REVIEW

Metastasis suppressor, NDRG1, mediates its activity through signaling pathways and molecular motors

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The metastasis suppressor, N-myc downstream regulated gene
1 (*NDRG1*), is negatively correlated with tumor progression in
multiple neoplasms, being a promising new target for cancer
treatment. However, the precise molecular effects of *NDRG1*
remain unclear. Herein, we summarize recent advances in
understanding the impact of *NDRG1* on cancer metastasis with
emphasis on its interactions with the key oncogenic nuclear
factor-kappaB, phosphatidylinositol-3 kinase/phosphorylated
AKT/mammalian target of rapamycin and Ras/Raf/mitogen-
activated protein kinase kinase/extracellular signal-regulated
kinase signaling pathways. Recent studies demonstrating the
inhibitory effects of *NDRG1* on the epithelial–mesenchymal
transition, a key initial step in metastasis, TGF-β pathway and
the Wnt/β-catenin pathway are also described. Furthermore,
NDRG1 was also demonstrated to regulate molecular motors
in cancer cells, leading to inhibition of F-actin polymerization,
stress fiber formation and subsequent reduction of cancer cell
migration. Collectively, this review summarizes the underlying
molecular mechanisms of the antimetastatic effects of *NDRG1*
in cancer cells.

The metastasis suppressor, N-myc downstream regulated gene 1

*N-myc downstream regulated gene 1 gene and regulation of its
expression*

N-myc downstream regulated gene 1 (*NDRG1*) (also known as *Drg1*,
RTP, *Rit42*, *PROXY-1* or *Cap43*) encodes a metastasis suppressor in a
number of cancers including colon, prostate and breast cancers (1–3).

Abbreviations: Arp 2/3, actin-related protein 2/3; ATF3, activating transcrip-
tion factor 3; EMT, epithelial–mesenchymal transition; ERK, extracellular
signal-regulated kinase; GFR, growth factor receptor; GSK3, glycogen syn-
thase kinase 3; GTP, guanosine triphosphate; HIF-1, hypoxia inducible factor
1; IKK, IκB kinase; LRP, lipoprotein receptor-related protein; MEK, mitogen-
activated protein kinase kinase; mRNA, messenger RNA; mTOR, mammalian
target of rapamycin; NDRG1, N-myc downstream regulated gene 1; NF-κB,
nuclear factor-kappaB; pAKT, phosphorylated AKT; PIP2, phosphatidylino-
sitol-2-disphosphate; PIP3, phosphatidylinositol-3-trisphosphate; PI3K, phos-
phatidylinositol-3 kinase; PTEN, phosphatase and tensin homolog; Rho, Ras
homolog; TβRI and II, type I and type II TGF-β receptors; TCF, T-cell fac-
tor; TGF-β, transforming growth factor-β; TSC, tuberous sclerosis complex;
WASP, Wiskott–Aldrich syndrome family protein.

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Homologs of this gene have been discovered in mice (NDR1 and
TDD5), *Caenorhabditis elegans* (ZK1073.1) and sunflowers (SF21),
which suggest that *NDRG1* is highly conserved between species (1,4).

The *NDRG1* gene has been mapped to chromosome 8q24.3 (8q24.2
in other studies) (5), which is approximately 60 kb in length (1,5,6).
This gene encodes a 3.0 kb messenger RNA (mRNA) that itself
encodes a very stable, 43 kDa NDRG1 protein (composed of 394
amino acids), which is highly conserved among multicellular organ-
isms (1,6–8). *NDRG1* contains a CpG island in its promoter and may
therefore be regulated by methylation (9).

The *NDRG1* gene is one of the members of the human NDRG
family, which comprises *NDRG2*, *NDRG3* and *NDRG4* that share a
53–64% homology with each other (7,10–12). The *NDRG1* mRNA is
ubiquitously expressed in most human tissues, but its levels are high
in the prostate, brain, kidney, placental and intestinal tissues (1,7).
However, the NDRG1 protein is mainly found in epithelial cells,
which may suggest that it has a specific function (1). Interestingly,
NDRG1 is also highly expressed in primary granulocytes (13). It is
well known that *NDRG2* is strongly expressed in the brain, heart,
skeletal muscle and the spinal cord, whereas its expression in other
organs was weak (7). The *NDRG3* gene is expressed in all organs but
more so in the testis (7). The *NDRG4* gene is uniquely expressed in
the brain and heart, with a number of isoforms being reported (i.e.
NDRG4, NDRG-4Bvar and NDRG-4H) (14). The *NDRG4B* mRNA
is detected in the brain only, whereas *NDRG4H* mRNA is more abun-
dant in the heart than in the brain (14).

The expression of the *NDRG1* gene is regulated by nickel com-
pounds, cobalt, hypoxia, oxidative stress, vitamins A and D, phorbol
esters, steroids, histone deacetylase-targeting drugs, homocysteine,
β-mercaptoethanol, tunicamycin, lysophosphatidylcholine, synthetic
retinoids, oncogene (N-myc/c-myc), tumor suppressor gene (p53 and
von Hippel-Lindau) and compounds that promote cell differentiation
(10,15,16). *NDRG1* was once known as 'reducing agents tunicamycin-
responsive protein' as it is upregulated by homocysteine, tunicamycin
and β-mercaptoethanol and is involved in the proteasome-mediated
endoplasmic reticulum-associated degradation of misfolded proteins
(17,18). This was supported by reports of its proteasomal localization
and interaction with endoplasmic reticulum chaperons and proteaso-
mal subunits (18). Overexpression of N-myc or c-myc downregulates
NDRG1 expression (1,19). The tumor suppressor gene, von Hippel-
Lindau has also been shown to downregulate *NDRG1* expression in
renal cancer cells by targeting hypoxia inducible factor 1 (HIF-1)
for degradation (1,20). On the other hand, hypoxia induces *NDRG1*
expression through an early growth response 1 binding motif present in
the *NDRG1* promoter (10). The *NDRG1* gene has three HIF-1 binding
sites, where one is situated at its promoter and the remaining two in its
3′ untranslated region (21). This implies that NDRG1 can be regulated
by HIF-1 through its binding sites in the untranslated region (22).

Protein structure of NDRG1

The NDRG family of proteins is included within the α/β hydrolase
group of enzymes, despite the fact that the NDRG1 protein lacks
a hydrolytic catalytic site and is deficient in enzyme function
(7,17,23,24). This suggests that the gene has been modified by
convergent evolution to obtain the same fold for a specific purpose (1).
The amino acid sequence of NDRG1 encodes three tandem repeats
of 10 hydrophilic amino acids (GTRSRSHTSE) in the C-terminal
(COOH) region that is involved in the binding of heavy metal ions
like nickel and copper (1,15,25,26). It lacks a transmembrane domain,
signal sequence or endoplasmic reticulum sequence (1,5).

Additionally, NDRG1 is a multiphosphorylated protein that contains five calmodulin kinase 2 phosphorylation sites, three serine phosphorylation sites, a casein kinase II site, five myristoylation sites, three protein kinase C phosphorylation sites, one thioesterase site, one tyrosine phosphorylation site and one phosphopantotheine attachment site (27). Phosphorylation of NDRG1 is mediated through serum/glucocorticoid regulated kinase 1 and glycogen synthase kinase 3 (GSK3) (which are essential Ser/Thr kinase family proteins) (28). The role of phosphorylation is uncertain, but may be related to the numerous physiological functions of NDRG1 (29). Indeed, a recent study demonstrated that the phosphorylation of NDRG1 was spatially and temporally controlled during the cell cycle (30). In fact, phosphorylated NDRG1 was found to co-localize with centromeres and β-tubulin bundles during cytokinesis (30). This indicates a functional role for phosphorylated NDRG1 in centromere function (30).

The N-terminus of NDRG1 consists of two myc boxes (MBI and MBII), which are crucial for the protein's function, whereas the central region consists of MBIII (involved in cell transformation and apoptosis) and MBIV (for apoptosis induction, transformation and G_2 arrest) (7). The existence of multifunctional domains in the NDRG1 sequence may implicate its many functional roles in cell biology. Currently, there is no evidence that NDRG1 acts as a transcription factor itself, as the sequence of NDRG1 is not indicative of a transcription factor and it also lacks a nuclear targeting sequence (31). However, it may affect the function of other transcription factors such as nuclear factor-kappaB (NF-κB), mothers against decapentaplegic homolog 4 (Smad4), and so on and these will be further discussed in the below section.

Distribution of NDRG1 in cells and tissues

Primarily, NDRG1 is a cytoplasmic protein expressed mostly in epithelial cells but its cellular localization depends upon the cell type (1,31). For intestinal and lactating breast epithelial cells, NDRG1 is associated with the plasma membrane; for prostate epithelial cells, NDRG1 is mainly localized to the nucleus; whereas for kidney proximal tubule cells, NDRG1 is associated with the mitochondrial inner membrane (1,31). This suggests that NDRG1 functions in a tissue-specific manner (1,31). However, other studies dispute this claim suggesting NDRG1 is not tissue-specific and claim that the protein is present in a wide variety of tissues (7,32). Analysis using the PSORTII software also predicts that NDRG1 is primarily a cytoplasmic protein (47.8%) followed by the nucleus (26.1%) and the mitochondrion (8.7%) (1). This suggests that NDRG1 has pleiotropic functions by possibly interacting with other proteins (33).

In certain cell types, the *NDRG1* gene is restricted to the nucleus despite lacking any nuclear localization signals in its protein structure (33). This indicates that interactions with nuclear protein(s) are necessary for the nuclear localization of this protein (34). One such nucleo-cytoplasmic transport protein, the heat-shock cognate protein (Hsc70) found in mast cells, was shown to associate with *NDRG1* allowing the gene to enter the nucleus (35). Furthermore, when DNA is damaged by DNA-damaging agents such as actinomycin D, NDRG1 is transcriptionally upregulated and translocates from cytoplasm to the nucleus (36,37). This may suggest that NDRG1 has a role in DNA repair and is acting as a stress response gene (1). The possible role of NDRG1 was inferred from transcription factors such as p53, which also translocates to the nucleus in response to DNA damage (38).

The membrane-associated NDRG1 protein was mostly found adjacent to adherens junctions, intermediate and microfilament bundles, which insert into these structures (31). This suggests that NDRG1 may play an important role in cell adhesion and motility by directly influencing other molecules involved in this processes, such as E-cadherin (1,31). The role of NDRG1 in cell adhesion and motility will be further examined in section 'NDRG1 and its effect on molecular motors involved in cancer cell migration'.

Expression in cancer

One of the most intensively studied functions of NDRG1 is its involvement in inhibiting cancer progression and metastasis (1). In particular,

NDRG1 mRNA and protein levels were found to be decreased in tumor tissues when compared with normal tissues (1). In fact, over-expression of NDRG1 in cancer cells may not only induce cellular differentiation (12) but also reduce invasion (39). Several studies have also reported a positive correlation between NDRG1 expression and patient survival, indicating that NDRG1 may be a prognostic bio-marker in cancer patients (5). Indeed, accumulating evidence shows that NDRG1 is negatively correlated with tumor progression and functions as a potent metastasis suppressor (39,40).

Paradoxically, some studies demonstrated that NDRG1 is highly expressed in hepatocellular carcinoma (10) and cervical cancer (34), with its levels being positively associated with vascular invasion, metastasis and poor prognosis. In these studies, silencing NDRG1 reduced proliferation, invasion and apoptosis *in vitro* while inhibiting tumor growth *in vivo* (10,34,41). Although genetic mutations of NDRG1 in these tissues could not be excluded, these latter findings may imply a cancer-type-dependent function of NDRG1.

Mechanism of action of NDRG1

Mechanistic studies at the molecular level revealed that NDRG1 can regulate key signaling pathways involved in oncogenesis, including: (i) the NF-κB pathway (42); (ii) the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling network (39); (iii) Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway (39); (iv) transforming growth factor-β (TGF-β) network (39) and (v) the Wnt pathway (40). Consequently, NDRG1 could interrupt many metastasis-associated biological functions, including the epithelial–mesenchymal transition (EMT), migration and invasion (39,40). Therefore, NDRG1 may act as a central regulator of multiple signaling pathways that modulate tumor progression. This review will focus on the recently described regulatory functions of NDRG1 in cancer cell signaling, particularly its role in regulating cancer metastasis through affecting key molecular effectors.

NDRG1 interacts with key signaling cascades

Recent studies have identified that NDRG1 interacts with the NF-κB (42,43) (Figure 1), PI3K/AKT/mTOR (39,44–47) (Figure 2) and the Ras/Raf/MEK/ERK pathways (39,47) (Figure 3). These interactions are outlined in the following sections.

NF-κB signaling pathway

The NF-κB signaling pathway plays a major role in metastasis, with NF-κB activation being tightly regulated *via* its localization (48) (Figure 1). In resting phase cells, the Rel-like domain-containing proteins, including RelA, NF-κB1/p105, NF-κB1/p50, RelB, NF-κB2/p100 and NF-κB2/p52, form homo- or hetero-dimeric NF-κB complexes in the cytoplasm (49). The NF-κB proteins are bound by inhibitors of NF-κB (IκB), including IκBα, IκBβ and IκBε, which inhibit the entry of the NF-κB complex into the nucleus (48,49).

When the membrane receptors (e.g. tumor necrosis factor receptor/tumor necrosis factor receptor-associated factor 3, lymphotoxin β receptor) are triggered, NF-κB signaling becomes activated and exerts its downstream effects through the classical (canonical) pathway initiated by NF-κB1 (p50/p105) and an alternative (non-canonical) pathway initiated by NF-κB2 (p52/p100) (48) (Figure 1). The canonical pathway involves activation of the IκB kinase (IKK) complex including IKKα, IKKβ and IKKγ (NEMO) (48) (Figure 1). Activation of the IKK complex leads to phosphorylation of IκB, which is then ubiquitinated and degraded through the 26S proteasome in the cytoplasm, leading to NF-κB1/p105 cleavage into NF-κB1/p50. NF-κB1/p50 binds to RelA and translocates into the nucleus (48) (Figure 1). On the other hand, the non-canonical pathway involves NF-κB-inducible kinase that mediates phosphorylation of NF-κB2/p100, leading to its cleavage into the p52 subunit. The cleaved p52 binds to RelB and translocates to the nucleus (48) (Figure 1). Once in the nucleus, RelA/p50 and RelB/p52 undergo a series of post-translational modifications including phosphorylation,
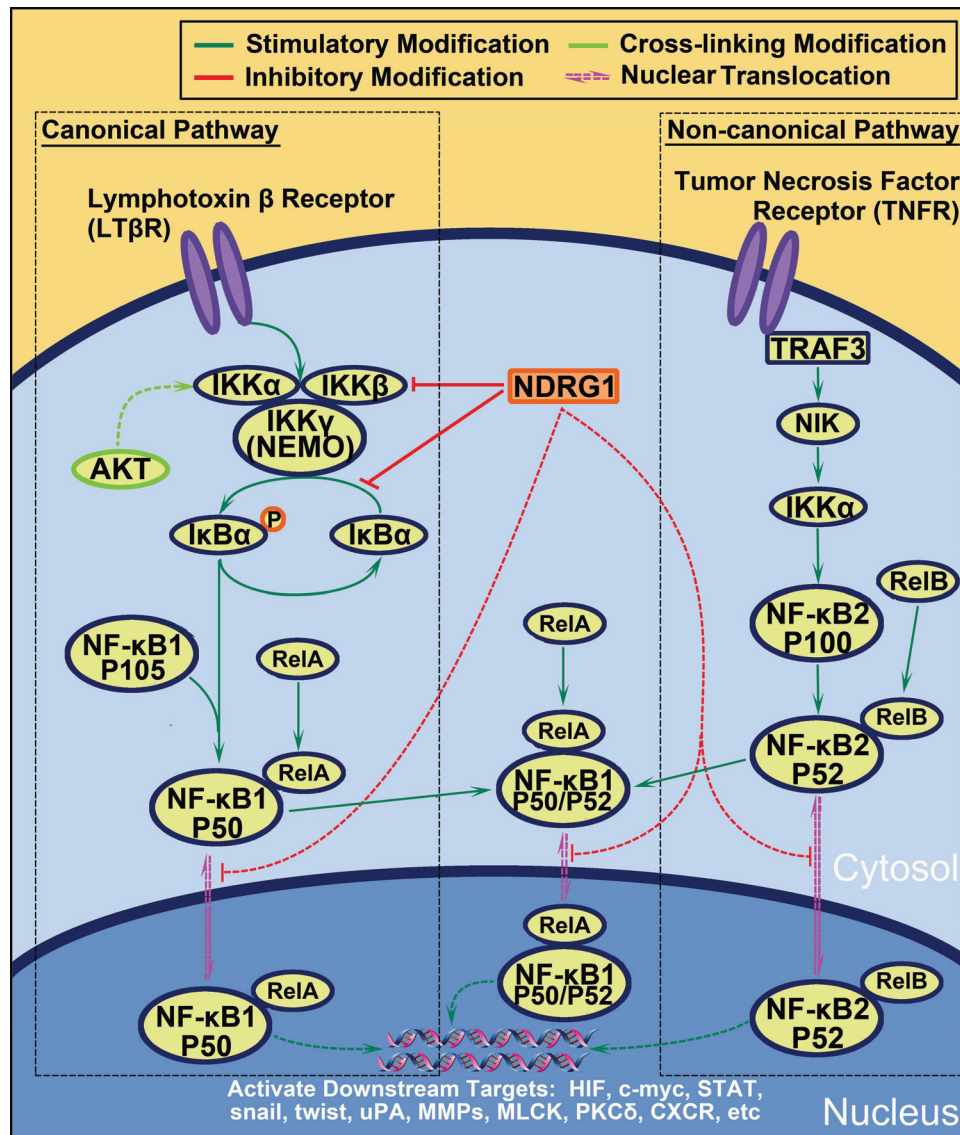
Fig. 1. NDRG1 is involved in NF-κB signaling for cancer metastasis. The NF-κB signaling pathway is involved in cancer metastasis. The canonical pathway involves activation of the IKK complex including IKKα, IKKβ and IKKγ (NEMO). Inhibitor of NF-κB (IκB) is phosphorylated, ubiquitinated and degraded in the cytoplasm, releasing NF-κB1/p105, which gets cleaved into NF-κB1/p50. NF-κB1/p50 binds to RelA and translocates into the nucleus, where it initiates transcription of gene products involved in metastasis. The alternative (non-canonical) pathway involves NF-κB-inducible kinase-initiated IKKα-mediated phosphorylation of NF-κB2/p100, leading to proteasome processing and creation of the NF-κB2/p52 subunit. The RelB/NF-κB2/p52 heterodimer then translocates into the nucleus to bind the promoter of genes whose products are important in metastasis. NDRG1 can attenuate the nuclear translocation of RelA and NF-κB1/p50 and their binding to the NF-κB binding motif by markedly suppressing the expression of IKKβ and also by blocking IκBα phosphorylation. Full lines represent direct modifications. Dashed lines represent indirect modifications.

acetylation and methylation followed by binding to specific κB sites to activate NF-κB target genes (48) (Figure 1). The NF-κB signaling pathway modulates the expression of a series of transcription factors, such as HIF-1 (50), c-Myc (51), signal transducers and activators of transcription (52), Snail (53) and Twist (54), all of which have significant roles in cancer metastasis (Figure 1).

NF-κB mediates cell adhesion through regulating urokinase-type plasminogen activator (55), matrix metalloproteinase/tissue inhibitor of metalloproteinase complexes (56), intercellular adhesion molecule-1/vascular adhesion molecule-1 (57), fibronectin (58) and caveolin 1 (59). Interestingly, NF-κB also activates stress fibers through myosin light chain (MLC) kinase, leading to increased cell migration (60). Other NF-κB targets include protein kinase C δ (40), p21 (61), vimentin (62) and the CXC chemokine receptor (63).

Recently, it was reported that NDRG1 can attenuate the nuclear translocation of RelA/p50 and its binding to the NF-κB motif by

blocking IκBα phosphorylation (43). This was found to occur as a result of suppressed IKKβ levels, which were downregulated by NDRG1 (Figure 1) *via* increasing the ubiquitination and proteasomal degradation of IKKβ (43). As a result, NDRG1 has further downstream effects on activating transcription factor 3 (ATF3), which also contains the NF-κB motif (42). The suppressed levels of ATF3 in prostate cancer cells were correlated with increased levels of the metastasis suppressor KAI-1, which was markedly upregulated in response to NDRG1 (42). Indeed, *KAI-1* is transcriptionally suppressed by ATF3 binding to its promoter (42). Hence, the ability of NDRG1 to mediate degradation of IKKβ has a 'ripple effect' on a number of downstream targets of the NF-κB pathway including IκBα, ATF3 and subsequently, KAI-1, which was found to be crucial for the antimetastatic effects of NDRG1 (42).

Interestingly, a recent study has reported that the phosphorylation of NDRG1 at Ser330 and Thr346 by serum/glucocorticoid regulated
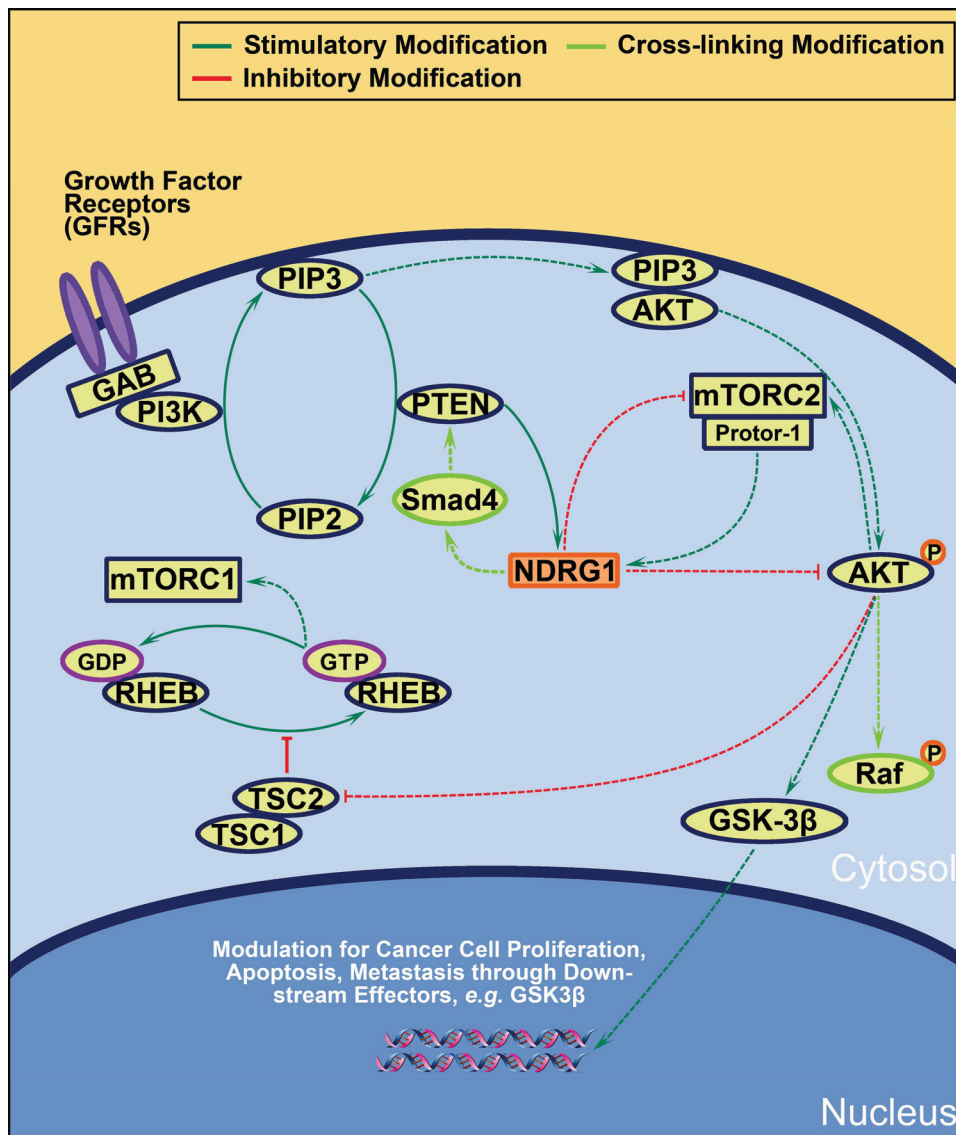
Fig. 2. NDRG1 is involved in PI3K/AKT/mTOR signaling for cancer metastasis. Activated PI3K phosphorylates PIP2 to generate membrane-bound PIP3, leading to AKT phosphorylation and activation. Phosphorylated AKT then activates downstream targets that promote metastasis. The lipid phosphatase PTEN dephosphorylates PIP3 to PIP2, leading to inhibition of AKT activity. A close link between NDRG1 and PTEN was demonstrated. Overexpression of PTEN significantly upregulates NDRG1, whereas NDRG1 can also increase PTEN protein expression in pancreatic cancer cells, suggesting a positive feedback loop between these two molecules. Moreover, NDRG1 also reduces expressions of pAKT and mTOR. Full lines represent direct modifications. Dashed lines represent indirect modifications.

kinase 1 was essential for it to inhibit the NF-κB pathway (64). This observation suggests that the functional roles of NDRG1 may be dictated by its phosphorylation, with certain phosphorylation states being able to activate this metastasis suppressor.

PI3K/AKT/mTOR signaling

The PI3K/AKT/mTOR signaling pathway regulates the activities of a broad range of downstream molecular effectors that modulate the metastatic behavior of cancer cells (65) (Figure 2). There are three classes of PI3K enzymes, among which class I is the most intensively investigated. Class I PI3K enzymes include p110α, β, γ and δ catalytic isoforms, which are controlled by coupling with their respective regulatory isoforms (p85 and p110) to affect their lipid kinase activity (66). Once activated by membrane receptors [e.g. growth factor receptors (GFRs)], the binding proteins (e.g. GFR-bound protein) cause PI3K enzymes to catalyze the phosphorylation of phosphoinositides, resulting in the generation of

phosphatidylinositol-3-trisphosphate (PIP3) from phosphatidylinositol-2-disphosphate (PIP2) (67) (Figure 2).

PIP3 acts as docking site for the recruitment of proteins with a pleckstrin homology domain such as phosphoinositide-dependent kinase 1 and AKT at the plasma membrane (Figure 2) (68). Activation of AKT by phosphorylation results in phosphorylation of its downstream targets, including mTOR (Figure 2). The mTORs are a family of Ser/Thr kinases involved in the PI3K pathway, which exist in two multiprotein complexes: namely, mTOR complex 1 and 2 (mTORC1 and mTORC2) (69). The phosphorylated AKT (pAKT) can also inhibit the tuberous sclerosis complex (TSC). Through its guanosine triphosphatase (GTPase)-inactivating protein activity toward Ras homolog (Rho) enriched in brain, the TSC1–TSC2 complex inhibits mTORC1 (70). Hence, pAKT promotes the activity of both mTORC1 and mTORC2.

Once activated, AKT regulates a wide array of downstream effectors that ultimately lead to cancer metastasis (Figure 2). For example, pAKT modulates GSK3-mediated cancer proliferation, inhibits apoptosis and promotes migration and invasion (65). This cascade

can be antagonized by the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a tumor suppressor that dephosphorylates PIP3 back to PIP2 and leads to decreased pAKT levels (65) (Figure 2).

Bandyopadhyay *et al.* (44) demonstrated a link between NDRG1 and PTEN. These investigators observed that overexpression of PTEN significantly upregulates the endogenous expression of NDRG1, whereas inhibition of PTEN by small interfering RNA decreases NDRG1 levels in a dose- and time-dependent manner. Interestingly, this latter study also revealed that the addition of a PI3K inhibitor, LY-294002, restored NDRG1 expression, indicating that PTEN controls NDRG1 by a PI3K/AKT-dependent pathway (44). On the other hand, NDRG1 can also increase PTEN protein expression in pancreatic cancer cells, suggesting a positive feedback loop between these two molecules (47) (Figure 2). The mechanism by which NDRG1 increases PTEN expression remains to be elucidated, although recent studies have demonstrated that NDRG1 does not increase *PTEN* mRNA levels (47). This observation suggests a post-transcriptional effect such as increased protein stability or reduced proteasomal degradation of PTEN.

Recently, we also demonstrated that NDRG1 reduces the expression of pAKT and its downstream target mTOR, and this is likely to occur as a result of the increased PTEN expression that inhibits pAKT (65). This finding further establishes the mechanistic interaction between NDRG1 and the PI3K-centered signaling network (Figure 2) (71).

NDRG1 also increased the expression of the key tumor suppressor, Smad4, in pancreatic cancer cells, although the mechanisms behind this effect remain to be established (39). Increased Smad4 expression can abolish the TGF-β-mediated suppression of PTEN, allowing this latter protein to inhibit PI3K/pAKT signaling (45). Interestingly, NDRG1 is also linked with a component of mTORC2, namely Protor-1, with mice bearing a Protor-1 knockout having markedly reduced NDRG1 levels in kidney tissues (46). Hence, NDRG1 may have multiple interactions with the PI3K/AKT/mTOR pathway, which could contribute to its antimetastatic effects (Figure 2).

Ras/Raf/MEK/ERK signaling

The Ras/Raf/MEK/ERK signaling pathway also has key roles in the transmission of proliferative signals from membrane-bound receptors (72) (Figure 3). Ras becomes activated after growth factor stimulation of corresponding receptors (e.g. receptor tyrosine kinases and insulin-like growth factor 1 receptor), which stimulates the GFR-bound protein 2/guanine nucleotide exchange factor Son of Sevenless complex (73,74).
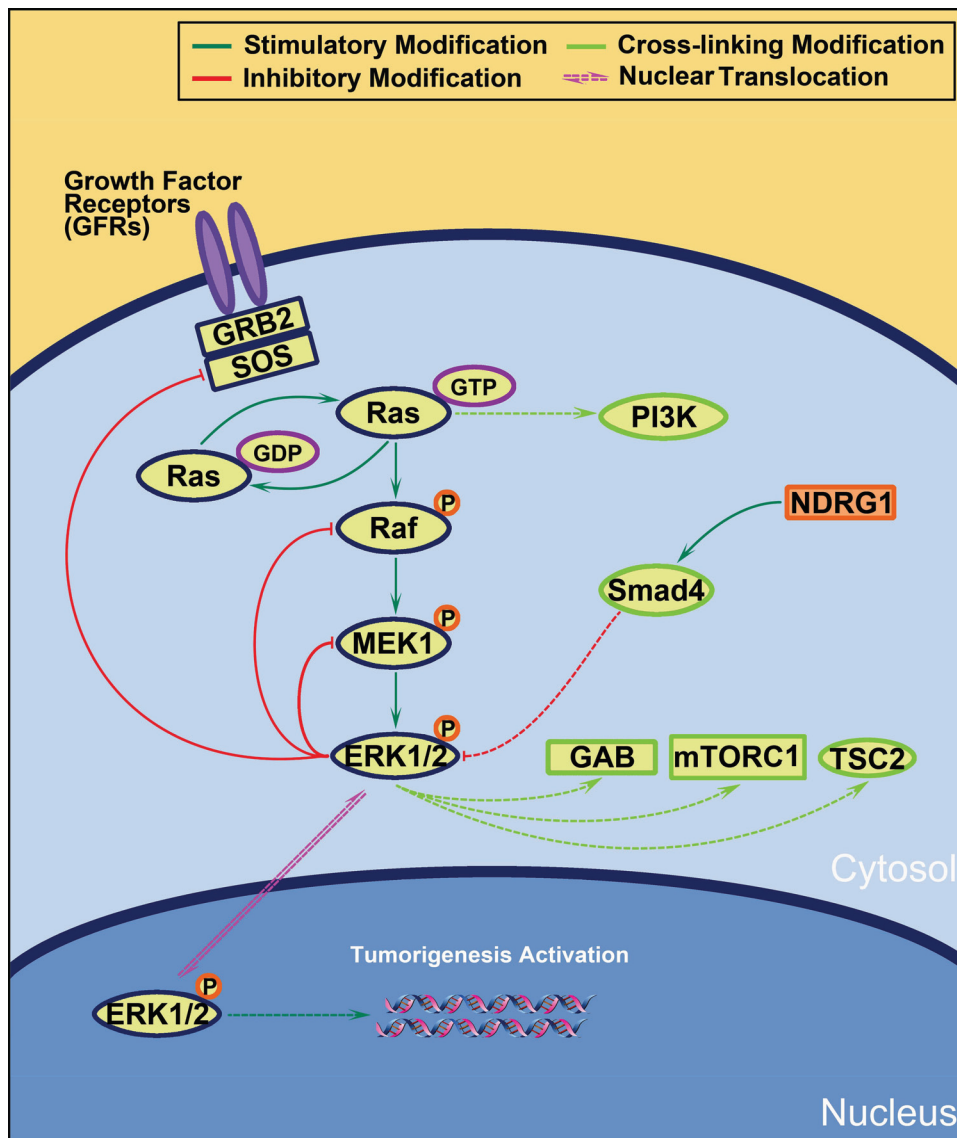
Fig. 3. NDRG1 is involved in Raf/MEK1/ERK signaling for cancer metastasis. Activation of GFRs triggers the binding of Ras with GTP. The active Ras:GTP then activates Raf/MEK1/ERK1/2 *via* phosphorylation, leading to activation of downstream targets. NDRG1 inhibits this pathway *via* the upregulation of Smad4. Full lines represent direct modifications. Dashed lines represent indirect modifications.

Once activated by GFR-bound protein 2/Son of Sevenless, Ras is triggered to bind with GTP, functioning as an guanosine diphosphate/GTP-regulated binary on-off switch that regulates cytoplasmic signal transduction (75) (Figure 3). The active Ras:GTP activates Raf *via* phosphorylation (76). Raf is then responsible for serine/threonine phosphorylation of MEK1, allowing this latter molecule to phosphorylate ERK1 and 2, leading to phosphorylation and activation of downstream cascades (Figure 3) (77).

In some cancer cells, Ras proteins contain single amino acid mutations (commonly at residuals 12, 13 or 61) that cause it to become persistently GTP bound and active, resulting in continuous activation of the downstream signaling cascade (78). In addition, loss of Smad4 expression enhances Ras signaling that can lead to progression to undifferentiated carcinoma (79). Interestingly, overexpression of NDRG1 increases Smad4 levels, which subsequently inhibits ERK phosphorylation (47). This indicates the involvement of NDRG1 in modulating the Ras/Raf/MEK/ERK pathway (Figure 3).

The three signaling pathways described above, including Ras/Raf/MEK/ERK, PI3K/AKT/mTOR and NF-κB pathways, interact to form a network that decides cell fate. The Ras/Raf/MEK/ERK and PI3K/AKT/mTOR pathways are able to cross-inhibit and cross-activate each other (80). In addition, while one pathway is blocked, the others can be effectively activated (80). This signaling co-ordination mainly involves GFR-bound protein phosphorylation by ERK (Figure 3) and Raf phosphorylation by AKT (Figure 2) (81,82). Components of the Ras/Raf/MEK/ERK pathway also positively regulate the PI3K/AKT/mTOR pathway through key integration points such as tuberous sclerosis protein 2 (TSC2) and mTOR1 (Figures 2 and 3) (83). NF-κB signaling is also targeted by the PI3K/AKT pathway (84). In fact, the activation of PI3K/AKT signaling could trigger the activation of NF-κB through the canonical pathway (84) (Figure 1). As NDRG1 is able to modulate each of these pathways, we hypothesize that NDRG1 could act as a regulatory molecule that bridges signaling networks.

NDRG1 affects the EMT

The EMT is a highly conserved process controlled by a series of transcription factors that lead epithelial-originated cells to undergo a shift to a mesenchymal phenotype (85). Cells that undergo EMT are characterized typically by a phenotype change from a polygonal/columnar shape with apico-basolateral polarization into a spindle shape with anterior–posterior polarization, leading to enhanced migratory potential (85).

Notably, EMT is a well-organized process, with one of the earliest events involving the disruption of tight junctions that connect epithelial cells and de-localization of tight junction proteins, including zonula occludens 1, claudin 1 and occludin (86). Adherens junction complexes, which contain E-cadherin and β-catenin, are also disrupted, with the actin cytoskeleton being re-organized into actin stress fibers that are anchored to the focal adhesion complexes (87). Upon EMT, cells lose their apical-basal polarities and express mesenchymal markers, including N-cadherin, vimentin, fibronectin, smooth muscle α-actin and fibroblast-specific protein-1 (88,89). The resultant mesenchymal cells exhibit reduced adhesive properties and increased expression and secretion of extracellular matrix metallopeptidases, leading to increased migratory and invasive capacity.

Modification of the EMT process is very complicated. In brief, there are two major signaling pathways that may regulate this process: the TGF-β pathway and the Wnt/β-catenin pathway (Figures 4 and 5).

NDRG1 and TGF-β pathway

The TGF-β pathway is the most important signaling cascade regulating EMT in various cancer cell types (90). When treated with TGF-β, many cancer cell lines show morphological alterations typical of EMT, including the loss of epithelial and gain of mesenchymal markers (39). The large latent TGF-β complex is formed by latent TGF-β binding proteins binding to the inactive, non-covalently associated small latent TGF-β complex (91) (Figure 4). Upon acidic

microenvironments and activation by proteolytic enzymes, such as insulin-like growth factor, matrix metalloprotease, thrombospondin-1 and integrin (91), large latent TGF-β complex is cleaved to release a mature, bioactive TGF-β that binds to cell-surface receptors to elicit signal transduction. TGF-β receptors are transmembrane serine/threonine kinases that include two major types: type I TGF-β receptors (TβRI) and type II TGF-β receptors (TβRII) (92). Binding of TGF-β to TβRII leads to the transactivation of TβRI (92) (Figure 4).

TGF-β signaling is then subsequently transduced through a group of small intracellular effector proteins known as Smads (Figure 4). Until now, three types of Smads have been identified: (i) receptor-activated Smads, including Smad1, 2, 3, 5 and 8; (ii) a common mediator Smad, i.e. Smad4 and (iii) inhibitory Smads, including Smads 6 and 7 (93). TβRI can phosphorylate Smad2 and 3, which forms functional heterotrimers with Smad4, respectively, and translocate into the nucleus, where they interact with DNA-binding transcription factors and either activate or repress transcription (94) (Figure 4). TGF-β/Smad signaling downregulates zonula occludens 1, claudin 1 and occludin, followed by degradation of tight junctions (95). The epithelial marker E-cadherin is also inhibited by TGF-β-mediated Snail1/2, high-mobility group AT-hook 2 and zinc finger E-box-binding homeobox 1/2 activation (96). Moreover, it was demonstrated that TGF-β attenuates cell junctions through repressing junctional adhesion molecule-A/B/C and tight junction protein 1/2/3 (97). Collectively, TGF-β/Smad signaling inhibits epithelial markers and degrades tight junctions in cancer cells, inducing EMT.

On the other hand, TGF-β also elicits signaling responses through other pathways that do not involve Smad signaling (96). The TGF-β-mediated activation of these non-Smad signaling pathways, e.g. ERK, c-jun N-terminal kinase/P38 and PI3K/AKT (98), is independent of Smad signaling (Figure 4). Direct activation of non-Smad signaling pathways by TGF-β occurs through interactions of signaling mediators either directly with the TβRII and/or TβRI receptors or through adaptor proteins (98). Among the non-Smad signaling responses, activation of ERK/mitogen-activated protein kinase, Rho GTPases and the PI3K/AKT pathways in response to TGF-β has been linked to TGF-β-induced EMT through their regulation of distinct processes, such as cytoskeleton organization, cell growth, survival, migration or invasion (96).

Recently, the inhibitory effect of NDRG1 on TGF-β signaling has been addressed (39). NDRG1 overexpression maintained epithelial markers E-cadherin and β-catenin and inhibited TGF-β-stimulated cell migration and invasion (39). Conversely, NDRG1 knockdown caused morphological changes from epithelial- to fibroblastic-like phenotype and also increased migration and invasion, demonstrating that a reduction in NDRG1 induced the EMT and enhanced the oncogenic effects of TGF-β (39). The mechanism behind this observation is likely to involve the downstream targets of the TGF-β pathway, namely Smad2, Smad3, Snail and Slug, all of which have been demonstrated to be suppressed at the protein level by NDRG1 expression (Figure 4) (39,47). Collectively, these studies demonstrate that NDRG1 can restrain TGF-β-induced EMT, preventing metastasis.

NDRG1 and Wnt/β-catenin pathway

Another important signaling pathway that is involved in regulating EMT is the Wnt/β-catenin pathway. Wnt proteins are ~40 kDa in size and are modified by lipids, which are important for Wnt secretion and activation of efficient signaling (99) (Figure 5). Wnt lipids can activate the cell membrane receptor Frizzled, leading to activation of downstream protein Dishevelled. Dishevelled is a key protein that binds to Frizzled-co-receptors namely, the low-density lipoprotein receptor-related protein (LRP) family to transduce signals into the cytosol (100). Activation of canonical Wnt signaling involves phosphorylation of LRP, which then recruits Axin to the receptor complex (101,102) (Figure 5). In addition, Wnt signaling also leads to dephosphorylation and subsequent inactivation of GSK3β (103). Under normal conditions, Axin and pGSK3β form a destruction complex with adenomatous polyposis coli, which functions to phosphorylate β-catenin, leading to its subsequent
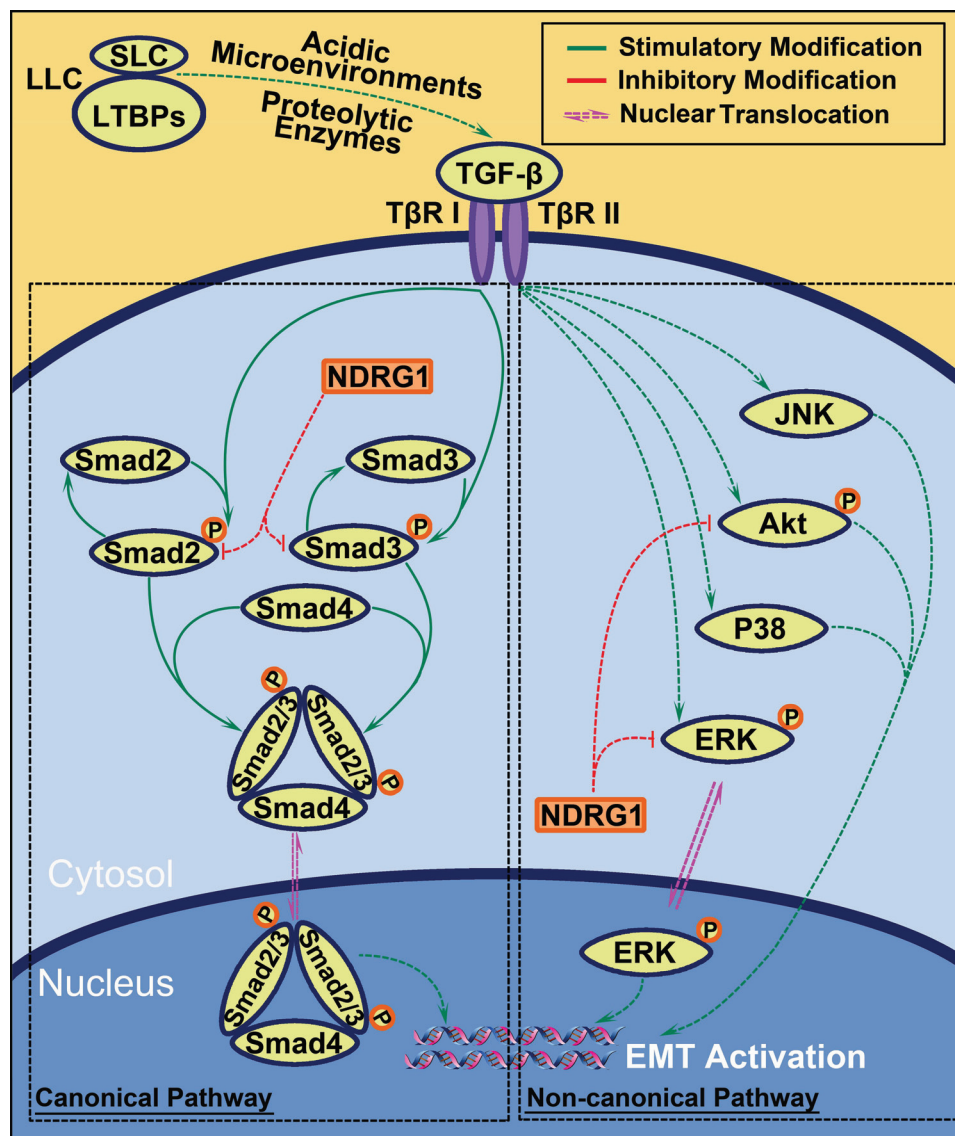
Fig. 4. NDRG1 is involved in EMT-associated TGF-β signaling. In the presence of acidic microenvironments or activation by proteolytic enzymes, bioactive TGF-β binds to cell-surface receptors to elicit signal transduction. There are two major types of TGF-β receptors: TβRI and TβRII. TGF-β signaling is then subsequently transduced through either the canonical or the non-canonical pathways. The canonical pathway involves the Smad proteins, which transport the TGF-β signal into the nucleus to induce transcription of genes relevant to EMT. On the other hand, the non-canonical TGF-β pathway also elicits a signaling response through other pathways that do not involve Smads, e.g. ERK, c-jun N-terminal kinase/P38, PI3K/AKT. Furthermore, NDRG1 antagonizes the TGF-β-activated Smad/Snail pathway to inhibit EMT. Full lines represent direct modifications. Dashed lines represent indirect modifications.

degradation *via* the proteasome (100) (Figure 5). However, upon Wnt activation, the recruitment of Axin to the membrane in addition to the dephosphorylation of GSK3β leads to dissociation of the destruction complex (Figure 5) (101–103). As a result, β-catenin escapes phosphorylation and translocates to the nucleus where it interacts with members of the T-cell factor (TCF)/lymphoid enhancer-binding factor family of transcription factors to regulate the expression of certain β-catenin/TCF-responsive target genes (102) (Figure 5). Indeed, β-catenin co-activates the transcription of target genes such as Myc, cyclin D1, TCF-1, peroxisome proliferator-activated receptor-δ, matrix metalloproteinase 7, Axin-2, CD44 and so on (102). Hence, β-catenin-dependent Wnt signaling governs cell differentiation, proliferation, apoptosis and EMT, depending on the cellular context and the surrounding microenviroment (101,102).

When expressed at the cell membrane, β-catenin can bind to E-cadherin to form an E-cadherin/β-catenin complex, which plays an important role in cell adhesion (104). In hepatic cell carcinoma

patients, the reduced expression of E-cadherin was accompanied by (partial) nuclear translocation of β-catenin and significantly correlated with intrahepatic metastasis and poor survival (105). This indicates the important role of nuclear β-catenin in facilitating disease progression.

It was recently demonstrated that NDRG1 interacts with the Wnt receptor, LRP6, leading to blocking of the Wnt signaling pathway (40) (Figure 5). Furthermore, the Wnt/NDRG1/LRP6 signature was also considered to be a strong predictable marker for recurrence-free survival of cancer patients (40). Hence, NDRG1 is proposed to be a novel negative master regulator of Wnt signaling during metastatic progression (40). It was also reported that NDRG1 can inhibit nuclear translocation of β-catenin while promoting its localization in the membrane (39,40). Moreover, NDRG1 was found to be a substrate for GSK3β, indicating its potential interactions with GSK3β-induced pathways in EMT (106) (Figure 5). Although the detailed mechanisms remain elusive, these studies clearly demonstrated that NDRG1 interacts with the Wnt/β-catenin pathway to regulate EMT.
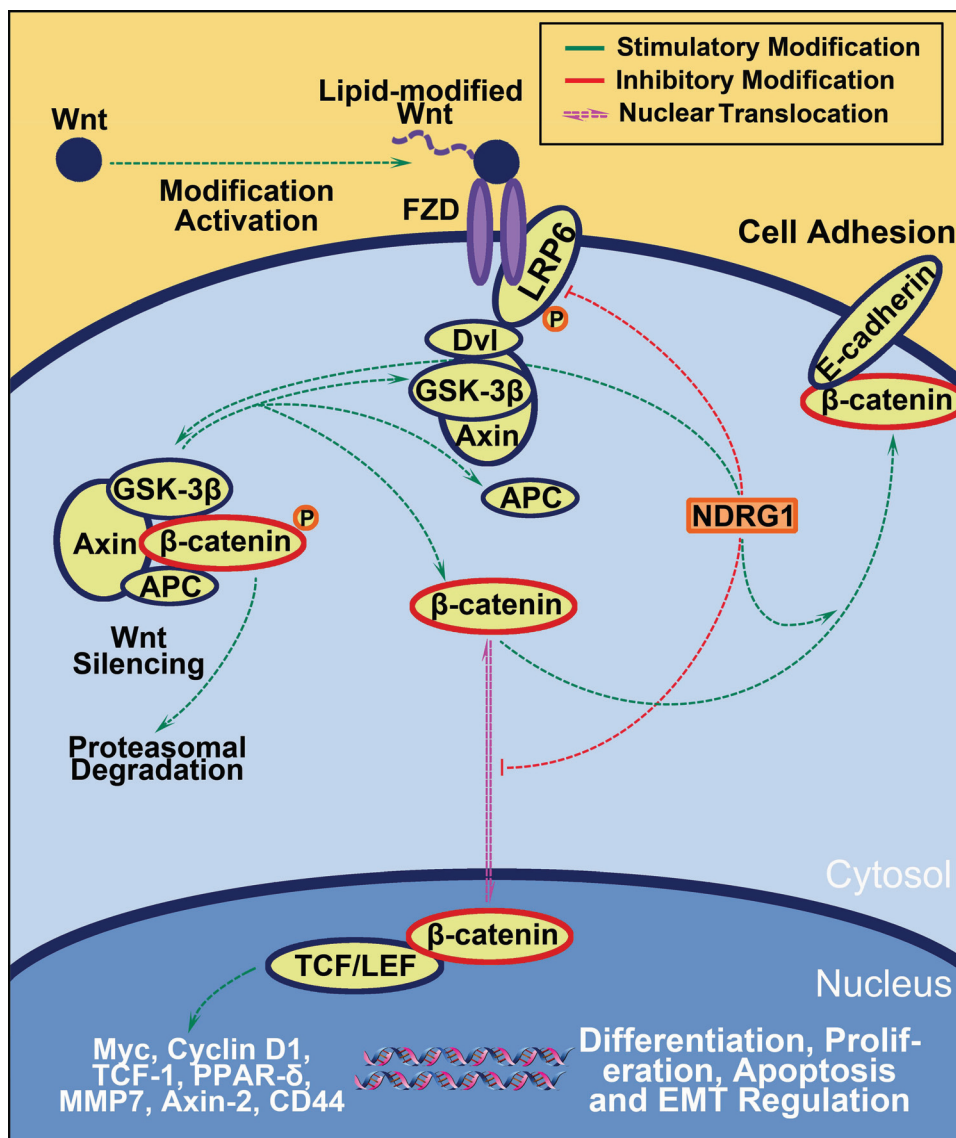
Fig. 5. NDRG1 is involved in EMT-associated Wnt/β-catenin signaling. Wnt signaling promotes the stabilization and nuclear localization of β-catenin. In the absence of a canonical Wnt signal, cytoplasmic β-catenin turns over rapidly, being phosphorylated by the constitutively active Axin/adenomatous polyposis coli/pGSK3β complex and subsequently degraded *via* the proteasome. Although Wnt is activated, cytoplasmic β-catenin escapes from phosphorylation and degradation. It then translocates to the nucleus, where it forms a complex with TCF/lymphoid enhancer-binding factor to regulate expression of genes that promote EMT and metastasis. Moreover, NDRG1 interacts with LRP6 and GSK3β, leading to blocking of the Wnt signaling pathway. Full lines represent direct modifications. Dashed lines represent indirect modifications.

NDRG1 and its effect on molecular motors involved in cancer cell migration

Cancer cell migration is a key process in cancer metastasis. It is driven by the activation of molecular motors that leads to polymerization of actin filaments, increased stress fiber synthesis and activation of actin-skeleton dynamics (107).

Actin-skeleton signaling and molecular motors
The cellular skeleton protein, actin, participates in cell motility, cell shape maintenance, polarity, cell junction function and chemotaxis, all of which are important events in cellular locomotion (108). Actin proteins exist in the cytoplasm in two different forms, namely the actin monomer (G-actin) and actin filament (F-actin). When G-actin exchanges bound adenosine diphosphate for adenosine triphosphate, it can be activated and polymerizes into F-actin bundles (108). Once actin monomers polymerize into filaments, a series of proteins and signaling pathways are activated to modify these filaments (Figure 6).

There are two groups of functional proteins regulating the polymerization process: polymerization promoters and capping proteins (109). Promoters include actin nucleators, e.g. formin and actin-related protein 2/3 (Arp 2/3) (109,110). Formins, regulated by adenomatous polyposis coli and Rho-guanine nucleotide exchange factors (111,112), are a group of multimodular proteins that bind to and regulate the stability of profilin, which is involved in the dynamic turnover and restructuring of the actin cytoskeleton (109) (Figure 6). The Arp 2/3 complex is a hetero-heptamer that promotes actin polymerization (110). The Arp 2/3 complex is activated by the Wiskott–Aldrich syndrome family protein (WASP) family of proteins (e.g. neural WASP), WASP family Verprolin-homologous protein (WAVE) and WASP and Scar homolog protein (WASH), which are activated by small Rho GTPases such as cell division control protein 42 homolog (Cdc42) and Ras-related C3 botulinum toxin substrate 1 *via* phosphorylation (113). On the contrary, actin capping proteins terminate elongation, thereby limiting polymerization of new filaments (114). The gelsolin and villin proteins cause calcium-dependent
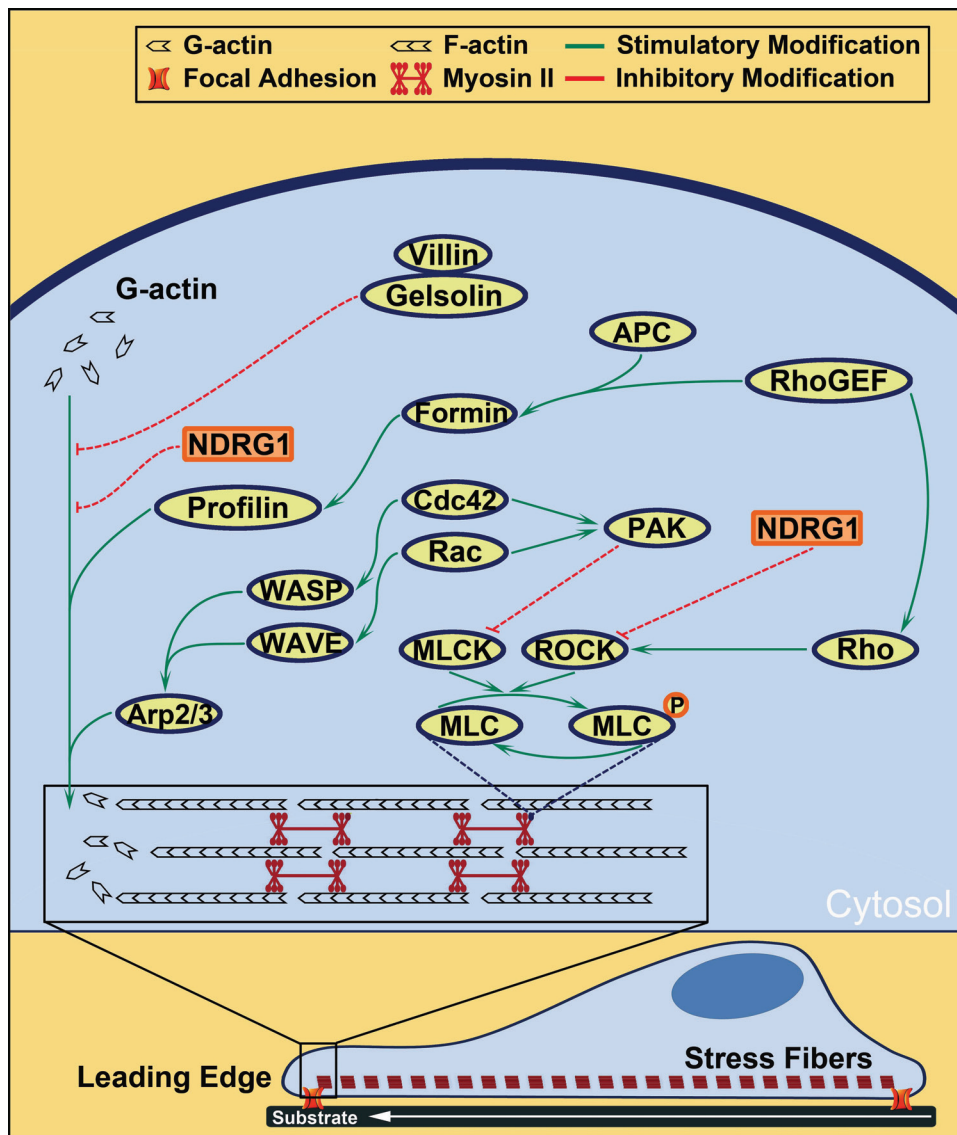
Fig. 6. Actin-skeleton dynamics and signaling pathway: potential cascades that NDRG1 may be involved in. Actin polymerization is regulated by a series of proteins and signaling pathways. There are two groups of functional proteins regulating the polymerization process: polymerization promoters (e.g. formins and Arps 2/3 complex) and capping proteins (e.g. gelsolin and villin). Moreover, actin filaments together with myosin II filaments form stress fibers, which play key roles in cancer cell motility. The key factor in the formation and contractility of the stress fibers is the phosphorylation of MLC. The small Rho-GTPase family is a typical molecular motor involved in regulating stress fiber formation, contraction and cell motility. Full lines represent direct modifications. Dashed lines represent indirect modifications.

fragmentation of actin filaments and are also capable of capping the filament barbed end (115).

Actin filaments together with myosin II filaments form contractile actomyosin structures in cells, regulating cell movement during the migration process (87). Stress fibers are one of the contractile actomyosin structures, which play key roles in cancer cell motility (87). When a cancer cell makes stable associations to a substrate or its potential metastasis signaling is triggered by activators (e.g. transcription factors, chemotactic factors and so on), stress fibers are rearranged and extend within the cell (116). The myosin interacts with F-actin to drive the movement of actin filaments past one another and the contraction of the fiber, causing alterations in cell shape and cellular migration (117). Furthermore, it is reported that the key factor in the formation and contractility of the stress fibers is the phosphorylation of MLC at Thr18/Ser19 (118) (Figure 6).

Hence, the signaling pathways that regulate MLC phosphorylation are able to mediate stress fiber formation and contractility (117) (Figure 6). The small Rho-GTPase family is a typical molecular motor involved in regulating stress fiber formation, contraction and cell motility (118). As a subgroup of the Ras superfamily of GTPases, Rho family GTPases have a close interaction with the PI3K signaling pathway (119). Rho-guanine nucleotide exchange factor activates monomeric GTPases by stimulating the release of guanosine diphosphate to allow binding of GTP to GTPase (120,121). In addition, GTPases, which were shown to regulate actin dynamics, were reported to be closely correlated with progression of many cancer types (122). There are three well-established members in the small Rho-GTPase family: Rho protein, cell division control protein 42 and Ras-related C3 botulinum toxin substrate 1 (123). Rho can activate its effector Rho-associated, coiled-coil containing protein kinase, which can induce membrane protrusions and cancer cell migration *via* phosphorylation of MLC (124). Both cell division control protein 42 and Rac can activate p21-activated kinase, which can inhibit MLC kinase-mediated phosphorylation (125,126). Collectively, the Rho-GTPase family acts as linking molecules in the signaling networks controlling cancer migration and metastasis.

NDRG1 and molecular motors

Recent studies have revealed a significant inhibitory effect of NDRG1 on cancer cell migration and invasion *via* antagonizing TGF-β in human prostate and colorectal cancer cells (39). NDRG1 may also interact with caveolin 1, which is involved in TGF-β-mediated EMT and integrin-associated cell adhesion in cancer (127). Although molecular motors drive cancer metastasis and NDRG1 functions as a metastasis suppressor, it is possible that NDRG1 can modulate the molecular motors mentioned above.

Indeed, our recent investigations demonstrated that NDRG1 inhibits actin filament polymerization, stress fiber assembly and cell migration *via* the Rho-associated, coiled-coil containing protein kinase 1/pMLC2 pathway in prostate and colorectal cancer cells (128). These results suggest that NDRG1 functions to inhibit metastasis by regulating the molecular motors and stress fiber assembly (Figure 6).

In summary, the metastasis suppressor, NDRG1, interacts with key signaling pathways to inhibit cancer progression and metastasis. Elucidating the molecular mechanisms that underlie the antimetastatic effects of NDRG1 may lead to the development of new therapies to inhibit cancer metastasis.

Acknowledgements

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