Interplay of the iron-regulated metastasis suppressor NDRG1 with epidermal growth factor receptor (EGFR) and oncogenic signaling

Published, Papers in Press, June 14, 2017, DOI 10.1074/jbc.R117.776393

Sharleen V. Menezes, Sumit Sahni 1, Zaklina Kovacevic 2, and Des R. Richardson 3
From the Molecular Pharmacology and Pathology Program, Department of Pathology, Bosch Institute, University of Sydney, Sydney, New South Wales 2006, Australia

Edited by F. Peter Guengerich

The iron-regulated metastasis suppressor N-myc downstream-regulated gene 1 (NDRG1) has been shown to inhibit numerous oncogenic signaling pathways in cancer cells. Recent findings have demonstrated that NDRG1 inhibits the ErbB family of receptors, which function as key inducers of carcinogenesis. NDRG1 attenuates ErbB signaling by inhibiting formation of epidermal growth factor receptor (EGFR)/human epidermal growth factor receptor 2 (HER2) and HER2/HER3 heterodimers and by down-regulating EGFR via a mechanism involving its degradation. Understanding the complex interplay between NDRG1, iron, and ErbB signaling is vital for identifying novel, more effective targets for cancer therapy.

Metastasis

Metastasis is a multistep process that remains the leading cause of cancer-related deaths worldwide (1). Beginning at the primary tumor site, cancer cells disseminate into the bloodstream and migrate to distant organs where they form secondary tumors (1). Metastasis of these cells can be triggered by a plethora of biochemical factors and molecules as well as the extracellular environment (1). Considering its key role in patient mortality, it is pertinent to understand how metastasis is regulated so as to design therapeutic modalities to inhibit cancer progression.

This review specifically addresses the regulation of the metastasis suppressor N-myc downstream-regulated gene 1 (NDRG1) by iron and its associated downstream signaling pathways that have also been linked to iron homeostasis. This is important, as iron is crucial for tumor cell proliferation and metastasis (2), and thus, it is vital to understand its role in oncogenic signaling.

Iron-regulated metastasis suppressor, NDRG1

The molecule NDRG1 is a well-known metastasis suppressor that results in decreased metastases and improved patient prognosis in several cancer types, including those of the breast, prostate, pancreas, and colon (3–5). Paradoxically, NDRG1 expression is demonstrated to be increased in tumors of the liver, esophagus and cervix, where it stimulates oncogenesis (6–8), although in the majority of studies, it is associated with metastasis suppression (3, 5). In fact, NDRG1 has been recognized for its involvement in cellular signaling, growth, differentiation, lipid biosynthesis, and the stress response (3, 5). Most importantly, in its role as a potent metastasis suppressor, NDRG1 has been of great interest due to its ability to inhibit tumor growth and to decrease cell proliferation, migration, invasion, and angiogenesis (9–19).

Recently, the roles of LSD1 and N-myc in the regulation of NDRG1 expression were investigated, and it was shown that LSD1 co-localizes with N-myc at the promoter region of the NDRG1 gene to inhibit its expression (20). Furthermore, it is well known that NDRG1 is a hypoxia-regulated gene and can be induced by hypoxic conditions via an HIF-1α (hypoxia-inducible factor-1α)-dependent mechanism (21). It has also been demonstrated that NDRG1 expression is regulated by intracellular iron in a wide variety of studies in vitro and in vivo (9, 13, 15, 16, 21–31). In fact, a decrease in cellular iron results in robust up-regulation of NDRG1 at the mRNA and protein level, whereas supplementation of iron leads to decreased NDRG1 expression (21, 22, 27). As an appropriate positive control, the same regulation was also reported for other classical iron-regulated molecules, e.g., the TR1 4 (transferrin receptor 1), VEGF1 (vascular endothelial growth factor 1), etc.,

The abbreviations used are: TR1, transferrin receptor 1; 311, 2-hydroxyl-1-naphthaldehyde isonicotinoyl hydrazone; BTC, betacellulin; DFO, desferrioxamine; Dp44mT, 2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; DpC, di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbzone; EGFR, epidermal growth factor receptor; EMT, epithelial to mesenchymal transition; NGF, nerve growth factor, p130<br>© 2017 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
confirming the physiological nature of the iron depletion reported (21, 22, 27). Critically, the up-regulation of NDRG1 occurs also in vivo in tumors after treatment of mice (intravenous) with the iron chelator, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) (26). This occurred concurrently in tumors with up-regulation of the TjR1 and also VEGF1 (26), both of which are classically known to be up-regulated by iron depletion (32). Dp44mT is a known iron chelator and results in cellular iron efflux and inhibits iron uptake from transferrin, leading to cellular iron depletion in vitro (33). Its in vivo effects were consistent with it binding iron in the tumor and forming an intracellular iron complex that sequestered or redistributed iron away from the cellular iron-sensing mechanisms, resulting in up-regulation of genes classically increased during iron depletion i.e. TjR1 and VEGF1 (25).

Furthermore, because of the treatment of mice with this novel chelator, there were higher iron levels in the liver, which corresponded with down-regulation of NDRG1 and also TjR1 and VEGF1 (26). This effect of increasing liver iron levels was a specific property of Dp44mT and also a related thiosemicarbazone, Triapine® (25), and it proved useful in demonstrating that NDRG1 was regulated by iron levels in vivo. Hence, the regulation of NDRG1 in vivo mirrors the regulation by iron observed in vitro in multiple studies performed by various independent investigators using many different protocols of iron depletion (9, 13, 15, 16, 21–31). Also consistent with this regulation are studies examining patients infected with hepatitis C virus, in which liver iron loading occurs, and NDRG1 is down-regulated (34). In summary, studies in vitro and in vivo clearly demonstrate that NDRG1 is regulated by cellular iron depletion (32). It is noteworthy that whereas iron has been shown to regulate the expression of NDRG1, further studies are required to confirm whether this regulation of NDRG1 levels occur due to a direct response to changes in physiological iron availability.

In terms of the iron-mediated regulation of NDRG1, studies by the authors first demonstrated that this occurred via both HIF-1α-independent and -dependent mechanisms (21). This is important to consider because iron plays an important role in oncogenesis, due to its role in cell cycle progression, DNA synthesis, and proliferation (35, 36). Of note, the iron chelators, desferrioxamine (DFO) and 2-hydroxyl-1-naphthaldehyde isonicotinoyl hydrazone (311), which cause cellular iron depletion, are of greatest interest, as they result in the biosynthesis of all of which are hallmarks of metastasis (11, 15, 16, 23, 24), and will be further discussed below.

An important oncogene that is regulated by NDRG1 is cellular Src (c-Src (15)). In fact, c-Src is known to be involved in promoting tumorigenesis by facilitating cellular migration and invasion and has been linked to the EMT (37). This process occurs when cells lose their tight junctions and epithelial phenotype, instead adopting a mesenchymal appearance with associated aggressive characteristics leading to metastasis (37). Although it has been suggested that non-mutated c-Src may not be oncogenic by itself, studies have indicated that c-Src can interact and activate receptor tyrosine kinases and other growth factor receptors (38, 39). These proteins include epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor, and platelet-derived growth factor receptor (PDGFR) (38, 39).

In light of this, it was recently discovered that c-Src signaling can be suppressed via up-regulation of NDRG1 (Fig. 1) (15). This mechanism occurred via inhibition of c-Src activation, with NDRG1 preventing the phosphorylation of Src at its activating site, Tyr-416 (15). This led to inhibition of its downstream targets, Crk-associated substrate (p130Cas) and Abelson murine leukemia viral oncogene homolog 1 (ABL1, also known as c-Abl), both of which participate in modulating Ras-related C3 botulinum toxin substrate 1 (Rac1), a key regulator of cell migration (15). Additionally, NDRG1 was able to inhibit the binding of EGFR to c-Src (15), which is known to be an important means by which c-Src activity is increased (40).

Another oncogenic signaling mechanism that is inhibited by NDRG1 is the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway, which is involved in solid tumor growth and is able to further cancer progression and angiogenesis (13, 25). PI3K signaling is commonly activated in cancer, particularly in prostate tumors, where the PI3K/Akt pathway is known to be the most predominant growth factor-activated pathway (41).

The PI3K enzymes are divided into three main classes based on their structure, distribution, and function (42). Class I PI3Ks are of greatest interest, as they result in the biosynthesis of phosphatidylinositol 3,4,5-trisphosphate, resulting in the phosphorylation of Akt by its upstream activator, phosphoinositide-dependent kinase 1 (PDK1) (43). When active, Akt has been correlated with poor patient prognosis due to malignant transformation (44). Interestingly, the tumor suppressor gene, phos-
phatase and tensin homologue deleted on chromosome 10 (PTEN), can inhibit the PI3K/Akt pathway by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate to phosphatidylinositol 4,5-bisphosphate (41). Acting through this pathway, PTEN can promote cell apoptosis and induce cell cycle arrest (45).

Of note, NDRG1 has been demonstrated to up-regulate PTEN expression leading to suppression of the Akt pathway (13, 46). This is important, as PTEN loses function when PI3K signaling is activated during oncogenesis (41). Interestingly, PTEN is also able to up-regulate NDRG1 expression (46). The role of NDRG1 in affecting the PI3K/Akt signaling cascade can also be mediated by its ability to decrease expression of the PI3K subunits, p-p85α and p-p55γ, as well as the levels of phosphorylated Akt (pAkt) (13). Notably, in glioma cells where PI3K/Akt signaling is linked to a poor prognosis, NDRG1 silencing increases pAkt levels (47). These findings are of significance, as the PI3K/Akt pathway has been implicated in many cancers by inducing the progression of the cell cycle and increasing the expression of pro-proliferative proteins such as cyclin D1 (48). These studies therefore further support the crucial role of NDRG1 in regulating molecules that are known to be major players in cancer metastasis.

The Ras/Raf/MEK/ERK signaling cascade is responsible for regulating the activity of various pro-oncogenic transcription factors (49) and is also targeted by NDRG1 (13, 25). Ras can become activated by growth factors leading to the recruitment of rapidly accelerated fibrosarcoma (Raf), which then activates mitogen-activated protein kinase kinase 1 (MEK1) and subsequently activates the extracellular signal-regulated kinase (ERK), leading to the promotion of cell migration. Interestingly, NDRG1 is able to halt this process by blocking MEK1 activation. Also contributing to the migratory capabilities of tumor cells is the c-Src pathway, which promotes Rac1 and PAK1 expression, contributing to cell migration. NDRG1 can inhibit this pathway by reducing c-Src levels and inhibiting its activation. Additionally, NDRG1 is able to inhibit the PI3K/Akt pathway. These latter pathways can promote STAT3, leading to the transcription of c-Myc and cyclin D1, both of which promote cell growth and proliferation. In addition to acting on these key molecules in the above-mentioned oncogenic pathways, NDRG1 is able to inhibit EGFR, HER2, and HER3 levels and negatively regulate their activation and dimerization.

ErbB family of receptor tyrosine kinases

The ability of NDRG1 to attenuate the plethora of oncogenic signaling pathways described above could be mediated by the ErbB family of receptors, which function as master regulators of cellular signaling (17, 50), and they were found to be inhibited by NDRG1 (17).

The ErbB family of receptors, which include EGFR, HER2 (human epidermal growth factor receptor 2), HER3 (human epidermal growth factor receptor 3), and HER4 (human epidermal growth factor receptor 4), are all receptor tyrosine kinases.
that are localized on the cell membrane (51). These receptors are known to be stimulated by numerous ligands, including the epidermal growth factor (EGF), betacellulin (BTC), transforming growth factor α (TGFα), and also several neuregulins (NRGs) (51, 52). Specifically, EGF has the ability to activate EGFR, HER2, and HER3; TGFα specifically activates HER3; NRG1–4 can bind HER3 and HER4; and BTC activates EGFR and HER4 (Fig. 1) (17, 51, 52). This binding of ligand to receptor occurs with high affinity and is rapidly followed by receptor homo- or heterodimerization (51). This event results in the activation of protein kinase activity, leading to phosphorylation of tyrosine residues (53). Notably, unlike the other ErbB receptors, HER2 does not have a ligand-binding site and instead relies on heterodimerization with other ErbB family members for subsequent activation (53). In fact, HER2 is the most preferred interacting partner of the ErbB family members in terms of heterodimerization (54).

The phosphorylation sites of the ErbB family serve as docking sites for different proteins that participate in the regulation and subsequent activation of various intracellular signaling cascades, such as c-Src, PI3K/Akt, and MAPK pathways (55). Specifically, upon heterodimer formation, the EGFR kinase can phosphorylate tyrosine sites of its dimerization partner (56). The site at which the ErbB receptor is phosphorylated is believed to be dependent on the dimerization partner, hence accounting for differences in cellular signaling (57). Although autophosphorylation of EGFR occurs at the carboxyl terminus to maintain an activated state, proteins such as Grb-2, Src, and Abl can directly dock to tyrosine residues at 1068, 891, and 1086, respectively, initiating various downstream signaling cascades (58). This is important, as the interaction between EGFR and Grb-2, for instance, is vital for the induction of the Ras/MAPK pathway (59).

Each of the oncogenic signaling pathways that can be inhibited by NDRG1 are also activated by the receptor tyrosine kinase, EGFR (38, 49, 60). Specifically, EGFR can interact with the regulatory subunit p85 to activate PI3K, as well as directly bind to c-Src to stimulate its activity (Fig. 1) (38, 60). Similarly, the Ras/Raf/MEK/ERK pathway is initiated by EGFR (49). These findings highlight the significance of EGFR as a crucial modulator of signaling pathways in cancer and point to its major role in the anti-oncogenic activity of NDRG1.

Of significance, NDRG1 was demonstrated to attenuate EGF-mediated activation of EGFR and its subsequent phosphorylation at Tyr-1068, Tyr-1086, and Tyr-1148 (17) (all of which participate in the activation of oncogenic pathways PI3K, RAS, and c-Src (Fig. 1) (38)).

In terms of the precise molecular mechanism by which NDRG1 can inhibit EGFR, it has been demonstrated that NDRG1 could play a role in promoting EGFR degradation (17). A recent study has explored the interaction of HER3 with the ubiquitin ligase NEDD4 (neural precursor cell expressed developmentally down-regulated gene 4), which facilitated the receptor’s degradation and showed that this binding was greater in the presence of NDRG1 (61). Interestingly, NDRG1 can up-regulate NEDD4 expression (13), which could promote the degradation of EGFR and would be consistent with the results of recent studies (17).

**EGFR and iron**

As cellular iron levels can regulate NDRG1 expression (21, 22), it is important to consider the regulation of EGFR by this metal ion. As discussed previously, it is well understood that iron is an essential requirement for cancer cell DNA synthesis and cell cycle progression (26). The TfR1 is responsible for the transport and internalization of iron bound to transferrin from the cell surface into the cell via receptor-mediated endocytosis (Fig. 2) (62).

Although little is known regarding EGFR’s role in iron metabolism, a recent study revealed that EGFR could regulate iron homeostasis by directly binding and re-distributing the TfR1 (Fig. 2) (63). In fact, in non-small cell lung carcinoma cells, it was demonstrated that inactivation of EGFR reduced cell surface TfR1 expression, causing a decrease in iron import, and cell cycle arrest (63).

The relationship between EGFR and iron metabolism is also further substantiated by recent studies examining PGRMC1 (progestosterone-receptor membrane component 1) (64). PGRMC1 is highly expressed in a variety of tumor types where it can promote proliferation and survival (65).

Until recently, the crucial role of iron in the heme prosthetic groups of PGRMC1 in terms of its structure and function were unknown (66). A recent study has revealed that in the presence of heme, PGRMC1 is able to form a unique dimeric structure between the heme moieties (66). Study of its crystal structure demonstrated that PGRMC1 is able to undergo dimerization through stacking interactions of heme prosthetic groups from each monomer (Fig. 2) (66).

In addition to this, it is understood that PGRMC1 is able to associate with EGFR and cytochrome P450, to regulate proliferation, chemo-resistance, and also sensitivity to EGFR inhibitors such as Erlotinib (64, 66). In fact, silencing of PGRMC1 led to a reduction of EGFR phosphorylation after binding to the EGF ligand (66). Downstream targets of EGFR were also negatively correlated with PGRMC1 silencing, resulting in a down-regulation of phosphorylated Akt and ERK (66).

Ahmed et al. (64) demonstrated that PGRMC1 may directly bind to EGFR and co-localize in endosomes, where PGRMC1 promotes the endosomal recycling of EGFR back to the cell membrane, in preference to its lysosomal degradation. It has been proposed that the heme-mediated dimerization of PGRMC1 enables its interaction with EGFR (Fig. 2) (66). This was validated through studies with succinylacetone, a known inhibitor of δ-aminolevulinic acid dehydratase, which is an essential enzyme in the heme synthesis pathway (66). This study demonstrated that incubation with succinylacetone significantly decreased both PGRMC1 dimerization and its binding to EGFR (66).

The association of PGRMC1 with EGFR can also be further explained by a recent study that showed inactivation of EGFR signaling when PGRMC1 was not maintained on the cell membrane (67). It was suggested that PGRMC1 acts as an adaptor protein to regulate EGFR membrane expression (67). Additionally, PGRMC1 can also be regulated by phosphorylation at Tyr-113, whereby membrane trafficking by PGRMC1 is required for co-localization with EGFR (66). Interestingly, Kabe et al. (66)
demonstrated that phosphorylation at this site could cause steric interference, thus discouraging heme binding. It was therefore suggested that heme binding and vesicular trafficking are mutually exclusive processes for PGRMC1 (68).

Although there is still no definitive evidence to suggest a direct link of iron regulation on EGFR, these findings are particularly pertinent considering the roles EGFR and PGRMC1 play in tumor and cancer progression (66).

Degradation mechanisms of EGFR

The link between the ErbB receptors and their signaling in cancer pathogenesis has been widely studied for decades. Considering their role in promoting cancer development, it is important to understand how ErbB family members may be degraded.

Upon ligand binding, EGFR is rapidly internalized into endosomes where it can either be recycled back to the cell surface or transported to lysosomes for degradation (Fig. 3) (69). Numerous studies have reported the role of c-Cbl (cellular casitas B-lineage lymphoma), an E3 ubiquitin ligase, in facilitating the endocytosis of EGFR (70). In fact, c-Cbl acts as a major substrate of tyrosine kinase phosphorylation, undergoing enhanced phosphorylation in response to ligand stimulation by EGF (70, 71).

In addition, c-Cbl was recently linked to the protein LRIG1 (leucine-rich repeat and immunoglobulin-like domain 1) in terms of regulating EGFR and its other family members (72). It was shown that following EGF stimulation, there is a recruitment of c-Cbl to simultaneously ubiquitinate EGFR and LRIG1, sorting them for degradation (Fig. 3) (72, 73). Furthermore, LRIG1 has also demonstrated the ability to suppress ErbB receptor levels by associating with EGFR, HER2, HER3, and HER4 to enhance ligand-stimulated ErbB receptor ubiquitination (73).

The MIG6 (mitogen-inducible factor 6) is also known as ERBB receptor feedback inhibitor 1, or the receptor-associated late transducer (74), and is involved in EGFR degradation (75). MIG6 acts to inhibit EGFR by associating with the activated receptor through a carboxyl-terminal binding domain (75). Specifically, a 25-residue epitope from MIG6 binds to the carboxyl-terminal lobe and blocks the formation of the activating dimer interface of EGFR that is required for its signaling (75). Therefore, although MIG6 has been reported to have several roles, including the regulation of stress responses

Figure 2. Relationship between EGFR and iron. Transferrin receptor 1 (TfR1) is responsible for the transport and internalization of iron bound to transferrin (Tf) and is brought into the cell via receptor-mediated endocytosis. After being transported to the endosome, iron is released from Tf and either utilized, stored, or exported out by the cell. Although the relationship between iron-bound transferrin receptor and EGFR is still not fully understood, it has been shown that EGFR may play a role in regulating iron homeostasis by redistributing TfR1 to the membrane to increase iron import for use by the cancer cell. In addition, it is believed that EGFR may play a role in iron metabolism through its association with the progesterone-receptor membrane component 1 (PGRMC1). PGRMC1 is able to form a dimeric structure with heme, and can bind directly to EGFR to promote tumor growth and proliferation.
and homeostasis (74), it has an important function as a tumor suppressor (76). Interestingly, the ability of MIG6 to inhibit tumor growth has also been linked to cellular iron depletion (27). Indeed, the high affinity iron chelators DFO and 311 both induce cellular iron depletion (77) and up-regulate MIG6 (27). Furthermore, it has also been demonstrated that \( \text{MIG6} \) is an HIF-1\( \alpha \)/HIF-2\( \alpha \) target gene, with this latter transcription factor directly binding to the \( \text{MIG6} \) promoter region, as confirmed by chromatin immunoprecipitation (ChIP) analysis (78, 79).

Considering its ability to promote EGFR degradation, MIG6 presents as a key anti-oncogenic molecule. In fact, MIG6 is able to increase EGFR internalization and trafficking to the lysosome (80). It is believed that EGFR endocytosis is initiated with the recruitment of c-Cbl by Grb-2 to further promote its lysosomal degradation (80). Furthermore, MIG6 can physically obstruct EGFR dimerization and bind to the proteins, syntaxin-8 and intersectin1/2, to foster lysosomal degradation (80). However, in tumor cells, the action of MIG6 to selectively target these receptors is hindered when it becomes phosphorylated.
ylated at Tyr-394 (Fig. 3) (81). A recent study showed that phosphorylation of MIG6 by EGFR at Tyr-394 and by Src at Tyr-395 can inhibit MIG6 activity (82). Hence, the ability of MIG6 to promote EGFR degradation is prevented under these conditions.

In light of the evidence that EGFR is regulated by NDRG1 (17), it is important to consider whether this metastasis suppressor causes its inhibition of EGFR by promoting the aforementioned mechanisms of degradation. It was recently demonstrated that in the presence of NDRG1, levels of EGFR monomer and dimer were both rapidly reduced in response to the ligand, EGF, suggesting that internalization and subsequent degradation of the receptor may occur (17).

We speculate here that NDRG1 may assist in the endosomal trafficking of EGFR to foster its degradation due to evidence of its role in processing of the low-density lipoprotein (LDL) receptor to the endosome (83). This was identified by a clear reduction of the LDL receptor at the cell surface and decreased accumulation in early endosomes in the absence of NDRG1 (83). Additionally, in NDRG1-depleted cells it was found that LDL receptor degradation was slowed, and this could be explained by NDRG1’s ability to interact with known regulators of degradation such as Rab GTPases (83). As such, the role of NDRG1 in LDL degradation could analogously be applied to EGFR, in ushering it to the endosome for eventual degradation.

Other metastasis suppressors

In addition to the metastasis suppressor, NDRG1, there are a host of other proteins that have been identified to perturb cancer progression by down-regulating molecules commonly implicated in metastasis (Fig. 4).

Although it is not as extensively studied as NDRG1, the Myc-repressed gene NDRG2, which also belongs to the NDRG family, has demonstrated tumor-suppressive functions in malignant carcinomas (84, 85). This was demonstrated by reduced overall survival of prostate cancer patients with low NDRG2 expression (84). The anti-tumorigenic activity of NDRG2 is mediated through its role in signal transduction pathways, whereby expression of NDRG2 inhibited STAT3 activation in a p38 MAPK-dependent manner, leading to decreased proliferation and survival of breast cancer cells (85). Similarly to NDRG1, NDRG2 is also regulated by iron levels, being up-regulated in response to iron depletion (86). In fact, studies examining hepatocellular carcinoma demonstrated that the iron chelator, Dp44mT (33), was able to up-regulate NDRG2, leading to reduced EMT and tumor metastasis via its effects on this latter molecule (86). Specifically, NDRG2 and Dp44mT reduced levels of the receptor gp130 and the activation of its downstream targets STAT3 and ERK1/2 (86).

The tetraspanin, KAI1, is another protein that acts as a metastasis suppressor through its ability to inhibit cancer cell motility and invasiveness (87). This is mediated by the ability of KAI1 to associate with proteins important for cell migration.
such as focal adhesion kinase (FAK) in its tetraspanin-enriched micro-domain, leading to the down-regulation of FAK function (87). This occurs by blocking the formation of the p130Cas–Crk complex, which is often described as a “molecular switch” for cell motility (88). In fact, as mentioned previously, NDRG1 is able to down-regulate p130Cas in a c-Src-dependent manner (15) and could therefore regulate KAII. Notably, it has been demonstrated that KAII is a downstream target of NDRG1 (89). Specifically, NDRG1 targets the ATF3 (activating transcription factor 3) (89), which can directly bind to the KAII promoter. Therefore, these results establish a functional connection between these two metastasis suppressors (89). Furthermore, loss of KAII expression is common in metastatic cancers (87), further supporting its significant role as a suppressor of metastasis.

Interestingly, KAII has been reported to modulate the activity of several receptor tyrosine kinases, including EGFR (90). The molecular mechanism involved was demonstrated to involve association of KAII with the EGFR membrane complex, resulting in more rapid clearance of the ligand-bound receptor from the cell’s surface (90). This event also led to a desensitization of the EGFR receptor, which is linked to an increased rate of receptor endocytosis (90).

Significantly, the classical tumor suppressor, p53, is able to increase the transcription of KAII (91). This occurs through a p53-responsive element and is of particular interest due to the known regulation of p53 by cellular iron depletion (91). In fact, HIF-1α, which is up-regulated upon iron depletion, leads to stabilization of transcriptionally active wild-type p53 (92). A link between p53, iron, and NDRG1 was also identified through the use of iron chelators (27). This latter study identified that p53 was necessary for the iron chelator-mediated up-regulation of NDRG1, with these agents failing to induce NDRG1 in p53-null H1299 cells (27). However, a more recent investigation has demonstrated that iron depletion markedly up-regulates NDRG1 irrespective of p53 status (93). This observation suggests that p53 may play some role in up-regulating NDRG1 under conditions of iron depletion depending on the cell type.

Iron-binding thiosemicarbazones that up-regulate NDRG1

Considering the aggressive nature of cancer, it is important to explore unique strategies for targeting molecules that function to inhibit key drivers of cancer progression and metastasis, such as the ErbB family of receptors. This can be achieved by agents such as the novel di-2-pyridylketone thiosemicarbazone (DpT) series, which bind cellular iron pools and potently up-regulate NDRG1 via HIF-1α-dependent and -independent mechanisms (21, 22).

These agents possess pronounced and selective anti-proliferative and anti-metastatic activity in vitro and in vivo (17, 18, 26, 28, 33, 86, 94, 95). The most active compounds of this series are Dp44mT and di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC), both of which have shown potency and selectivity in vitro and in vivo against a broad spectrum of cancer types (26, 94–96). Although Dp44mT showed evidence of cardiotoxicity at high, non-optimal doses in mice (26), the recently developed DpC analog demonstrated potent anti-tumor activity in vivo, with no evidence of toxicity even at much higher doses (28, 94, 95). DpC was also demonstrated to be highly potent against the aggressive pancreatic cancer in vivo, almost completely inhibiting tumor growth and being more effective than both Dp44mT and the current gold standard for pancreatic cancer treatment, gemcitabine (17, 28).

These compounds elicit their anti-cancer activity by binding intracellular metal ions such iron and copper, leading to the generation of cytotoxic reactive oxygen species (97–99). These agents also markedly up-regulate the metastasis suppressor, NDRG1 (21, 26, 28), which has been shown to play a major role in their anti-cancer activity (15, 16, 18, 23, 24).

Because of their ability to up-regulate NDRG1, these agents can also potently inhibit the EGFR, Src, WNT, FAK, and PI3K/Akt pathways (14–17, 25). In particular, regarding the ErbB family of receptors, it was recently shown that Dp44mT and DpC significantly reduced EGFR levels and inhibited its activation in response to EGF (17). Additionally, DpC decreased the levels and activation of the oncoproteins, HER2 and HER3 (17). This further supports the potential of these thiosemicarbazones as a novel strategy for the treatment of cancer.

Conclusions

It is clear that the iron-regulated metastasis suppressor NDRG1 plays a vital role in regulating oncogenic signaling. This was shown by NDRG1’s inhibition of the ErbB family of receptor tyrosine kinases, especially considering that they promote metastasis, a major factor in the death of cancer patients (17). In elucidating the link between NDRG1 and EGFR with iron, a scaffold for controlling and inevitably deterring EGFR-dependent cancers can be identified. Through examination of this intricate and complex relationship, we have prompted further investigation into understanding the mechanisms by which NDRG1 exerts its potent anti-cancer activity.

Acknowledgments—We acknowledge Kyung Chan Park and Leyla Fouani (Molecular Pharmacology and Pathology Program, Department of Pathology, University of Sydney) for their critical evaluation of the manuscript prior to submission.

References

1. Weigelt, B., Peterse, J. L., and van’t Veer, L. J. (2005) Breast cancer metastasis: markers and models. Nat. Rev. Cancer 5, 591–602
2. Fouani, L., Menezes, S. V., Paulson, M., Richardson, D. R., and Kovacevic, Z. (2017) Metals and metastasis: exploiting the role of metals in cancer metastasis to develop novel anti-metastatic agents. Pharmacol. Res. 115, 275–287
3. Fang, B. A., Kovaˇcevic, Ž., Park, K. C., Kalinowski, D. S., Jansson, P. J., Lane, D. J., Sahni, S., and Richardson, D. R. (2014) Molecular functions of the iron-regulated metastasis suppressor, NDRG1, and its potential as a molecular target for cancer therapy. Biochim. Biophys. Acta 1845, 1–19
4. Ellen, T. P., Ke, Q., Zhang, P., and Costa, M. (2008) NDRG1, a growth and cancer related gene: regulation of gene expression and function in normal and disease states. Carcinogenesis 29, 2–8
5. Kovacevic, Z., and Richardson, D. R. (2006) The metastasis suppressor, Ndrg-1: a new ally in the fight against cancer. Carcinogenesis 27, 2355–2366
6. Chua, M. S., Sun, H., Cheung, S. T., Mason, V., Higgins, J., Ross, D. T., Fan, S. T., and So, S. (2007) Overexpression of NDRG1 is an indicator of poor prognosis in hepatocellular carcinoma. Mod. Pathol. 20, 76–83
MINIREVIEW: Iron, NDRG1, and metastasis suppression

7. Nishio, S., Ushijima, K., Tsuda, N., Take moto, S., Kawano, K., Yamaguchi, T., Nishida, N., Kakuma, T., Tsuda, H., Kasamatsu, T., Sasaki, Y., Kage, M., K u wano, M., and Kamura, T. (2008) Cap43/NDRG1/Drg-1 is a molecular target for angiogenesis and a prognostic indicator in cervical adenocarcinoma. Cancer Lett. 264, 36–43

8. Ai, R., Sun, Y., Guo, Z., Wei, W., Zhou, L., Liu, F., Hendricks, D. T., Xu, Y., and Zhao, X. (2016) NDRG1 overexpression promotes the progression of esophageal squamous cell carcinoma through modulating Wnt signaling pathway. Cancer Biol. Ther. 17, 943–954

9. Kovacevic, Z., Fu, D., and Richardson, D. R. (2008) The iron-regulated metastasis suppressor, Ndrg-1: Identification of novel molecular targets. Biochim. Biophys. Acta 1728, 1981–1992

10. Kovacevic, Z., Sivagurunathan, S., Mangs, H., Chikhan i, S., Zhang, D., and Richardson, D. R. (2011) The metastasis suppressor, N-Myc downstream-regulated gene 1 (NDRG1), upregulates p21 via p53-independent mechanisms. Carcinogenesis 32, 732–740

11. Chen, J., Chen, J. K., Nagai, K., Plieth, D., Tan, M., Lee, T. C., Threadgill, D. W., Neilson, E. G., and Harris, R. C. (2012) EGFR signaling promotes TGFβ-dependent renal fibrosis. J. Am. Soc. Nephrol. 23, 215–224

12. Sun, J., Zhang, D., Bae, D. H., Sahni, S., Jansson, P., Zheng, Y., Zhao, Q., Yue, F., Zheng, M., Kovacevic, Z., and Richardson, D. R. (2013) Metastasis suppressor NDRG1 mediates its activity through signaling pathways and molecular motors. Carcinogenesis 34, 1943–1954

13. Kovacevic, Z., Chikhan i, S., Lui, G. Y., Sivagurunathan, S., and Richardson, D. R. (2013) The iron-regulated metastasis suppressor NDRG1 targets NEDD4L, PTEN, and SMAD4 and inhibits the PI3K and Ras signaling pathways. Antioxid. Redox Signal. 18, 874–887

14. Jin, R., Liu, W., Menezes, S., Yue, F., Zheng, M., Kovacevic, Z., and Richardson, D. R. (2014) The metastasis suppressor, NDRG1, modulates β-catenin phosphorylation and nuclear translocation by mechanisms involving FRAT1 and PAK4. J. Cell. Sci. 127, 3116–3130

15. Liu, W., Yue, F., Zheng, M., Merlot, A., Bae, D. H., Huang, M., Lane, D., Jansson, P., Lui, G. Y., Richardson, V., Sahni, S., Kalinowski, D., Kovacevic, Z., and Richardson, D. R. (2015) The proto-oncogene c-Src and its downstream signaling pathways are inhibited by the metastasis suppressor, NDRG1. Oncotarget 6, 8851–8874

16. Wang pu, X., Lu, J., Xi, R., Yue, F., Sahni, S., Park, K. C., Menezes, S., Huang, M. L., Zheng, M., Kovacevic, Z., and Richardson, D. R. (2016) Targeting the metastasis suppressor, N-Myc Downstream Regulated Gene-1, with novel di-2-pyridylketone thiosemicarbazones: suppression of tumor cell migration and cell-collagen adhesion by inhibiting focal adhesion kinase/paxillin signaling. Mol. Pharmacol. 89, 521–540

17. Kovacevic, Z., Menezes, S. V., Sahni, S., Kalinowski, D. S., Bae, D. H., Lane, D. J., and Richardson, D. R. (2016) The metastasis suppressor, N-Myc downstream-regulated gene-1 (NDRG1), down-regulates the ErBb family of receptors to inhibit downstream oncogenic signaling pathways. J. Biol. Chem. 291, 1029–1052

18. Liu, W., Xing, F., Iizumi-Gairani, M., Okuda, H., Watabe, M., Pai, S. K., Pandey, P. R., Hirota, S., Kobayashi, A., Mo, Y. Y., Fukuda, K., Li, Y., and Watabe, K. (2012) Role of ribonucleotide reductase in inhibition of hepatic adenoma growth in the N-myc transgenic mouse. Mol. Carcinogenesis 51, 30–38

19. Hagi st, S., Hüttmann, H., Möllmann, G., Helling, U., Kieslich, D., Kuner, R., Balaguer, S., Seitz, H. K., Poustka, A., and Mueller, S. (2009) In vitro-targeted gene identification in patients with hepatitis C using a genome-wide microarray technology. Hepatology 49, 378–386

20. Nyholm, S., Mann, G. I., Johannson, A. G., Bergeron, R. J., Gräslund, A., and Thelander, L. (1993) Role of ribonucleotide reductase in inhibition of mammary-cell growth by potent iron chelators. J. Biol. Chem. 268, 26200–26205

21. Kalinowski, D. S., and Richardson, D. R. (2005) The evolution of iron chelators for the treatment of iron overload disease and cancer. Pharmacol. Rev. 57, 547–583

22. Nagathihalli, N. S., and Merchant, N. B. (2012) Src-mediated regulation of E-cadherin and EMT in pancreatic cancer. Front. Biosci. 17, 2059–2069

23. Leu, T. H., and Maa, M. C. (2003) Functional implication of the interaction between EGF receptor and c-Src. Front. Biosci. 8, S28–S38
41. Cantley, L. C., and Neel, B. G. (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase AKT pathway. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 4240–4245

42. Domin, J., and Waterfield, M. D. (1997) Using structure to define the function of phosphoinositide 3-kinase family members. *FEBS Lett.* **410**, 91–95

43. Song, G., Ouyang, G., and Bao, S. (2005) The activation of Akt/PKB signaling pathway and cell survival. *J. Cell. Mol. Med.* **9**, 59–71

44. Wegiel, B., Bjartell, A., Cugil, Z., and Persson, J. L. (2008) Interleukin-6 induces STAT-3 and STAT-5 activation in prostate cancer; role of lateralsignaling. *Proteomics* **8**, 4540–4548

45. Lu, X. X., Cao, L. Y., Chen, X., Xiao, J., Zou, Y., and Chen, Q. (2016) PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase family members. *Cancer Lett.* **331–340

46. Ahmed, I. S., Rohe, H. J., Twist, K. E., and Craven, R. J. (2010) Pgrmc1 (Progesterone Receptor Membrane Component 1) associates with epidermal growth factor receptor and regulates erlotinib sensitivity. *J. Biol. Chem.* **285**, 24775–24782

47. Cahill, M. A., Jazayeri, J. A., Catalano, S. M., Toyokuni, S., Kovacevic, Z., and Richardson, D. R. (2016) The emerging role of progesterone receptor membrane component 1 (PGRMC1) in cancer biology. *Biochim. Biophys. Acta* **1866**, 339–349

48. Kabe, Y., Nakane, T., Koike, I., Yamamoto, T., Sugura, Y., Harada, E., Sugase, K., Shimamura, T., Ohmura, M., Muraoka, K., Yamamoto, A., Uchida, T., Iwata, S., Yamaguchi, Y., Krayukhina, E., et al. (2016) Haem-dependent dimerization of PGRMC1/Sigma-2 receptor facilitates cancer proliferation and chemoresistance. *Nat. Commun.* **7**, 11030–11042

49. Aizen, J., and Thomas, P. (2015) Role of Pgrmc1 in estrogen maintenance of meiotic arrest in zebrafish oocytes through GPER1/Gfr. *J. Endocrinol.* **225**, 59–68

50. Cahill, M. A., Jazayeri, J. A., Kovacevic, Z., and Richardson, D. R. (2016) PGRMC1 regulation by phosphorylation: potential new insights in controlling biological activity! *Oncotarget* **7**, 50822–50827

51. Mizuno, E., Iura, T., Mukai, A., Yoshimori, T., Kitamura, N., and Komada, M. (2005) Regulation of epidermal growth factor receptor down-regulation by UBPY-mediated dequiquitination at endosomes. *Mol. Biol. Cell* **16**, 5163–5174

52. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998) c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev.* **12**, 3663–3674

53. Galisteo, M. L., Dikic, I., Batzer, A. G., Landdon, W. Y., and Schlessinger, J. (1995) Tyrosine phosphorylation of the c-Cbl protooncogene protein product and association with epidermal growth-factor (Egf) receptor upon Egf stimulation. *J. Biol. Chem.* **270**, 20242–20245

54. Gur, G., Rubin, C., Katz, M., Amit, I., Citri, A., Nilsson, J., Amargiolo, N., Henriksson, R., Rechavi, G., Hedman, H., Wides, R., and Yarden, Y. (2004) LRIG1 restricts growth factor signaling by enhancing receptor ubiquitylation and degradation. *EMBO J.* **23**, 3270–3281

55. Laederich, M. B., Funes-Duran, M., Yen, L., Ingalla, E., Wu, X., Carraway, K. L., 3rd, and Sweeney, C. (2004) The leucine-rich repeat protein LRIG1 is a negative regulator of ErbB family receptor tyrosine kinases. *J. Biol. Chem.* **279**, 47050–47056

56. Anastasi, S., Sala, G., Huiqing, C., Caprini, E., Russo, G., Iacovelli, S., Lucini, F., Ingvarsson, S., and Segatto, O. (2005) Loss of RALT/MIG-6 expression in ERBB2-amplified breast carcinomas enhances ErbB-2 oncogenic potency and favors resistance to herceptin. *Oncogene* **24**, 4540–4548

57. Zhang, X., Pickin, K. A., Bose, R., Jura, N., Cole, P. A., and Kuriyan, J. (2007) Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface. *Nature* **450**, 741–744

58. Yu, X. D., Yang, R., and Leng, C. J. (2016) Truncation, modification, and optimization of MIG6(segment) (2) peptide to target lung cancer-related EGFR. *Comput. Biol. Chem.* **61**, 251–257

59. Chastan, T. B., Lovejoy, D. B., Watts, R. N., and Richardson, D. R. (2003) Examination of the antiproliferative activity of iron chelators: multiple cellular targets and the different mechanism of action of triapine com...
pared with desferrioxamine and the potent pyridoxal ionesicnolyl hydra-
zone analoge 311. Clin. Cancer Res. 9, 402–414
78. Saarikoski, S. T., Rivera, S. P., and Hankinson, O. (2002) Mitogen-induc-
gene 6 (MIG-6), adipophilin and tuftelin are inducible by hypoxia. FEBs Lett. 530, 186–190
79. Schödel, J., Okonomopoulos, S., Ragoussis, J., Pugh, C. W., Ratcliffe, P. J., and Mole, D. R. (2011) High-resolution genome-wide mapping of HIF-
binding sites by ChiP-seq. Blood 117, e207–e217
80. Frosi, Y., Anastasi, S., Ballarò, C., Vassilev, B., Bäck, N., Zelcer, N., and Ikonen, E. (2013) NDRG1 functions in LDL receptor trafficking by regulating endosomal recycling and degradation. J. Cell Biol. 197, 587–597
81. Wang, Z., Raines, L. L., Hoo, R. M., Roberson, H., Leahy, D. J., and Cole, P. A. (2013) Tyrosine phosphorylation of mig6 reduces its inhibition of the epidermal growth factor receptor. ACS Chem. Biol. 8, 2372–2376
82. Park, E., Kim, N., Ficarro, S. B., Zhang, Y., Lee, B. I., Cho, A., Kim, K., Park, A. K., Park, W. Y., Murray, B., Meyerson, M., Bittman, R., Marto, J. A., Cho, J., and Eck, M. J. (2015) Structure and mechanism of activity-based inhibition of the EGFR receptor by Mig6. Nat. Struct. Mol. Biol. 22, 703–711
83. Pietiäinen, V., Vassilev, B., Blom, T., Wang, W., Nelson, J., Bittman, R., Bäck, N., Zelcer, N., and Ikonen, E. (2013) NDRG1 functions in LDL receptor trafficking by regulating endosomal recycling and degradation. J. Cell Sci. 126, 3961–3971
84. Ren, G. F., Tang, L., Yang, A. Q., Jiang, W. W., and Huang, Y. M. (2014) Prognostic impact of NDRG2 and NDRG3 in prostate cancer patients undergoing radical prostatectomy. Histol. Histopathol. 29, 535–542
85. Park, Y., Shon, S. K., Kim, A., Kim, K. I., Yang, Y., Cho, D. H., Lee, M. S., and Lim, J. S. (2007) SOCS1 induced by NDRG2 expression negatively regulates STAT3 activation in breast cancer cells. Biochem. Biophys. Res. Commun. 363, 361–367
86. Wang, J., Yin, D., Xie, C., Zheng, T., Liang, Y., Hong, X., Lu, Z., Song, X., Song, R., Yang, H., Sun, B., Bhatta, N., Meng, X., Pan, S., Jiang, H., and Liu, L. (2014) The iron chelator Dp44mT inhibits hepatocellular carcinoma metastasis via N-Myc downstream-regulated gene 2 (NDRG2)/gp130/STAT3 pathway. Oncotarget 5, 8478–8491
87. Liu, W. M., and Zhang, X. A. (2006) KA11/CD82, a tumor metastasis suppressor. Cancer Lett. 240, 183–194
88. Zhang, X. A., He, B., Zhou, B., and Liu, L. (2003) Requirement of the p130Cas-Crk coupling for metastasis suppressor KA11/CD82-mediated inhibition of cell migration. J. Biol. Chem. 278, 23739–23738
89. Liu, W., Iizumi-Gairani, M., Okuda, H., Kobayashi, A., Watabe, M., Pai, S. K., Pandey, P. R., Xing, F., Fukuda, K., Modur, V., Hirota, S., Suzuki, K., Chiba, T., Endo, M., Sugai, T., and Watabe, K. (2011) KA11 gene is engaged in NDRG1 gene-mediated metastasis suppression through the ATF3-NFκB complex in human prostate cancer. J. Biol. Chem. 286, 18849–18859
90. Odintsova, E., Sugiu, T., and Berditchevski, F. (2000) Attenuation of EGFR receptor signaling by a metastasis suppressor, the tetraspanin CD82/KAI-1. Curr. Biol. 10, 1009–1012
91. Mashimo, T., Watabe, M., Hirota, S., Hosobe, S., Miura, K., Tegtmeyer, P. J., Rinker-Saeffer, C. W., and Watabe, K. (1998) The expression of the KA11 gene, a tumor metastasis suppressor, is directly activated by p53. Proc. Natl. Acad. Sci. U.S.A. 95, 11307–11311
92. An, W. G., Kanekal, M., Simon, M. C., Maltepe, E., Blagosklonny, M. V., and Neckers, L. M. (1998) Stabilization of wild-type p53 by hypoxia-inducible factor 1α. Nature 392, 405–408
93. Moussa, R. S., Kovacevic, Z., and Richardson, D. R. (2015) Differential targeting of the cyclin-dependent kinase inhibitor, p21(CIP1/WAF1), by chelators with anti-proliferative activity in a range of tumor cell-types. Oncotarget 6, 29694–29711
94. Lovejoy, D. B., Sharp, D. M., Seebacher, N., Obeidy, P., Prichard, T., Stefani, C., Basha, M. T., Sharpe, P. C., Jansson, P. J., Kalinowski, D. S., Bernhardt, P. V., and Richardson, D. R. (2012) Novel second-generation di-2-pyridyldiketone thiosemicarbazones show synergism with standard chemotherapeutics and demonstrate potent activity against lung cancer xenografts after oral and intravenous administration in vivo. J. Med. Chem. 55, 7230–7244
95. Guo, Z. L., Richardson, D. R., Kalinowski, D. S., Kovacevic, Z., Tan-Un, K. C., and Chan, G. C. (2016) The novel thiosemicarbazone, di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC), inhibits nevoblastoma growth in vitro and in vivo via multiple mechanisms. J. Hematol. Oncol. 9, 98–113
96. Kalinowski, D. S., and Richardson, D. R. (2007) Future of toxicology—iron chelators and differing modes of action and toxicity: the changing face of iron chelation therapy. Chem. Res. Toxicol. 20, 715–720
97. Richardson, D. R., Sharpe, P. C., Lovejoy, D. B., Senaratne, D., Kalinowski, D. S., Islam, M., and Bernhardt, P. V. (2006) Dipyridyl thiosemicarbazone chelators with potent and selective antitumor activity form iron complexes with redox activity. J. Med. Chem. 49, 6510–6521
98. Kalinowski, D. S., Sharpe, P. C., Bernhardt, P. V., and Richardson, D. R. (2007) Design, synthesis, and characterization of new iron chelators with anti-proliferative activity: structure-activity relationships of novel thiohydrazido analogues. J. Med. Chem. 50, 6212–6225
99. Lovejoy, D. B., Jansson, P. J., Brunk, U. T., Wong, J., Ponka, P., and Richardson, D. R. (2011) Antitumor activity of metal-chelating compound Dp44mT is mediated by formation of a redox-active copper complex that accumulates in lysosomes. Cancer Res. 71, 5871–5880