Published in final edited form as:

Cancer Res. 2009 August 1; 69(15): 6115–6121. doi:10.1158/0008-5472.CAN-08-3024.

Differentiation-Related Gene-1 decreases Bim stability by Proteasome-mediated Degradation

Grazia Ambrosini, Sharon L. Seelman, and Gary K. Schwartz¹

Laboratory of New Drug Development, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York.

Summary

Drg1 was identified as a differentiation-related, putative metastatic suppressor gene in human colon and prostate cancer. Its expression is associated with resistance to irinotecan (CPT-11) therapy in preclinical colorectal cancer models both *in vitro* and *in vivo*. However, the functional significance of Drg1 in these processes is unknown. We have demonstrated for the first time that Drg1 directly binds to the BH3-only pro-apoptotic protein Bim. Depletion of Drg1 by siRNA induced up-regulation of Bim and its accumulation in the mitochondria, which correlated with loss of mitochondrial membrane potential and induction of apoptosis in cells exposed to SN-38. Further analyses revealed that Drg1 promotes degradation of Bim through the Cullin2/ElonginB-CIS ubiquitin-protein ligase complex. Conversely, in the absence of Drg1, Bim was stabilized and bound more abundantly to Hsp70. These results demonstrate that Drg1 renders cancer cells more resistant to chemotherapy through enhanced proteasome-mediated Bim degradation.

Keywords

Drg1; Bim; DNA damage; apoptosis

Introduction

Drg1/Cap43/NDRG1 is a member of the NDRG family of four genes that share 57-65% amino acid identity. While these proteins have been proposed to possess the alpha/beta hydrolase fold, they do not have hydrolase activity (1), (2). Drg1 contains three unique tandem repeats of 10 hydrophilic amino acids near the C-terminus. It was previously identified through differential screening techniques during stress response, hormone responses, cell growth and differentiation (3) (4) (5) (6) (7). The gene for Drg1 has been localized to the chromosome 8q24.3 (8). Mutations in the Drg1 gene are linked to hereditary motor sensory neuropathy (9, 10) and Drg1-deficient mice exhibited a progressive demyelination of peripheral nerves (11). Several *in vitro* studies indicated this gene to be the target of multiple regulatory pathways. For example, the enforced expression of Drg1 in the metastatic colon cancer cell line SW620 has been shown to induce morphological changes that are indicative of differentiation, and reduced *in vitro* invasion through Matrigel and *in vivo* liver metastasis in nude mice (5). An inverse correlation has been reported between Drg1 expression and the metastatic potential of prostate cancer cells (12) (13). Drg1 has been also shown to play an important role in the context of human cancer progression. Kurdistani et al., showed that Drg1 mRNA cycles with cell division, peaking at G1 and G2-M, with lower expression in S phase, and this biphasic expression of Drg1 mRNA was absent in tumor cells (8). In contrast, other reports demonstrated

¹To whom correspondence should be addressed. 1275 York Ave, New York, NY 10021. E-mail: schwartzg@mskcc.org.

that Drg1 is highly expressed in human cancers (14), (15), and its high expression is an indicator of poor prognosis in hepatocellular carcinoma (16). Also, Drg1 was observed to increase during colorectal carcinogenesis suggesting that Drg1 might play a role in tumor progression (17).

We have previously reported that in Hct116 human colon cancer cells Drg1 is transcriptionally induced by SN-38, the active metabolite of the topoisomerase I poison irinotecan (CPT-11) (18). The suppression of Drg1 in Hct116 cells using specific anti-sense constructs rendered these cells sensitive to SN-38 in vitro and to CPT-11 in vivo with enhanced apoptosis. In contrast, overexpression of Drg1 in SW620 colon cancer cells, which have low endogenous expression of Drg1, resulted in resistance to SN-38 in vitro and to CPT-11 in vivo. In a clinical analysis of 131 patients with metastatic colon cancer, 40% of the tumor cells in the liver lesions staining positively for Drg1. In a subset analysis of patients who went on to received CPT-11 therapy, patients with low Drg1 expression (< 30% cells staining) in their liver metastases remained on irinotecan based therapy for 9.3 months, whereas as those with high Drg1 expression (> 30%) remained on therapy for 6.8 months, suggesting an increased sensitivity to CPT-11 in the Drg1 low patient population (19). These results indicate that Drg1 is highly expressed in both primary and metastatic colon cancer. Furthermore, Drg1 may play a functional role in the sensitivity to CPT-11, such that increased Drg1 expression results in a decrease in CPT-11 sensitivity. However, the mechanism by which Drg1 renders these cells resistant to CPT-11 remains unknown. Here we report that Drg1 interacts with the proapoptotic BH3-only protein Bim (20), and negatively regulates Bim protein stability by mediating its binding to the proteasome. Therefore, downregulation of Drg1 stabilizes Bim and results in increased sensitivity to CPT-11.

Experimental Procedures

Cell culture

Human Hct116 colon carcinoma cells were purchased from ATCC and cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin, and maintained at 37°C in 5% CO₂. The human gastric cancer cell line MKN74 was supplied by Dr. E. Tahara (Hiroshima University, Hiroshima, Japan). SK-Mel-173 and SK-Mel-19 melanomas were obtained from Dr. A. Houghton, and OCM3 ocular melanoma was from Robert Folberg (University of Illinois, Chicago, IL). Cells were treated with SN-38 (5 mM stock; supplied by Pfizer Inc., Groton, CT). For protein stability experiments, cells were treated with 10µM cycloheximide (CHX) (Sigma) for up to 5 hours.

RNAi-mediated gene knockdown

Small interfering RNA against Drg1 and Bim (21) were purchased from Dharmacon Inc. The Drg1 siRNA sequences were GGAGUCCUUAACAGUUUG (22) and GCAUUAUUGGCAUGGGAAC (23). Control siRNA, which consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA, and Hsp70 siRNA were from Santa Cruz Biotechnology. They were transfected in Hct116 cells using Lipofectamine RNAiMAX reagent (Invitrogen) following the manufacturer's instructions. The HA-tagged Drg1 siRNA recognition site silent mutant (called HA-Drg1-M) was generated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) with the oligonucleotide GCTTCCTGGAGTACTCCAGCAGTTTGGGCTG. The plasmid was authenticated by DNA sequencing, and transfected in cells 24 hours after the siRNAs with FuGENE 6 (23). The empty HA-CMV vector (Clontech) was used as control.

Apoptosis assays

Apoptosis was measured using Annexin V-FITC Apoptosis detection Kit II (BD Pharmingen) following manufacturer's instructions. Both adherent and floating cells were collected and

resuspended in Annexin-V binding buffer. The Annexin-V positive population was analyzed on a FACScan (Becton Dickinson) and data were analyzed using FlowJo software (Tree Star Inc.). One representative experiment is shown. Apoptosis was also measured by quantitative fluorescence microscopy (QFM) with 4',6'-diamidino-2-phenylindole (DAPI, Sigma Chemical Co.) for nuclear chromatin staining. Mitochondrial membrane potential was monitored using MitoProbe™ DiIC1(5) Assay Kit (Molecular Probes, Invitrogen Detection Technologies) and analyzed by flow cytometry. The cyanine dye DiIC1(5) at 100nM penetrates the cytosol of eukaryotic cells and accumulates primarily in mitochondria with active membrane potentials. Flow cytometry analysis reveals a decrease in fluorescence in cells with disrupted mitochondrial membrane potential.

Immunoblotting and immunoprecipitation

Cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail tablets (Complete Mini, Roche Diagnostics) and 1 mM NaVO₃. Total protein concentration of the lysates was measured by Bio-Rad protein assay (Bio-Rad Laboratories), and equal amounts of protein were loaded on 4-12% PAGE gels (Invitrogen). The membranes were probed with Drg1 (graciously supplied by Therese Combes, University Montpellier II, Montpellier Cedex, France), Bim (Calbiochem), Bak (Pharmingen), Bid (Cell Signaling), Bax, ubiquitin, and Elongin B (Santa Cruz Biotechnology), Noxa (Imgenex), Hsp70 (Stressgen), Cullin 2 (Abcam), and α -tubulin (Upstate Biotechnology) antibodies. For immunoprecipitation experiments, equivalent amount of proteins from the cleared lysates were incubated with antibodies for 3 hours at 4 °C. Then, 30 μ l of agarose beads (Upstate Biotechnology) were added for an additional hour. Immunocomplexes were washed in lysis buffer and suspended in 4xSDS sample buffer and Western analysis of bound proteins. The mitochondrial and cytoplasmic fractions were prepared using the Mitochondria Isolation Kit (Pierce Biotechnology).

GST binding assays

The map of the various Drg1 deletion constructs used in these studies is shown in Fig. 5C. For these experiments, the full-length Drg1 cDNA was amplified by PCR (Supplemental Methods). The products were digested with EcoRI and XhoI, and directionally cloned in pGEX-6P-2 vector (Pharmacia). The expression of the GST-Drg1 fragments was confirmed by SDS-gel electrophoresis and Coomassie Blue staining. In binding assays, purified GST fusion proteins bound to glutathione beads were incubated with recombinant Bim protein (R&D Systems) for 2 hours at 4 °C in 250 μ l of binding buffer containing 20 mM Tris (pH 8.0), 100 mM NaCl, 1mM EDTA, 0.05% Tween 20, and 1mM DTT. GST-beads were used as control. The beads were washed three times with binding buffer and collected by centrifugation. The bound Bim protein was eluted in SDS sample buffer, subjected to SDS- PAGE and detected by immunoblotting.

Results

Downregulation of Drg1 induces Bim and sensitizes Hct116 cells to SN-38

It has been previously shown that inhibition of endogenous Drg1 expression in Hct116 cells by stable expression of an antisense Drg1 cDNA increased the sensitivity of cells to undergo apoptosis by the active metabolite of CPT-11, SN-38 (18). A small interfering RNA-based approach was used to selectively inhibit Drg1 expression in Hct116 cells before exposure to 250 nM SN-38 for 24 hours, and examined for apoptosis by Annexin V/propidium iodide staining. Under these conditions, apoptosis increased from 19.1% with control siRNA to 35.5% (Fig. 1A) following selective suppression of Drg1 (Fig. 1D). This was further evaluated by DAPI staining in QFM assays. Apoptosis was induced by SN-38 in a time- and dose-dependent fashion in Hct116 cells transfected with Drg1 siRNA compared to control siRNA (Fig. 1B and 1C). While Drg1 expression was decreased with two non-overlapping siRNA, we also found

a substantial increase in the expression of the pro-apoptotic protein Bim, especially the BimEL form (Fig. 1D). This effect on Bim was reversed by reintroducing Drg1 in cells transfected with siRNA using a HA-tagged Drg1 construct (HA-Drg1-M) carrying third-base silent mutations within the siRNA recognition site, thus excluding off-target interactions of the siRNA (Fig. 1E). The expression of other pro-apoptotic proteins including Bax, Bid, Bak and Noxa did not change after Drg1 siRNA transfection (Fig. 2A), suggesting a relative degree of specificity for Bim induction. Also, Drg1 siRNA did not affect the expression of anti-apoptotic proteins Bcl-2 and Mcl-1, and SN-38 treatment did not change the expression of either (Fig. 2B). Bim expression was also analyzed in a panel of different cancer cell lines. Downregulation of Drg1 induced Bim only in Drg1-expressing cells (MKN-74 and Mel-173), while no induction of Bim was observed in cells with undetectable Drg1 (Mel-19 and OCM3) (Fig. 2C), further confirming the specificity of the Drg1 siRNA. To determine whether Bim expression mediated the increase in apoptosis by SN-38 following Drg1 downregulation, a specific Bim siRNA was transfected in Hct116, alone or together with Drg1 siRNA (Fig. 2D, right). Bim knockdown inhibited the induction of apoptosis by 50% in cells co-transfected with Drg1 siRNA, while it did not significantly change the apoptosis induced by SN-38 when Bim siRNA was transfected alone (Fig. 2D, left). These results suggest that Bim induction plays a direct role in the sensitivity to SN-38 only when Drg1 is downregulated.

BH3-only proteins have been reported to localize in different cellular compartments including the mitochondria, cytosol and microtubules to sense different sources of stress (24). However, mitochondrial targeting of Bim is required for induction of apoptosis (25). Thus, we analyzed the sub-cellular localization of Bim in Hct116 cells with or without Drg1. As shown in Fig. 3A, with Drg1 suppression there was a marked increase of Bim in the mitochondria. Oxphos is shown to confirm equal loading and purity of the mitochondrial fractions. This induction of Bim correlated with a 2-fold increase in mitochondrial membrane depolarization when Drg1-siRNA cells were exposed to SN-38 treatment, as compared to control-siRNA cells (Fig. 3B).

Drg1 promotes Bim degradation via the proteasome.

In order to determine whether Drg1 affects Bim expression at the transcriptional level, we performed real-time PCR in Hct116 cells 48 hours after siRNA transfections. This analysis revealed an average increase in Bim mRNA levels of 1.2-fold (data not shown), which could not account for the 5-fold increase of Bim observed at the protein level. Furthermore, we also examined the RNA stability of Bim by treating siRNA-transfected cells with Actinomycin D, and we found no difference in the kinetics of Bim RNA degradation (data not shown). Hence, we concluded that Drg1 depletion does not affect RNA transcription or stability of Bim.

Next, we tested whether Drg1 loss affected Bim protein stability. Hct116 cells were transfected with the siRNAs for 48 hours, then treated with cyclohexamide (CHX) and analyzed for Bim protein expression over the time. While in Drg1-expressing control cells Bim was degraded after 3 hours of treatment, in Drg1-depleted cells Bim levels were more stable (Fig. 4A and Supplement S1B and S1C), suggesting that the presence of Drg1 favors Bim protein degradation. It has been previously reported that Bim is regulated by posttranslational mechanisms, such as ERK-mediated phosphorylation, leading to increased Bim ubiquitination and proteosomal degradation (26). We found no change in the phosphorylation of Bim after Drg1 downregulation (data not shown). Therefore, we investigated whether Drg1 could affect Bim ubiquitination. Cell lysates from siRNA-transfected Hct116 cells were immunoprecipitated with a Bim antibody, followed by immunoblotting using ubiquitin or Bim antibodies. Bim was ubiquitinated in control-siRNA transfected cells. However, the ubiquitinated forms decreased in Drg1-depleted cells, especially 72 hours after transfection, while immunoprecipitated Bim was elevated (Fig. 4B, bottom panel). Hence, the lack of Drg1 directly accounts for the decrease in Bim ubiquitination and its rate of protein degradation.

In order to identify Bim interacting partners involved in its degradation, we performed a series of immunoprecipitation experiments. First, we found that Bim coimmunoprecipitated with Drg1, but not with a control IgG antibody (Fig. 4C, upper panel). Previous findings demonstrated that Drg1 interacted with the heat shock cognate protein 70 (Hsc70) (27). Thus, we tested whether Bim could also bind to the heat-shock family members Hsc70/Hsp70, and whether Drg1 could affect Bim stability through these interactions. We found a remarkable increase in the binding of Bim to Hsp70 in cells transfected with Drg1 siRNA (Fig. 4C, bottom right). Also, the inverse immunoprecipitation with a Bim antibody revealed binding to Hsp70 (Supplement S1A). We could also demonstrate that downregulation of Hsp70 by siRNA induced a decrease in Bim stability when cells were treated with CHX, especially in Drg1 siRNA cotransfected cells (Supplement S1B and S1C). However, while Hsp70 appeared to protect Bim in the absence of Drg1, it does not explain how Bim is degraded in the presence of Drg1. More recently, it has been reported that RACK1 promotes Bim degradation in paclitaxel-treated cells through the interaction with the ElonginB/C-Cullin2-CIS ubiquitin-protein ligase complex (28). Based on these findings, we performed immunoprecipitation assays using antibodies against these endogenous proteasome subunits in cell lysates of Hct116 cells, after transfection with control or Drg1 siRNA. As shown in Fig. 4D (left panel), both Bim and Drg1 co-immunoprecipitated with Cullin2 in control cells, while binding of Bim to Cullin2 was decreased in Drg1 siRNA-transfected cells. In addition, endogenous Elongin B and CIS co-immunoprecipitated with Bim, and these interactions were greatly decreased in the absence of Drg1 (Fig. 4D, right). As a control, the total amount of immunoprecipitated Bim is also shown (Fig. 4D, bottom right). These results demonstrated that Drg1 mediates the binding of Bim to the proteasome, thus promoting its degradation.

Bim directly binds to Drg1 at the carboxy-terminus

To further characterize the Drg1-Bim interaction, Drg1 was expressed as a GST fusion protein and used in binding assays with recombinant Bim. As shown in Figure 5A, recombinant Bim bound to immobilized GST-Drg1 in a dose dependent manner, but not to GST alone. Next, a series of GST-tagged Drg1 deletion mutants were generated (Fig. 5B) to identify the region of the Drg1 sequence responsible for the interaction with Bim. In agreement with the immunoprecipitation experiments, Bim bound to full length Drg1 and also to fragment lacking the amino-terminus (Δ N-Drg1), while it did not bind to Δ 1-Drg1, Δ 2-Drg1, Δ 3-Drg1 or GST (Fig. 5C). These results suggest that Bim directly interacts with Drg1 at the amino acids 338-394.

Discussion

We have previously reported that suppression of Drg1 increases sensitivity to CPT-11 in Hct116 colon cancer bearing xenografts and results in increased sensitization to SN-38-induced apoptosis in this same cell line (18). Its relevance to CPT-11 resistance in colon cancer therapy has also been suggested by several recent studies (19) (29). The pan-CDK inhibitor flavopiridol has been shown to suppress the transcriptional induction of Drg1 by SN-38 and enhance SN-38-induced apoptosis in Hct116 colon cancer cells in vitro and increase the efficacy of CPT-11 in vivo (18). The importance of Drg1 down-regulation by flavopiridol to the sensitivity to CPT-11 has been evaluated in a phase I clinical trial (30). The results from serial biopsies indicated that clinical benefit to therapy was only observed in patients who exhibited a decrease or no induction in Drg1 protein expression. These findings are consistent with emerging data that Drg1 plays an essential role in the resistance to CPT-11 (31), and suppressing its expression both by pharmacologic or molecular means could increase sensitization to this agent both in vitro and in vivo across a spectrum of tumor types. Nevertheless, the function of this protein relative to chemotherapy resistance has remained essentially unknown.

Here we provide the first evidence of the mechanism of action of Drg1 in the inhibition of apoptosis. Our study suggests that Drg1 plays a critical role in facilitating Bim turnover by mediating its binding to the proteasome. As summarized in Fig. 6, we found that Drg1 mediated the degradation of Bim through the interaction to the ElonginB/Cullin2-CIS ubiquitin-protein ligase complex (28), promoting resistance to chemotherapy. The suppression of Drg1 using siRNA results in Bim stabilization, allowing its association with Hsp70, its mitochondrial localization, and the SN-38-mediated induction of apoptosis. Bim knockdown reversed the effect of SN-38 in Drg1 siRNA-transfected cells, while it did not affect the basal level of apoptosis in control cells. BH3-only pro-apoptotic proteins interact with pro-survival Bcl-2-like proteins inactivating their functions (32). Hence, Bim expression levels have a prominent role in mediating cell death (33).

Previous reports demonstrated that Drg1 is highly expressed in human cancers (14), (15) (17). Looking at the expression levels of Drg1 and Bim in the five cell lines we have tested, it appears that there is a negative correlation between these two proteins. However, more tumor-derived cells need to be analyzed to further confirm and validate the significance of Drg1-mediated regulation of Bim. Recently it has been reported that RACK1 reduced Bim protein levels in paclitaxel-treated cells through a proteasome-dependent pathway (28). Similarly to RACK1, knockdown of Drg1 by siRNA induced Bim expression in treated cells. We did not test whether Drg1 differentially regulates Bim in the presence of paclitaxel. However, by immunoprecipitation experiments, we could demonstrate that Drg1 binds to a Cullin2/ElonginB-CIS E3 ubiquitin ligase complex in untreated cells. While this novel interaction needs further characterization, we have also shown that Bim binds to the proteasome through Drg1. Conversely, when Drg1 is downregulated, Bim preferentially bound to Hsp70. In support of this, we could also demonstrate that Drg1 competed with Hsp70 for the binding of Bim in vitro (Supplement S2). Hsp70 proteins function as chaperons by assisting the folding and assembly of multi-protein complexes, as well as participate in the transport of proteins across cellular membranes (34) (35). We did not find any changes in the expression of Hsp70, while its interaction with Bim increased after Drg1 depletion, suggesting a role of Hsp70 in Bim stability and /or localization under these conditions.

Bim also directly bound to the carboxy-terminus region of Drg1, which may provide the basis to design peptides or small molecules to interrupt this specific interaction. Indeed, finding a way to stabilize and increase Bim levels may provide the means to increase sensitivity to CPT-11-based therapy. In view of the high expression of Drg1 in both primary and metastatic colon cancer to liver, this could result in new treatment paradigms for patients with this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by RO1 CA 67819 (NCI).

References

1. Shaw E, McCue LA, Lawrence CE, Dordick JS. Identification of a novel class in the alpha/beta hydrolase fold superfamily: the N-myc differentiation-related proteins. *Proteins* 2002;47:163–8. [PubMed: 11933063]
2. Ellen TP, Ke Q, Zhang P, Costa M. NDRG1, a growth and cancer related gene: regulation of gene expression and function in normal and disease states. *Carcinogenesis* 2008;29:2–8. [PubMed: 17916902]

3. Agarwala KL, Kokame K, Kato H, Miyata T. Phosphorylation of RTP, an ER stress-responsive cytoplasmic protein. *Biochem Biophys Res Commun* 2000;272:641–7. [PubMed: 10860807]
4. Lin TM, Chang C. Cloning and characterization of TDD5, an androgen target gene that is differentially repressed by testosterone and dihydrotestosterone. *Proc Natl Acad Sci U S A* 1997;94:4988–93. [PubMed: 9144177]
5. Guan RJ, Ford HL, Fu Y, Li Y, Shaw LM, Pardee AB. Drg-1 as a differentiation-related, putative metastatic suppressor gene in human colon cancer. *Cancer Res* 2000;60:749–55. [PubMed: 10676663]
6. Piquemal D, Joulia D, Balaguer P, Basset A, Marti J, Commes T. Differential expression of the RTP/ Drg1/Ndr1 gene product in proliferating and growth arrested cells. *Biochim Biophys Acta* 1999;1450:364–73. [PubMed: 10395947]
7. van Belzen N, Dinjens WN, Diesveld MP, et al. A novel gene which is up-regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms. *Lab Invest* 1997;77:85–92. [PubMed: 9251681]
8. Kurdistani SK, Arizti P, Reimer CL, Sugrue MM, Aaronson SA, Lee SW. Inhibition of tumor cell growth by RTP/rit42 and its responsiveness to p53 and DNA damage. *Cancer Res* 1998;58:4439–44. [PubMed: 9766676]
9. Hunter M, Bernard R, Freitas E, et al. Mutation screening of the N-myc downstream-regulated gene 1 (NDRG1) in patients with Charcot-Marie-Tooth Disease. *Hum Mutat* 2003;22:129–35. [PubMed: 12872253]
10. Kalaydjieva L, Gresham D, Gooding R, et al. N-myc downstream-regulated gene 1 is mutated in hereditary motor and sensory neuropathy-Lom. *Am J Hum Genet* 2000;67:47–58. [PubMed: 10831399]
11. Okuda T, Higashi Y, Kokame K, Tanaka C, Kondoh H, Miyata T. Ndr1-deficient mice exhibit a progressive demyelinating disorder of peripheral nerves. *Mol Cell Biol* 2004;24:3949–56. [PubMed: 15082788]
12. Bandyopadhyay S, Pai SK, Gross SC, et al. The Drg-1 gene suppresses tumor metastasis in prostate cancer. *Cancer Res* 2003;63:1731–6. [PubMed: 12702552]
13. Ulrix W, Swinnen JV, Heyns W, Verhoeven G. The differentiation-related gene 1, Drg1, is markedly upregulated by androgens in LNCaP prostatic adenocarcinoma cells. *FEBS Lett* 1999;455:23–6. [PubMed: 10428464]
14. Cangul H, Salnikow K, Yee H, Zagzag D, Commes T, Costa M. Enhanced expression of a novel protein in human cancer cells: a potential aid to cancer diagnosis. *Cell Biol Toxicol* 2002;18:87–96. [PubMed: 12046693]
15. Nishie A, Masuda K, Otsubo M, et al. High expression of the Cap43 gene in infiltrating macrophages of human renal cell carcinomas. *Clin Cancer Res* 2001;7:2145–51. [PubMed: 11448934]
16. Chua MS, Sun H, Cheung ST, et al. Overexpression of NDRG1 is an indicator of poor prognosis in hepatocellular carcinoma. *Mod Pathol* 2007;20:76–83. [PubMed: 17170744]
17. Wang Z, Wang F, Wang WQ, et al. Correlation of N-myc downstream-regulated gene 1 overexpression with progressive growth of colorectal neoplasm. *World J Gastroenterol* 2004;10:550–4. [PubMed: 14966915]
18. Motwani M, Sirotak FM, She Y, Commes T, Schwartz GK. Drg1, a novel target for modulating sensitivity to CPT-11 in colon cancer cells. *Cancer Res* 2002;62:3950–5. [PubMed: 12124325]
19. Shah MA, Kemeny N, Hummer A, et al. Drg1 expression in 131 colorectal liver metastases: correlation with clinical variables and patient outcomes. *Clin Cancer Res* 2005;11:3296–302. [PubMed: 15867226]
20. Willis SN, Adams JM. Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol* 2005;17:617–25. [PubMed: 16243507]
21. Liang M, Graham R, Hulley PA. Bim, Bak, and Bax regulate osteoblast survival. *J Bone and Mineral Res* 2008;23:610–20.
22. Chen B, Nelson DM, Sadovsky Y. N-myc down-regulated gene 1 modulates the response of term human trophoblasts to hypoxic injury. *J Biol Chem* 2006;281:2764–72. [PubMed: 16314423]
23. Stein S, Thomas EK, Herzog B, et al. NDRG1 is necessary for p53-dependent apoptosis. *J Biol Chem* 2004;279:48930–40. [PubMed: 15377670]

24. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 1999;3:287–96. [PubMed: 10198631]
25. Weber A, Paschen SA, Heger K, et al. BimS-induced apoptosis requires mitochondrial localization but not interaction with anti-apoptotic Bcl-2 proteins. *J Cell Biol* 2007;177:625–36. [PubMed: 17517961]
26. Ley R, Ewings KE, Hadfield K, Cook SJ. Regulatory phosphorylation of Bim: sorting out the ERK from the JNK. *Cell Death Differ* 2005;12:1008–14. [PubMed: 15947788]
27. Sugiki T, Taketomi Y, Kikuchi-Yanoshita R, Murakami M, Kudo I. Association of N-myc downregulated gene 1 with heat-shock cognate protein 70 in mast cells. *Biol Pharm Bull* 2004;27:628–33. [PubMed: 15133235]
28. Zhang W, Cheng GZ, Gong J, et al. RACK1 and CIS mediate the degradation of BimEL in cancer cells. *J Biol Chem* 2008;283:16416–26. [PubMed: 18420585]
29. Yu J, Shannon WD, Watson MA, McLeod HL. Gene expression profiling of the irinotecan pathway in colorectal cancer. *Clin Cancer Res* 2005;11:2053–62. [PubMed: 15756032]
30. Shah MA, Kortmansky J, Motwani M, et al. A phase I clinical trial of the sequential combination of irinotecan followed by flavopiridol. *Clin Cancer Res* 2005;11:3836–45. [PubMed: 15897584]
31. Azrak RG, Yu J, Pendyala L, et al. Irinotecan pharmacokinetic and pharmacogenomic alterations induced by methylselenocysteine in human head and neck xenograft tumors. *Mol Cancer Ther* 2005;4:843–54. [PubMed: 15897249]
32. Strasser A, Puthalakath H, Bouillet P, et al. The role of bim, a proapoptotic BH3-only member of the Bcl-2 family in cell-death control. *Ann N Y Acad Sci* 2000;917:541–8. [PubMed: 11268382]
33. Bouillet P, Cory S, Zhang LC, Strasser A, Adams JM. Degenerative disorders caused by Bcl-2 deficiency prevented by loss of its BH3-only antagonist Bim. *Dev Cell* 2001;1:645–53. [PubMed: 11709185]
34. Shi Y, Thomas JO. The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Mol Cell Biol* 1992;12(5):2186–92. [PubMed: 1569948]
35. Murakami H, Pain D, Blobel G. 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. *J Cell Biol* 1988;107(6 Pt 1):2051–7. [PubMed: 3058716]

Figure 1.

Drg1 silencing sensitizes Hct116 cells to SN-38. A, Hct116 cells were transfected with control (siCtr) or Drg1 siRNA (siDrg), treated with 250 nM SN-38 for 24 hours, and analyzed by FACS after Annexin V/propidium iodide staining. Bottom left quadrant, accumulation of viable cells in the four-quadrant gate; bottom right quadrant, early apoptotic cells, staining with Annexin V only; top right quadrant, double-positive staining cells undergoing late apoptosis and early necrosis. The numbers represent the percentage of the cell population in each of the respective gates. The sum of the Annexin V-positive quadrants provides the total percentage of apoptotic cells. B, Apoptosis was measured by QFM after siRNA transfections of Hct116 cells treated with increasing concentrations of SN-38; bars, $\pm$ SD; *, **, $P < 0.004$, versus treated control siRNA, or C, after 24 and 48 hours of treatment with 250 nM SN-38; bars, $\pm$ SD; *, **, $P < 0.005$, **, $P < 0.0001$ versus treated control siRNA. D, Two non-overlapping Drg1 siRNA were used to transfect Hct116 cells, and cell lysates were analyzed by Western blot for Drg1 and Bim expression. E, Induction of Bim is a specific effect of Drg1 down-regulation. Hct116 cells were subjected to two rounds of transfections with the siRNA first, then with empty HA-cmv vector or HA-Drg1-M carrying the silent mutations. Blots for the expression of various proteins are shown.

Figure 2.

Drg1 silencing induces Bim. A, Hct116 transfected cells were analyzed for expression of Drg1, Bim, Bax, Bid, Bak, and Noxa. B, Western blot analysis of siRNA-transfected Hct116 cells treated with 250 nM SN-38 for Bcl-2 and Mcl-1 expression. C, Four cell lines were transfected with control and Drg1 siRNA and analyzed for Drg1 and Bim expression. Tubulin is shown to confirm equal protein loading. Each blot is representative of multiple independent experiments. D, Hct116 cells were transfected with the Drg1 and Bim siRNAs alone or combined, and analyzed by immunoblotting (inlet). Apoptosis was measured by QFM after 24 hours of SN-38 treatment. Columns, mean of three independent experiments performed in duplicates. *, $P < 0.003$ versus Drg1 siRNA alone.

Figure 3.

Induced Bim localizes into the mitochondria and increases mitochondrial membrane depolarization. A, Hct116 cells were transfected with siRNA, and then subjected to cellular fractionation. Cytosolic and mitochondrial fractions were analyzed by Western blotting for Drg1 and Bim. Purity of the fractions was confirmed using the mitochondrial specific protein OxPhos, and tubulin. B, siRNA-transfected cells were treated with 250nM SN-38, then stained with MitoProbe DiIC1(5) and subjected to flow cytometry analysis. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as positive control to uncouple mitochondria (dotted line). Cells with depolarized mitochondria show a decrease in the mitochondrial distribution of the dye. One of two representative experiments is shown.

Figure 4.

Drg1 downregulation increases Bim protein stability. A, Hct116 cells were transfected with control and Drg1 siRNA. After 48h, cells were treated with 10 μ g/ml cyclohexamide (CHX) for up to 5 hours. Cell lysates were then analyzed by Western blotting for Drg1 and Bim expression. B, siRNA-transfected cells were immunoprecipitated (IP) with a Bim antibody after 48 and 72 hours from transfection and immunoblotted with ubiquitin (top panels) or Bim (bottom panels) antibodies. Bim binds to Drg1 and Hsp70. C, Hct116 cell lysates were immunoprecipitated with Drg1 or control IgG antibodies (top panel), and Hsp70 (bottom right panel) and immunoblotted (IB) with a Bim antibody. Whole cell lysates are shown on the left. D, Cullin2 (left) and Bim (right) were immunoprecipitated, and then blotted with the indicated antibodies. Each experiment was repeated at least three times.

Figure 5.

Bim binds to Drg1 in vitro. A, GST and GST-Drg1 bound to GSH-agarose beads were incubated for 2 hours at 4°C with increasing amounts (10, 20, 40 ng) of recombinant Bim. Beads were washed with binding buffer, and captured proteins were analyzed by SDS-PAGE and immunoblotting with a Bim antibody. B, Schematic illustration of Drg1 full-length and deletion fragments generated to determine the region involved in the interaction with Bim. The dashed area represents the three tandem repeats. C, Purified GST alone or GST-Drg1 full-length and deletion fragments bound to GSH-agarose beads were incubated for 2 hours with Bim (15 ng), washed and subjected to immunoblotting with a Bim antibody.

Figure 6.

A model for the function of Drg1 in the regulation of Bim protein stability. Drg1-bound Bim is ubiquitinated and degraded by the proteasome (left). In the absence of Drg1, Bim is stabilized and binds to Hsp70. Increased levels of Bim reduce the mitochondrial membrane potential (Δ_m) and sensitize cells to drug-induced apoptosis (right).