was available for 69 of the 83 genes in the SAM-derived gene list and these were used to search for enriched gene ontology terms by Gominer [28]. The cellular compartments identified in the Gominer analysis using a cutoff for significant GO categories at p≤0.005, are listed in table S2; vesicle coat (3 genes), organelle membrane (13 genes), extracellular space (10 genes), membrane coat (3 genes), integral to organelle membrane (4 genes), and endoplasmic reticulum (11 genes), in total adding up to 26 of the 69 genes (as several of the genes are present in more than one category). Motivated by the Gominer results, we further reviewed the literature regarding the remaining genes in the SAM-derived gene list. The products from an additional 8 genes were found to be involved in ER to Golgi, Golgi to ER or endosomal transport, and 2 were found to be involved in endocytosis and exocytosis. In summary, of the 69 genes with functional information in the SAM-derived gene list, a total of 21 genes were shown to be involved in processes connected to vesicle transport (indicated in orange in Figure 5). Nineteen of these 21 genes showed an opposite expression pattern compared to NDRG1; i.e. they were up-regulated in the cell populations where the NDRG1 level was reduced by shRNAs, and down-regulated in ZR-75-1 with ectopic NDRG1 expression (indicated in orange in Figure 5). These results suggest that induced changes in NDRG1 expression affect the expression of genes involved in vesicle transport.

Discussion

NDRG1 Exerts its Effect through Different Stress-signalling Pathways

Hypoxic stress has been shown to induce NDRG1 expression in a variety of cells [29], but NDRG1’s roles during hypoxia are not fully understood. We identified a similar expression pattern of only seven genes, including NDRG1, when comparing the global gene expression profile in ZR-75-1 cells grown under hypoxia with cells ectopically over-expressing NDRG1. Thus, NDRG1 does not act as a sensor of intracellular oxygen tension. The elevated NDRG1 expression is rather a secondary effect of hypoxic stress.

NDRG1 is induced both by ectopically expressed and endogenously increased TP53, the NDRG1 promoter contains a putative TP53 binding site [25,30], and NDRG1 has been shown to be necessary for TP53-dependent apoptosis in colon and pancreatic cells [25,26]. In line with this, we detected decreased apoptosis in doxorubicin-treated breast epithelial cells expressing NDRG1 shRNAs under normoxia. Other studies show no correlation between NDRG1 expression and apoptosis despite the up-regulation of TP53 [15,21,30,31], suggesting different responses depending on the cell types and conditions studied. Jung et al. [32] demonstrated that hypoxia-induced NDRG1 expression can mediate doxorubicin resistance, suggesting that NDRG1 might increase cellular survival under hypoxia. We observed strongly elevated NDRG1 levels under hypoxia and in cells subjected to acute DNA-damage by doxorubicin treatment. Under hypoxia we detected an even cytoplasmic NDRG1 signal, whereas strongly elevated NDRG1 levels under hypoxia and in cells subjected to acute DNA-damage by doxorubicin treatment. Under hypoxia we detected an even cytoplasmic NDRG1 signal, whereas strongly elevated NDRG1 levels under hypoxia and in cells subjected to acute DNA-damage by doxorubicin treatment. Under hypoxia we detected an even cytoplasmic NDRG1 signal, whereas strongly elevated NDRG1 levels under hypoxia and in cells subjected to acute DNA-damage by doxorubicin treatment. Under hypoxia we detected an even cytoplasmic NDRG1 signal, whereas strongly elevated NDRG1 levels under hypoxia and in cells subjected to acute DNA-damage by doxorubicin treatment. Under hypoxia we detected an even cytoplasmic NDRG1 signal, whereas strongly elevated NDRG1 levels under hypoxia and in cells subjected to acute DNA-damage by doxorubicin treatment. Under hypoxia we detected an even cytoplasmic NDRG1 signal, whereas strongly elevated NDRG1 levels under hypoxia and in cells subjected to acute DNA-damage by doxorubicin treatment.

Global Gene Expression Analysis Links NDRG1 to Endomembranes and Vesicular Transport

Several of the genes identified in the current global expression profiling encode proteins located in the ER [(NFE2L3, ASPHD1,
CKAP4, HSD17B2, TMPRSS3, KSR1 and RDH16, proteins required for endosome to Golgi trafficking (ARL1), for transport through Golgi (GOLM1), for the retrograde Golgi-to-ER transport (COPE), and for vesicle budding from the ER (SEC24D). The microarray results also revealed genes encoding proteins involved in the targeting of proteins to the ER (SRP54), ER membrane translocation (SSR3), and proteins needed for chaperone function (DNAJC12 and TMEM132A). Previous studies have suggested that NDRG1 might be a client protein of the chaperone HSP90, or that NDRG1 is a chaperone protein itself [19]. In our microarray experiments we detected CD47 and NECAP1, the latter inhibits endocytosis when over-expressed [34], and CD47 trigger exocytosis when activated [35,36]. In total, 21 of the 69 differently expressed genes detected in the gene expression profiling in cells with manipulated NDRG1 expression encode proteins that are linked to ER, Golgi, endosomes or vesicular transport between these compartments (indicated in orange in Figure 5). Nineteen of these 21 genes are up-regulated in cell populations with NDRG1 knock down, and down-regulated in cell populations with ectopic over-expression of NDRG1, which indicates that NDRG1 facilitates a down-regulation of several genes encoding proteins in the ER to endosome axis. In stressful situations with lack of energy, such as under hypoxia or during ER-stress, a down-regulation of some activities in the secretory and/or endocytic routes of the cell might conserve energy and thus contribute to restore cellular homeostasis. It is tempting to speculate that NDRG1 might increase cell survival during hypoxia through such a mechanism.

CDC42 belongs to the Rho family of GTPases and influences vesicle trafficking at many levels, anterograde ER to Golgi, post-Golgi, exocytosis as well as endocytosis and further retrograde transport via interaction with Wiskott-Aldrich syndrome protein (N-WASP) and the Arp2/3 complex leading to altered actin dynamics [37–39]. Three of the genes detected in our microarray analysis, CDC42EP, DOCK11 and ARHGEF16 encode proteins that modulate the activity of CDC42. While CDC42EP negatively regulates CDC42 function, both DOCK11 and ARHGEF16

**Figure 3. Immunofluorescence staining of SUM102 cell cultures.** Colors were applied artificially for graphical purposes. (A) NDRG1 (green) and calnexin (red) in untreated, hypoxia and doxorubicin treated cells. Bar: 5 μm. (B) NDRG1 (green) and EEA1 (red) in hypoxia treated cells. Bar: 10 μm. doi:10.1371/journal.pone.0087268.g003

**Figure 4. Knock down of NDRG1 expression by shRNAs.** Western blots detect reduced NDRG1 expression in the breast cell lines ME16C2 and SUM102 expressing the shRNAs NDRG1si4 (si4) and NDRG1si7 (si7), transduced in parallel experiments (A and B) compared to control cells (C) transduced with the empty vector pSiRPG [27]. α-tubulin was used as loading control. doi:10.1371/journal.pone.0087268.g004
specifically activate CDC42 [40–43]. In cells over-expressing NDRG1, the levels of CDC42EP, DOCK11 and ARHGEF1 detected on the micro arrays are shifted in a direction that is in agreement with an induction of increased CDC42 activity, concievably resulting in increased vesicle trafficking. Thus, NDRG1 may indirectly play a role in vesicle trafficking and in maintenance of cellular polarity, by stimulating CDC42. Interestingly, hyperactivation of CDC42 can contribute to cellular transformation and tumor invasion and metastasis, through generation of plasma membrane protrusions, so-called invadopodia [39]. Further investigations are needed to clarify the putative interplay between NDRG1 and CDC42 signalling pathways.

In humans, Alaskan Malamute and Greyhound, germline NDRG1 mutations cause the demyelinating disorder CMT4D [9,44] and Ndrg1 deficient mice show defects connected to myelin sheath maintenance [16,45]. Generally, many genes affected in Charcot-Marie-Tooth disease include proteins involved in regulation of endocytosis and vesicle transport [46–48]. In a study of an early onset demyelinating disease, classified as CMT4H in one Lebanese and one Algerian family, Delague and colleagues [49] identified novel mutations in a protein directly influencing the activity of CDC42. The protein, known as FRABIN (FGD1-related F-actin binding protein) is a specific regulator of CDC42 activity and it was postulated that the mutations led to loss of CDC42 activity, leading to reduced F-actin positive filipodia protrusions in cell cultural experiments. Whether CDC42 activity is compromised also in CMT4D, due to NDRG1 inactivation, is presently unknown, and might thus provide a starting-point for further studies into the molecular pathogenesis of CMT4D.

Recently, cell death signals have been shown to induce trafficking of recycling endosomes through a pathway involving CDC42 [50], and CDC42 was shown to be involved in FAS-enhanced membrane trafficking [51]. Also, TP53 can induce apoptosis through death receptors by increased expression of FAS [52]. NDRG2 silencing has been shown to inhibit TP53-mediated apoptosis [53], and inactivation of NDRG2 may elicit resistance against FAS-mediated cell death [54]. Our microarray results may indicate an involvement of NDRG1 in trafficking of recycling endosomes. Additional research is required to explore if increased NDRG1 levels could result in TP53-mediated apoptosis through increased vesicle formation, internalization and recycling of FAS through activation of CDC42. Furthermore, the reported tumor-promoting role of NDRG1 [11,12] and the role in metastasis suppression [13–15], which are apparently contradictory may be explained by involvement of NDRG1 both in cell survival under hypoxia and in doxorubicin-induced apoptosis under normoxia. In summary, the pleotropic roles of NDRG1 reported in apoptosis, cell survival, myelin sheath maintenance and enhanced exocytosis in mast cells, and in the cellular responses to hypoxia, heavy metals, and androgenes may converge by NDRG1 influencing vesicular trafficking.

**Materials and Methods**

**Cell Culture and Generation of Stable Cell Populations**

The breast epithelial cell lines ME16C2 [55], and SUM102, a gift from Steve Ethier of Wayne State University (Asterand, MI, USA), and their derivatives were grown in TCF-EGM developed by the Tissue Culture Facility at the University of North Carolina at Chapel Hill [http://www.unclineberger.org/tcf/index.asp].
The luminal breast cancer cell lines ZR-75-1 (CRL 1500; ATCC, Manassas, VA, USA) and MCF-7 (HTB-22; ATCC) and their derivatives were grown in RPMI1640 with phenol red (Lonza, Walkersville, MD, USA) supplemented with 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA) and 10% FBS (Invitrogen). HCT116 (CCL-247; ATCC) was grown in McCoy’s 5a medium (Lonza) supplemented with 10% FBS. For the hypoxia experiments cells were grown in a hypoxic chamber, MIC-101, (Billups-Rothenberg, Del Mar, CA, USA) at 1% O₂.

Transductions were performed as previously described [27]. Stably transduced cell populations were established by selection with puromycin (2 μg/mL; Invitrogen) for 2–5 weeks. All cell populations were analyzed by Western blotting for expression of NDRG1. Cell populations transduced with the vector pSiRPG without insert were also created as controls.

Western Blotting

Cells harvested in PBS were lysed in M-PER (Thermo Fisher Scientific, Rockford, IL, USA) containing Halt Protease Inhibitor and 5 mM EDTA (Thermo Fisher Scientific). Protein concentrations were quantified using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) or with a dye-binding assay (Bio-Rad, Hercules, CA, USA). Protein electrophoresis, western blotting and immunodetection were performed as previously described [27]. The blots were incubated over night with the NDRG1 antibody (diluted 1:100; Clarient, Aliso Viejo, CA, USA). A monoclonal α-tubulin antibody (diluted 1:1000; SIGMA Aldrich, St. Louis, MO, USA) was used as loading control. Secondary antibody was horseradish peroxidase-conjugated anti-rabbit or anti-mouse, respectively (diluted 1:5000; GE Healthcare, Buckinghamshire, United Kingdom), which was detected by enhanced chemiluminescence (ECL Plus, GE Healthcare). Blots in Figure 1 were visualized with alkaline phosphatase-conjugated secondary antibodies (Bio-Rad) and fluorescence was recorded with a variable mode imager (Typhoon 9200, GE Healthcare).

Global Gene Expression Analysis

Total RNA was isolated from all cell populations using RNAasy (Qiagen, Hilden, Germany). Cells grown under hypoxic conditions (24 h and 48 h) and control cells incubated at normoxia were harvested in lysis buffer at the same time points. RNA was labeled with Cy3- or Cy5-CTP [56]. To minimize the dye effects, three of the cell populations, ZR-75-1/NDRG1 A/B/C, were labeled with both Cy3- and Cy5-CTP and mixed with the control RNA labeled with the opposite dye. The three remaining ZR-75-1 populations were labeled once, but population E was labeled with the opposite dyes compared to population D and F. For ZR-75-1 and MCF-7 grown under hypoxia, all parallels were labeled once, but two of the parallels harvested after 48 hours were labeled with the opposite dyes compared to the other samples. For the cell populations expressing shRNAs, each sample was labeled with both dyes in a dye flip set-up. Samples were hybridized to either GenePix Oligo Microarrays (G41120) or Human Whole Genome Oligo Microarrays (G4112A) (Agilent, Santa Clara, CA, USA). Scanning were performed using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) adjusting the PMT settings for each channel and each array so that the count ratio was close to 1 and the intensities of the spots did not exceed the maximum detection limit. Image analysis was performed using GenePix Pro 6 (Molecular Devices). Loess Normalization was performed to adjust microarray data for variation between the samples.

All microarray data reported in this paper is MIAME compliant and the raw data has been deposited in the GEO database (GEO accession number GSE33439).

Statistical Analysis

Both one class and two class SAM (SAM version 3.02) was performed using 100 permutations and 10 k-nearest neighbors [57]. The SAM delta value was adjusted to give a false discovery rate (FDR) less than 5%. The SAM derived gene lists were clustered using the average linkage hierarchical cluster analysis in the Cluster program [58] sorted on samples, and the data were visualized in Treeview [59]. The SAM derived gene list was analyzed for the cellular component gene ontology using High-Throughput GoMiner [60]. All genes represented on the microarray were entered as a background file. A one sided p-value was calculated for each category based on a Fishers exact test. The threshold of significance for a category was defined as p≤0.05.

Measuring Apoptosis by TUNEL Assay

SUM102 seeded at 200,000 cells per cm² adhered for 24 h before treatment with doxorubicin (1 μM; Sigma-Aldrich) for 24 hours. Duplicates were harvested and washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 15 min at 4°C. Cells were pelleted by centrifugation and permeabilized in pure methanol for at least 20 min at −20°C. The cells were washed twice with PBS and apoptotic cells detected by terminal deoxynucleotidyl transferase mediated dUTP end-labeling (TUNEL) assay using the In situ cell death detection kit, Fluorescein (Roche Applied Science, Mannheim, Germany). The cells were incubated for 60 min at 37°C with TdT and fluorescein, centrifuged and stained with Hoechst 33258 (2 μg/ml; Invitrogen) for 15 min on ice. The cells were filtered through a nylon mesh and 10000 events were analyzed for each sample using FACS DIVa Vantage Cell Sorter (Becton Dickinson, Heidelberg, Germany) and data collection carried out using the CellQuest 3.3 software (BD Biosciences, Franklin Lakes, NJ, USA).

Immunofluorescence

SUM102 cells were plated on glass chambered slides and grown to 60–80% confluence. The cells were fixed in 2% paraformaldehyde in PBS, washed and treated with 0.2% Triton X-100 in PBS for permeabilization. After blocking (2% BSA, 0.2% Tween 20, 7% Glycerol, 2% Goat serum, in PBS), cells were incubated with NDRG1 antibody (rabbit, diluted 1:200; Clarient) or isotype control antibodies (Invitrogen) overnight. Secondary antibody was Alexa-405 anti-rabbit (diluted 1:250; Invitrogen A31556). Early endosomes were marked with the EEA1- antibody (mouse monoclonal IgG1 diluted 1:100; Abcam ab70521), and detected with secondary anti-mouse IgG1 antibody Alexa-633 (diluted 1:1000; Invitrogen A21126). Endoplasmic reticulum was marked with an anti-calnexin antibody (mouse monoclonal IgG1 diluted 1:1000; Abcam ab31290) and secondary anti-mouse IgG1 antibody Alexa-488 (diluted 1:200; Invitrogen). Propidium iodide (PI), was used in a 1:10 dilution for nuclear contrast. Images were acquired using a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Lasers and fluorescent filters were optimized for 405 nm (NDRG1), 488 nm (calnexin), 560 nm (PI), and 633 nm (EEA1) excitation maxima. To facilitate signal interpretation, pseudocolors were applied digitally, using the Zen Lite 2011 software package (Carl Zeiss, Jena, Germany). The aquired images were assembled in Adobe Photoshop Elements 10 (Adobe Systems Inc., San Jose, California, USA).
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