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N-myc downstream regulated gene 1 modulates Wnt-β-catenin signalling and pleiotropically suppresses metastasis

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Wnt signalling has pivotal roles in tumour progression and metastasis; however, the exact molecular mechanism of Wnt signalling in the metastatic process is as yet poorly defined. Here we demonstrate that the tumour metastasis suppressor gene, NDRG1, interacts with the Wnt receptor, LRP6, followed by blocking of the Wnt signalling, and therefore, orchestrates a cellular network that impairs the metastatic progression of tumour cells. Importantly, restoring NDRG1 expression by a small molecule compound significantly suppressed the capability of otherwise highly metastatic tumour cells to thrive in circulation and distant organs in animal models. In addition, our analysis of clinical cohorts data indicate that Wnt+/NDRG−/LRP+ signature has a strong predictable value for recurrence-free survival of cancer patients. Collectively, we have identified NDRG1 as a novel negative master regulator of Wnt signalling during the metastatic progression, which opens an opportunity to define a potential therapeutic target for metastatic disease.

INTRODUCTION

Cancer is becoming a curative disease due to the screening for early detection and significant advancement of treatment measures; however, more than 90% of cancer deaths are still attributed to metastatic disease. Although metastasis has been considered as a stepwise culmination of neoplastic progression, recent discoveries from both clinical and experimental studies indicate that metastatic progression is an independent process from primary tumour development and acquisition of metastatic competence can occur at an early stage of tumour progression (Kim et al, 2009; Klein, 2009; Norton and Massague, 2006; Riethmüller and Klein, 2001). Nevertheless, metastasis is a complex process and needs dysregulation of a series of genes and related signalling for proliferation, survival, invasion, angiogenesis and growth at distant sites. Many studies have demonstrated that characteristic changes of oncogene pathways for primary tumorigenesis such as Wnt signalling are also integral parts of the metastatic progression (Khew-Goodall and Goodall, 2010; Nguyen et al, 2009; Zhang et al, 2009). Wnt pathway is an essential signalling for embryogenesis and normal physiology; however, it also plays a major role in the primary tumorigenesis and the metastatic progression (Clevers, 2006; Klaus and Birchmeier, 2008; Logan and Nusse, 2004; Moon et al, 2004). Binding of Wnt ligands to its co-receptors, Frizzled and low density lipoprotein receptor-related protein 5/6 (LRP5/6), prevents the degradation of β-catenin which then accumulates in the nucleus and serves as an active transcription factor with T-cell specific transcription factor/Lymphoid enhancer-binding factor (TCF/LEF) followed by activation of various pro-metastatic genes (Logan & Nusse, 2004). Wnt signalling is also capable of inducing epithelial-to-mesenchymal transition (EMT), which plays a critical role in metastatic progression of epithelial cancers (Vincan & Barker, 2008). Although aberrant expression of Wnt signalling has been well documented in various cancers, the exact molecular mechanism by which the dysregulated Wnt

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signalling and its down-stream targets coordinate the transformation and metastatic progression is as yet poorly understood.

Another important evidence for the notion that metastasis is distinct from tumorigenesis came from the studies of metastasis suppressors, which inhibit metastasis but do not affect the primary tumour growth (Berger et al, 2005; Nash & Welch, 2006; Shevde & Welch, 2003; Steeg, 2003, 2006). N-myc downstream regulated gene-1 (NDRG1) was originally isolated as a novel gene that was strongly induced during differentiation of colon carcinoma cell lines (Van Belzen et al, 1997), and it was shown to be controlled by various neoplastic factors and microenvironment (Ellen et al, 2008; Kovacevic & Richardson, 2006). Recent studies provide compelling evidence showing that the NDRG1 is a tumour metastasis suppressor and its status may serve as a diagnostic and prognostic marker (Iiizumi et al, 2008; Kitowska & Pawełczyk, 2010). However, how NDRG1 exerts its metastasis suppressor function is largely unknown. To gain mechanistic insight into the role of NDRG1, we took two different approaches. One is to identify downstream effectors of NDRG1 by microarray analysis, by which we found that NDRG1 inhibited metastasis by suppressing the expression of activating transcription factor3 (ATF3) gene, a stress-inducible transcription factor (Bandyopadhyay et al, 2006a). Secondly, we performed a protein–protein interaction screening using yeast two-hybrid screening system and found that NDRG1 is capable of interacting with LRP6, a co-receptor of Wnt signalling. In this report, we investigated the molecular mechanism by which cross-talk of NDRG1 and Wnt pathways modulates metastatic process and also explored a possibility of restoring the NDRG1 expression by small molecule compound as a potential therapeutic intervention for metastatic disease.

RESULTS

NDRG1 interacts with the Wnt co-receptor LRP6 and blocks the Wnt-β-catenin signalling

After performing the yeast two-hybrid screening using a complementary DNA (cDNA) library of normal human prostate tissues, we found that LRP6 showed the strongest interaction with NDRG1 (Fig 1A). To verify the interaction of these proteins in mammalian cells, NDRG1 and LRP6 were tagged with Flag and HA, respectively. Flag-NDRG1was then subcloned into a tetracycline (Tet) inducible vector followed by establishing a cell line using PC3mm and MCF7 cells (PC3mm/Tet-Flag-NDRG1 and MCF7/Tet-Flag-NDRG1, referred to as PC3mm/Tet and MCF7/Tet). Our results of Co-IP showed that NDRG1 was pulled down with LRP6 and *vice versa*, suggesting that these two proteins are indeed interacting in human prostate cancer cells (Fig 1B, Supporting Information Fig S1A). We also found that up to 116 bases and 3929 bases from each N-terminal of NDRG and LRP6, respectively, are dispensable for the interaction (Supporting Information Fig S1B). Because Wnt is known to induce phosphorylation of LRP6 at multiple sites (Thr^{1479}, Ser^{1490} and Thr^{1493}) (Davidson et al, 2005; Tamai et al, 2004; Zeng et al, 2005) followed by activation of downstream signalling, we examined the effect of NDRG1 on LRP6 and found that phosphorylation of LRP6 (Ser^{1490}) induced by Wnt signalling was substantially decreased by NDRG1, while it did not affect the total protein level of LRP6 (Fig 1C), suggesting that NDRG1 interacts with LRP6 and prevents its activation by Wnt ligands. To further examine the effect of NDRG1 on Wnt signalling pathway, we performed a phospho-site profiling analysis and found that ectopic expression of NDRG1 strongly increased the phosphorylated level of glycogen synthase kinase 3β (GSK3β) ($Tyr^{279/216}$) and also de-phosphorylated Akt at Thr^{308}, while NDRG1 did not affect their mRNA expression (Fig 1D, Supporting Information Fig S1C and D). On the other hand, knocking down of LRP6 alone did not alter the phosphorylation of Akt, suggesting that NDRG1 can de-phosphorylate Akt independently from the Wnt pathway (Supporting Information Fig S1D). GSK3β is a key signal mediator of the Wnt pathway, which is constitutively active in the absence of Wnt ligand and forms a scaffold complex with adenomatous polyposis coli (APC), axin and CK1α to stimulate ubiquitination of β-catenin for its degradation (Logan & Nusse, 2004; Moon et al, 2004). Wnt signalling leads to de-phosphorylation of GSK3β to inactivate this protein followed by stabilization of β-catenin and activation of downstream targets. It should be also noted that Akt inactivates GSK3β by phosphorylating it at $Serine^{9/21}$ residues (Cross et al, 1995). Therefore, our results suggest that NDRG1 interacts with LRP6 and blocks silencing signals of Wnt pathway, leading to re-activation of GSK3β. Furthermore, we performed TopFlash reporter assay and found that NDRG1 significantly blocked Wnt-induced reporter activity in both prostate and breast cancer cells, while knocking down of NDRG1 by small hairpin RNA (shRNA) significantly augmented the Wnt reporter activity in RWPE1 which is an immortalized human prostate cell line and has a high level of NDRG1 (Fig 1E, Supporting Information Fig S1E). We also found that induction of NDRG1 significantly down-regulated β-catenin expression (Fig 1F and G). The down-regulation is likely due to the degradation of this protein because NDRG1 strongly increased phosphorylation of β-catenin at Ser33/Ser37/Thr41 residues that are the targets of GSK3β for the subsequent degradation of β-catenin (Supporting Information Fig S1F). These results strongly indicate that NDRG1 interacts with and inactivates LRP6 followed by blocking de-phosphorylated inactivation of GSK3β and subsequent activation of Wnt signalling. NDRG1 was previously shown to be down-regulated by N-myc and C-myc genes that are known targets of Wnt signalling. Therefore, we examined the expression of NDRG1 in the presence or absence of Wnt after knocking down N-myc or C-myc. We found that knock-down of myc genes indeed significantly up-regulated the NDRG1 expression; however, Wnt treatment significantly abrogated this effect (Supporting Information Fig S1G). Therefore, our results suggest that there are other inhibitory factors of NDRG1 as Wnt downstream targets and that NDRG1 expression is controlled by an intricate balance between Wnt-mediated expression of NDRG1 inhibitors and suppressive effect of NDRG1 on the Wnt signalling.

NDRG1 inhibits ATF3 through modulating Wnt-β-catenin signalling

Previously, we demonstrated that NDRG1 was able to modulate the metastasis-promoting gene ATF3, and therefore, we further

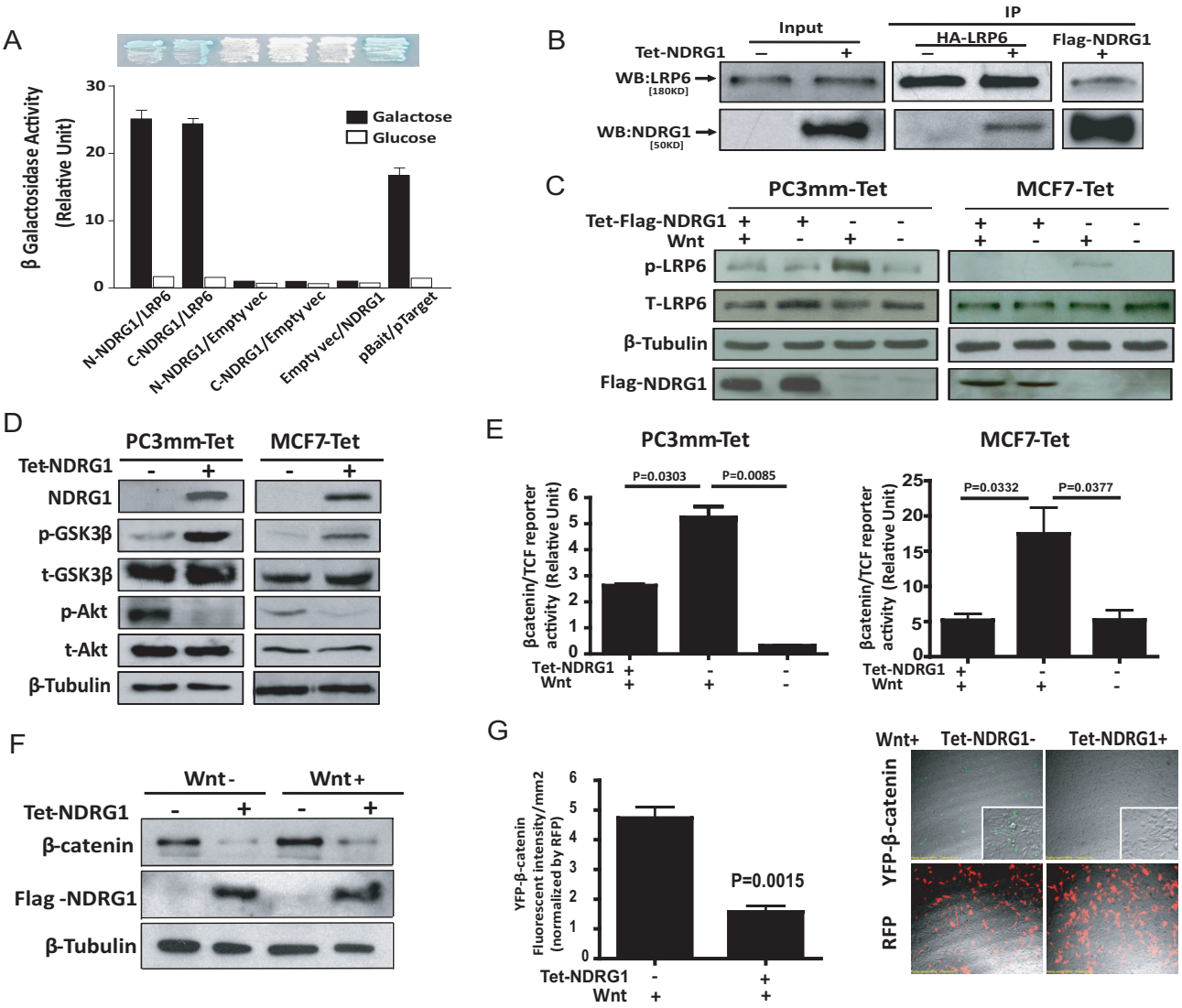


Figure 1. NDRG1 interacts with the Wnt co-receptor, LRP6, and blocks Wnt-β-catenin signalling by re-activating GSK3β.

A. NDRG1 was fused with LexA DNA-binding domain at N- or C-terminal, and LRP6 was fused with B42 activation domain. Appropriate combinations of expression plasmids were transformed in the same yeast strain EGY48. The resultant transformants were grown and assayed for the β-galactosidase activity of the reporter LacZ gene. pBait and pTarget are a pair of positive controls. Inset image shows transformants grown in an agar plate containing X-gal.

B. Co-IP analysis for the interaction of NDRG1 and LRP6 in mammalian cells. PC3mm/Tet cells were transfected with the expression plasmid of HA-LRP6, treated with tetracycline to induce NDRG1. Cell lysates were precipitated with anti-HA for LRP6 (lanes 3 and 4) or anti-Flag for NDRG1 (lane 5) followed by Protein-G agarose. Western blot was performed to detect NDRG1 and LRP6.

C. PC3mm/Tet and MCF7/Tet cells were treated with (lanes 1 and 2) or without (lanes 3 and 4) tetracycline to induce NDRG1 in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of Wnt. Cell lysates were subjected to Western blot with antibodies against phospo-LRP6, total-LRP6, Flag-NDRG1 and β-tubulin.

D. Western blot analysis to confirm results from phospho-site profiling analysis for phospho-GSK3β ($Tyr^{279/216}$), GSK3β, phospho-Akt (Ser^{473}) and Akt expression in PC3mm/Tet and MCF7/Tet cells that were treated with or without tetracycline.

E. PC3mm/Tet and MCF7/Tet cells were transfected with the TOPflash reporter plasmid in the presence or absence of Wnt followed by treatment with tetracycline or not. Luciferase activity of cell lysates was assayed and normalized by internal Renilla luciferase activity. Values are from three independent experiments in triplicate. *p* values were calculated by two-sided Student's *t*-test.

F. Western blot analysis of β-catenin expression in PC3mm/Tet cells treated with or without tetracycline prior to the addition of Wnt.

G. PC3mm/Tet-Flag-NDRG1 cells were transfected with YFP-β-catenin and RFP expression plasmids in the presence or absence of tetracycline. After 48 h, the fluorescence intensity of β-catenin was determined under a confocal microscope. Each value was normalized by the RFP fluorescence intensity. Right panels show representative microscopic photos for each experimental group. Values are from three independent experiments in triplicate. *p* values were calculated by two-sided Student's *t*-test.

explored a possibility that ATF3 expression was regulated by Wnt signalling. We found that Wnt strongly increased the nuclear expression of ATF3 and that the induction of NDRG1 strongly abrogated this effect (Supporting Information Fig S1H). Consistently, ectopic expression of β-catenin and TCF also significantly promoted the expression of ATF3 at both mRNA and protein levels in prostate and breast cancer cells (Fig 2A). Moreover, a deletion of β-catenin/TCF binding consensus sequence of the ATF3 promoter significantly blocked the suppressive effect of NDRG1 (Fig 2B), suggesting that down-regulation of ATF3 by NDRG1 depends directly on the β-catenin/TCF binding site in the ATF3 promoter. In agreement with this result, we found that Wnt treatment led to a significant increase in the ATF3 promoter activity of wild type but not mutant (Fig 2C), and that Wnt substantially augmented the level of ATF3 protein which was decreased upon induced NDRG1 expression (Fig 3B). In addition, our results of ChIP assay by precipitating β-catenin/TCF-chromatin complex clearly showed

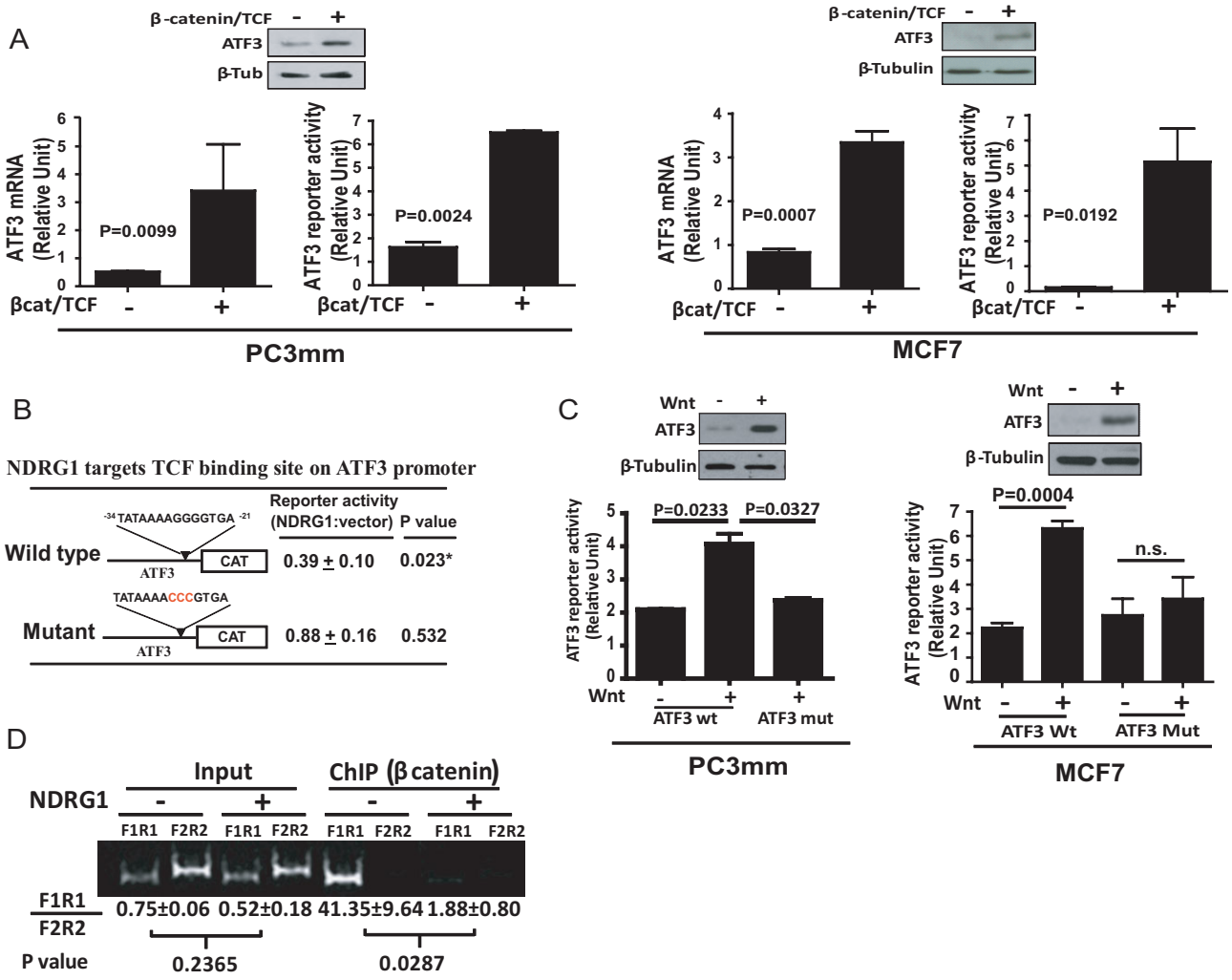


Figure 2. NDRG1 blocks Wnt-β-catenin signalling followed by inhibiting ATF3 expression.

A. The expression of ATF3 was examined by qRT-PCR or ATF3 reporter assay in PC3mm (left panel) or MCF7 (right panel) that were transfected with or without the expression plasmid of β-catenin/TCF. Western blot was also performed to confirm the ATF3 expression in the presence of β-catenin/TCF. Values are from three independent experiments in triplicate.

B-C. Three-base mutation was introduced into the consensus sequence of β-catenin/TCF binding site on the ATF3 gene promoter of the CAT-reporter plasmid. Wild-type and mutant reporter plasmids were then transfected into PC3mm/Tet or MCF7/Tet cells in the presence or absence of tetracycline (**B**) or Wnt (**C**). CAT activity of the cell lysates was assayed and normalized by internal Renilla luciferase activity. Western blot analysis of ATF3 expression was also performed. Values are means ± SD of triplicate measurements.

D. ChIP assay for PC3mm/Tet cells treated with or without tetracycline for 48 h. Precipitated DNA was subjected to q-PCR using primers specific for the β-catenin binding consensus sequence on the ATF3 promoter (F1R1) as well as a control primer set (F2R2). The ratio of the precipitated DNA was calculated based on cyclic threshold value for each reaction. Values are means ± SD of triplicate measurements. For all experiment, *p* values were calculated by two-sided Student's *t*-test.

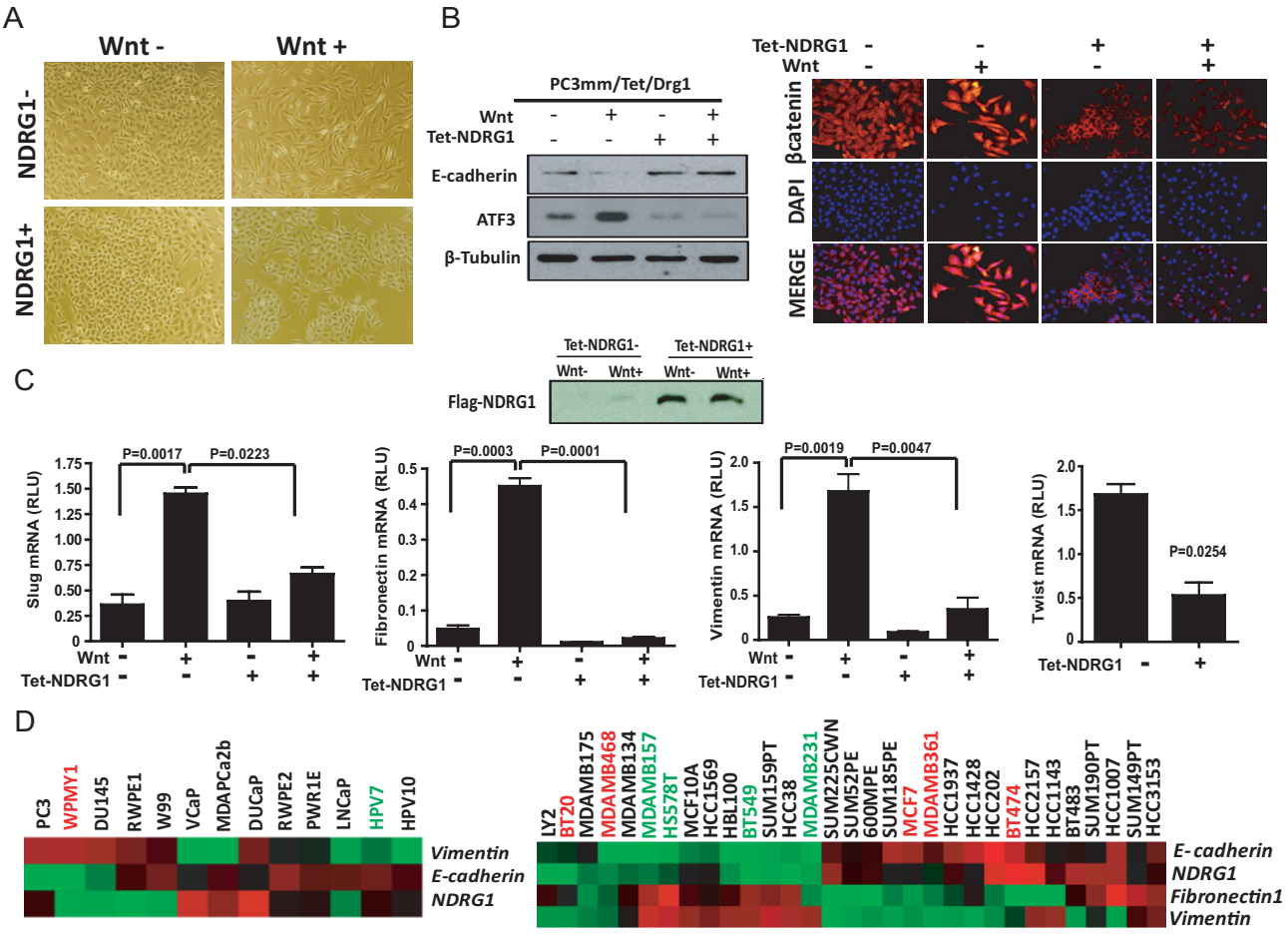


Figure 3. NDRG1 suppresses Wnt-mediated EMT.

A. EMT-like morphological changes of PC3mm/Tet cells that were treated with or without Wnt in the presence or absence of tetracycline.

B. Western blot analysis for E-cadherin and ATF3 expression (left panel) and immuno-fluorescence staining of β-catenin (right panel) in PC3mm/Tet cells that were treated with or without tetracycline prior to the addition of Wnt.

C. PC3mm/Tet-Flag-NDRG1 cells were cultured in the presence or absence of Wnt with or without the treatment of tetracycline. RNA were then prepared and subjected to qRT-PCR analysis for the expression of *Slug*, *Fibronectin*, *Vimentin* and *Twist*. Values are from three independent experiments in triplicate. *p* values were calculated by two-sided Student's *t*-test. Western blot analysis was also performed to confirm the expression of NDRG1 (inserted fig).

D. *NDRG1* expression correlates with epithelial traits in a panel of prostate (left panel, GSE9633) and breast (right panel, Neve et al, 2006) cancer cell lines. Heatmap depicts the relative expression of normalized expression values of *E-cadherin*, *NDRG1*, *Fibronectin1* and *Vimentin* for each cell lines. Green colour indicates low expression and red colour indicates high expression. Representative cell lines exhibiting epithelial-like and mesenchymal morphologies were marked with red and green colours, respectively.

that β-catenin/TCF binds to the predicted region of ATF3 promoter, while expression of NDRG1 led to more than 95% decrease in binding efficiency (Fig 2D), which provided direct evidence that NDRG1 suppresses ATF3 expression by blocking Wnt-β-catenin signalling.

NDRG1 suppresses Wnt/β-catenin-induced mesenchymal traits and metastatic-relevant activities

Because Wnt/β-catenin signalling affects various stages of tumour progression, we next examined the effect of NDRG1 on metastasis cascade through blocking the Wnt signalling. We found that over-expression of NDRG1 did not affect cell viability in monolayer culture; however, it significantly suppressed the colony-forming ability of tumour cells under the anchorage-independent condition (Supporting Information Fig S2A), suggesting that NDRG1 affects invasiveness and outgrowth of tumour cells when they infiltrate into distant organs. The Wnt-β-catenin signalling also plays a critical role in the EMT, a process vital for endowing malignant tumour cells to acquire motility and invasive as well as survival abilities during dissemination (Polyak and Weinberg, 2009). We found that an addition of Wnt to PC3mm/Tet cells, which normally exhibited a characteristic epithelial cobblestone-like morphology, converted them to be more fibroblastic morphology whereas induction of NDRG1 expression partially reverted these cells to epithelial morphology (Fig 3A). This morpholo-

gical change was accompanied by up-regulation of E-cadherin and membrane β-catenin (Fig 3B), as well as down-regulation of EMT-regulatory factors *Slug*, *Twist*, *Vimentin* and *Fibronectin* (Fig 3C). Besides its role as a hub of Wnt-mediated transcriptional events, β-catenin at the cytoplasmic membrane serves as a core component of cadherin–catenin adherent junctions between adjacent epithelial cells (Heuberger and Birchmeier, 2010). As shown in Fig 3B (right panel), we indeed observed that Wnt treated cells showed relocalization of β-catenin from cytoplasm to nucleus compared with the non-treated control cells; however, induction of the NDRG1 expression substantially decreased cytoplasmic/nuclear β-catenin, suggesting that NDRG1 re-activates GSK3β, thereby promoting proteasome-mediated degradation of cytosol β-catenin. We also found that NDRG1 suppressed Wnt-induced mesenchymal traits in MCF7/Tet cells (Supporting Information Fig S2B-E). Moreover, we demonstrated that high expression of *NDRG1* gene was positively correlated with the expression of *E-cadherin* but not with *Vimentin* in a panel of prostate and breast cancer cell lines, reflecting the correlation of NDRG1 expression with epithelial phenotype (Fig 3D). These data indicate negative regulatory role of NDRG1 in EMT process through modulation of Wnt signalling, which partly account for the metastasis suppressor function of NDRG1. As shown in Fig 4A, the activation of Wnt signalling led to more than fourfold increase in the invasivenesss of PC3mm/Tet cells, which was significantly blocked by NDRG1 expression. Notably, majority of NDRG1-expressing cells showed cortical actin distribution whereas Wnt-treated cells showed condensed stress fibres and invadopodia-like structures around the periphery of cells (Supporting Information Fig S2F). We also found that PC3mm/Tet cells cultured in suspension exhibited anoikis-mediated cell death; however, addition of Wnt significantly enhanced cell viability, while NDRG1 expression restored the occurrence of anoikis (Fig 4B). Beside the intrinsic ability to evade anoikis, metastatic tumour cells also have an ability of adhering to endothelial cells which provides critical support for survival during dissemination. We found that, when PC3mm/Tet cells were co-cultured with mouse lung microvessel endothelial cells, Wnt treatment significantly increased the number of PC3mm/Tet cells to the endothelial cells by more than threefold compared to the non-treated cells whereas the ectopic expression of NDRG1 significantly decreased the number of attached cells even in the presence of Wnt (Fig 4C, left panel). Furthermore, consistent with the notion that NDRG1 expression is correlated with epithelial cell type, we found that NDRG1-expressing cells attached to endothelial cells showed apparently more differentiated phenotype with tight intracellular adhesion (Fig 4C, right panel).

Because the abilities of invasion, anoikis-resistance and adhesion are all essential propensities of disseminating tumour cells in early stage of metastasis, we speculated that NDRG1 exerts its metastasis suppressor function by blocking these abilities of tumour cells thereby restricting them to thrive in distant sites. To test this possibility, PC3mm/Tet cells with or without NDRG1 expression were labelled with cell-tracker dye and they were intravenously injected into Nude mice. We found that tumour cells quickly lodged onto lung endothelium after 2 h regardless of the status of NDRG1 expression (Supporting Information Fig S2G). However, 48 h after implantation, the number of NDRG1-expressing tumour cells in the lungs was significantly diminished by 60% compared with cells without induction of NDRG1 expression, and they were detected exclusively within vessels as a single cell (Fig 4D). Forced activation of Wnt signalling by ectopic expression of β-catenin and TCF significantly enhanced survival of the tumour cells in the lungs, but it failed to overcome the suppressive effect of NDRG1. Taken together, our observations from assessing multiple processes of the metastasis cascade provide compelling evidence that NDRG1, by modulating Wnt pathway, imposes a metastasis-suppressive network.

Ability to modulate Wnt signalling is essential for NDRG1 to suppress metastasis *in vitro* and *in vivo*

To address the question whether the ability of NDRG1 to inhibit metastasis depends on its ability to modulate Wnt signalling, we employed the MMTV-Wnt mouse model. The mouse carries the Wnt transgene which is driven by the mouse mammary tumour virus LTR and develops extensive ductal hyperplasia early in life and mammary adenocarcinoma later (Li et al, 2000). We first isolated tumour cells from the mammary tumour of this mouse and established a cell line (WB1-1) which was then infected with lentivirus encoding luciferase gene (Supporting Information Fig S3A). By introducing a Flag-tagged NDRG1 expression vector and/or a dominant active β-catenin (the ΔN90 non-degradable mutant, Bahmanyar et al, 2008) into WB1-1, we further established a series of WB1-1 cell lines (WB1-1/NDRG1, WB1-1/ΔN90-β catenin and WB1-1/ΔN90-β catenin/NDRG1, referred to as NDRG1, ΔN90, ΔN90/NDRG1). Consistent with our results of prostate cancer cells, WB1-1cells expressing NDRG1 (WB1-1/NDRG1) showed drastically decreased level of β-catenin and ATF3 (Supporting Information Fig S3B), and significantly down-regulated Wnt signalling activity as well as compromised metastatic abilities compared to the parental WB1-1 cells (Supporting Information Fig S3C-H). Strikingly, however, WB1-1 cells with constitutively active β-catenin (ΔN90 and ΔN90/NDRG1) exhibited similar, if not more aggressive abilities of migration, anoikis resistance, adhesion as well as survival in the lungs despite over-expression of NDRG1. These data further support our notion that NDRG1 exerts its metastasis suppressor activity by inhibiting Wnt signalling.

We next implanted WB1-1 cell series into the mammary fat pads of SCID mice and monitored the primary and metastatic tumour growth for 6 weeks. Consistent with the results of our *in vitro* study, all cell lines formed primary tumours with similar growth rate and histological morphology; however, they exhibited vastly different metastatic behaviors (Fig 5A and B, Supporting Information Fig S4A). Despite the same rate of metastatic incidence, mice inoculated with ΔN90-expressing cells (N90 and N90/NDRG1) developed distant metastases at a much earlier stage compared to the parental WB1-1 cells (Fig 5A and D left panel, Supporting Information Fig S4B), and they also formed significantly larger metastatic lesions (Fig 5C, D right panel). These observations indicate that constitutively active

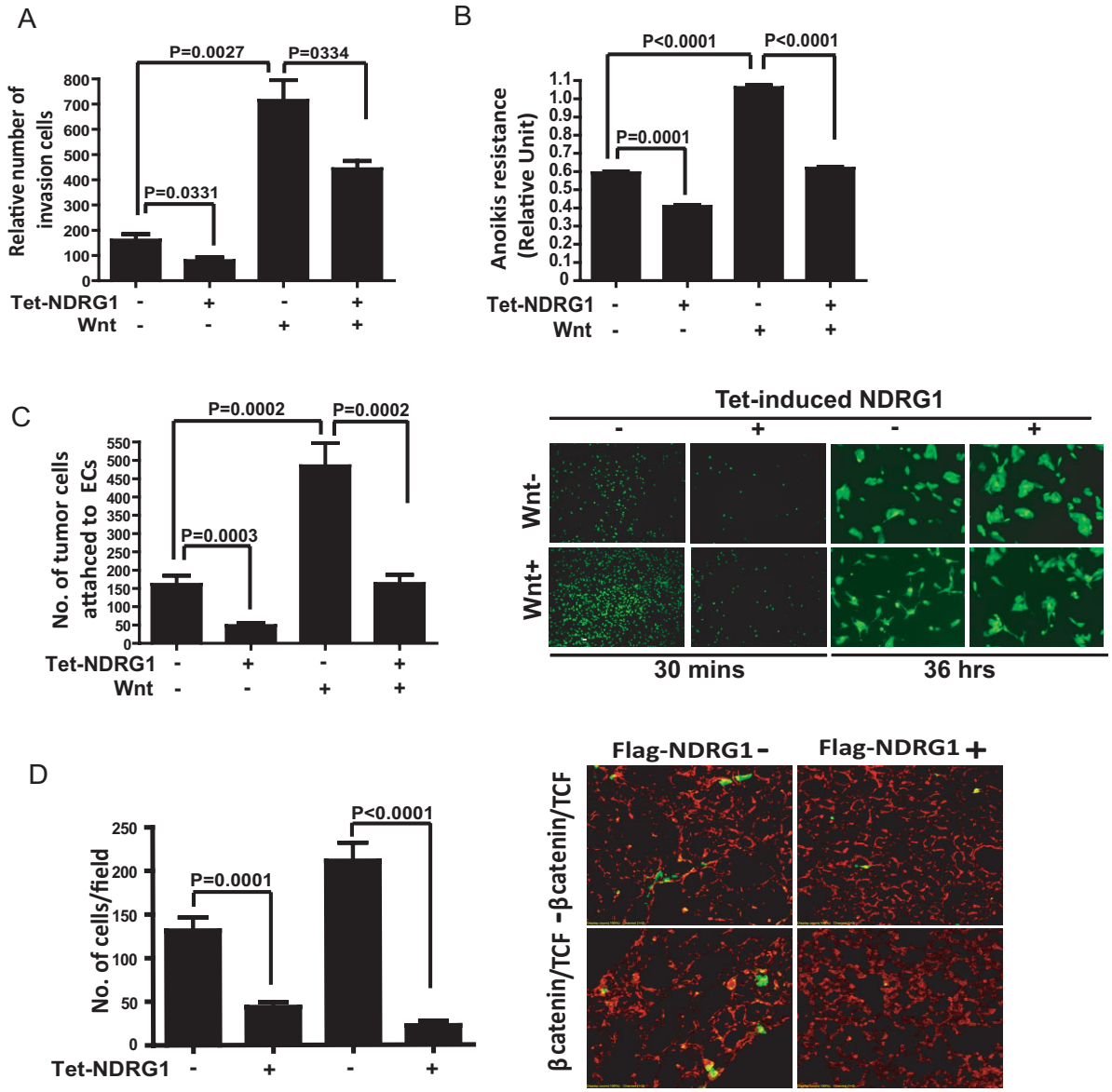


Figure 4. NDRG1 suppresses Wnt-mediated multiple metastatic traits.

A-C. PC3mm/Tet cells were treated with or without Wnt followed by treatment of tetracycline or not, and were subjected to assays for invasion (**A**), anoikis resistance (**B**) and adhesion assays (**C**).

D. PC3mm/Tet cells were transfected with the expression plasmids of β-catenin/TCF or vector control plasmids followed by treatment of tetracycline or not. Cells were then stained with CellTracker Green and injected into the lateral vein of nude mice ($n = 3$). Lungs were removed after 48 h and sectioned followed by counting the number of tumour cells under the confocal microscope. Lung vasculature was visualized by staining with rhodamine-conjugated lectin. For all experiment, values are from three independent experiments in triplicate. *p* values were calculated by two-sided Student's *t*-test.

β-catenin augmented the Wnt signalling, which promoted tumour cells to invade from local site and to infiltrate into distant organs followed by colonization. On the other hand, NDRG1-exression did not affect the primary tumour growth but suppressed the local invasion and significantly reduced overall metastasis by 80% compared to the parental cells, which is in accordance with our previous observations in an animal model of prostate cancer (Bandyopadhyay et al, 2003) (Fig 5A and B, Supporting Information Fig S4C). However, the metastasis-suppressive effect of NDRG1 was drastically overcome by constitutively active β-catenin, which provided direct evidence that the ability to antagonize Wnt-β-catenin signalling is required for NDRG1-mediated metastasis suppression. We further assessed the status of Wnt signalling in primary tumours by immunostaining for β-catenin and its downstream target ATF3. As expected, β-catenin was found to be mostly localized

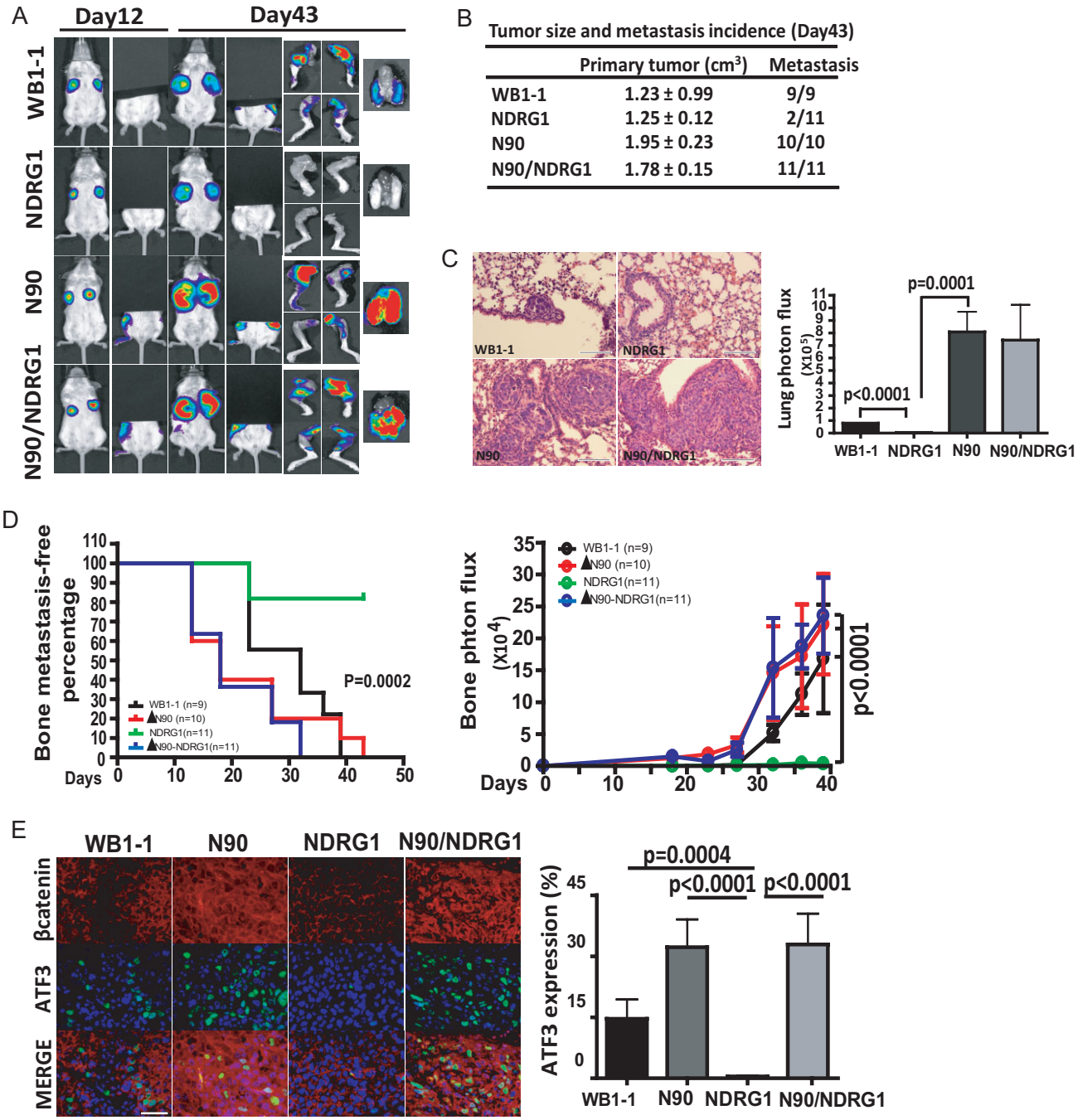


Figure 5. NDRG1 suppresses metastasis by blocking Wnt pathway *in vivo*.

A. To test tumorigenic and metastatic ability of WB1-1cell lines, 5×10^5 cells of each line were injected into the mammary fat pad of nude mice. Non-invasive BLI was performed to monitor the primary tumour growth and the development of metastasis. At the time of necropsy, lungs and bones from individual mice were dissected and imaged *ex vivo* for bioluminescence. Shown are representative bioluminescent images of primary tumour and metastasis of individual mice ($n \geq 9$).

B. Summary of the tumour size and metastatic incidence at day 43.

C. H&E-stained sections of mouse lungs from each group at the endpoint (left panel). The scale bar represents 100 μm. Metastatic growth of tumours in the lungs was also measured by BLI after necropsy (right panel). Data are presented as the mean photon flux. Error bars represent the SEM. *p* values were calculated by two-sided Student's *t*-test.

D. Incidence of hind limb bone metastasis was represented by Kaplan–Meier curves, and their metastatic growth was measured by BLI. *p* values were calculated by Log-rank test and two-sided Student's *t*-test.

E. Immuno-fluoresence staining of β-catenin and ATF3 in mouse primary tumours. The percentage of ATF3 positive tumour cells were quantified for each group. *p* values were calculated by two-sided Student's *t*-test.

at the membrane site of cell–cell junctions in NDRG1-expressing tumour which was accompanied with minimally detectable level of ATF3. On the contrary, ΔN90-expressing tumours showed robust expression of cytoplasmic/nuclear β-catenin as well as ATF3 (Fig 5E). Notably, most ATF3 was found at the margins of the invasive front in primary tumours formed by ΔN90-expressing cells, which provided further evidence for a role of ATF3 in promoting invasion and metastasis (Supporting Information Fig S4D).

Correlation of NDRG1 with Wnt signalling and metastatic status of cancer patients

To corroborate our *in vitro* results in clinical settings, expression of NDRG1, β-catenin and ATF3 were examined by immunohistochemistry with tumour specimens from 29 prostate and 33 breast cancer patients. As shown in Fig 6A and B, NDRG1was strongly expressed in normal prostate and breast epithelium but was significantly down-regulated in high-grade cancers. The expression of β-catenin was mostly localized in membrane/cytoplasm in normal tissue, but it was shifted to cytoplasm/nucleus in high grade tumours. Moreover, positive correlations between membranous β-catenin and NDRG1 as well as cytoplasmic/nuclear β-catenin and ATF3 were observed, which is consistent with the notion that NDRG1 suppresses ATF3 expression by blocking Wnt-β-catenin signalling. We also examined the expression of NDRG1 and β-catenin in prostate ($n = 52$) and breast ($n = 66$) tissue microarrays including normal, malignant or metastatic tissues. As shown in Supporting Information Fig S5A, expressions of NDRG1 and membrane β-catenin were significantly down-regulated in prostate and breast cancers. Moreover, membranous β-catenin and NDRG1were positively correlated in both cancers (Supporting Information Fig S5B).

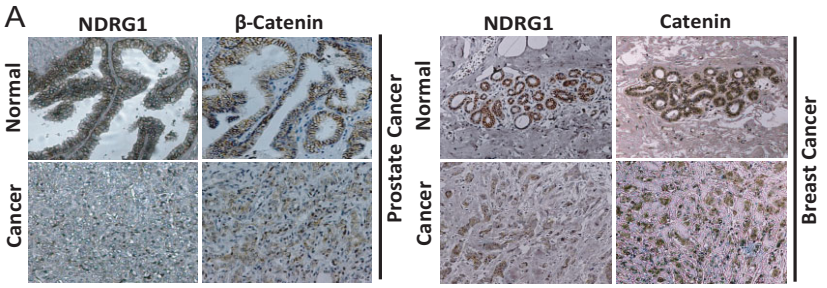
To examine whether expression of these genes predicts clinical outcomes, we analyzed existing multiple cohort data sets of prostate cancer in GEO database. We found that the expression of the genes for LRP6, ATF3 and various Wnt ligands were all significantly up-regulated in patients with metastatic disease compared to those without metastasis (Fig 6C, Supporting Information Fig S5C and D). Notably, tissue from distant metastases showed even higher expression of Wnt than that from primary tumours (Supporting Information Fig S5C and D, left panel). In contrast, NDRG1 expression was significantly down-regulated in metastatic tumours and in distant metastatic sites. These results suggest a multifaceted role of Wnt signalling in promoting cancer metastasis, which can be achieved by the loss of NDRG1 expression. Moreover, we found that patients positive for Wnt or negative for NDRG1 had significantly worse prognosis of metastasis-free survival than their counterpart (Supporting Information Fig S5D, *lowert panels*). To further examine the prognostic value of these markers, we stratified prostate cancer patients into different risk groups based on a combined gene signature (Wnt/LRP6/NDRG1) and analyzed for their relapse-free survival. The results of Cox regression analysis showed that patients in the high- (Wnt+/NDRG1− or LRP6+/NDRG1−) and medium-risk (Wnt−/NDRG1−, Wnt+/NDRG1+, LRP6−/NDRG1− or LRP6+/NDRG1+) groups had a significantly increased risk of recurrent disease compared to the patients in the low-risk group (Wnt−/NDRG1+, LRP6−/NDRG+, Fig 6D).

A small compound restored NDRG1 expression and blocked the metastatic ability of human breast cancer cells *in vivo*

We next explored the possibility that restoration of NDRG1 expression may prevent metastasis *in vivo*. Dp44mT is a highly selective agonist of NDRG1 which enhances NDRG1 mRNA and protein expression (Le & Richardson, 2004; Whitnall et al, 2006). To evaluate an efficacy of Dp44mT on tumour metastasis, we first examined the effect of Dp44mT on the expression of NDRG1 in culture and found that Dp44mT treatment significantly up-regulated NDRG1 by 12–800-fold in mouse (WB1-1) and human cancer cells (MDA-MB231-BoM and PC3mm), while it did not affect the expression in cells carrying the silencing shRNA for NDRG1 (Fig 7A, Supporting Information Fig S6A and C). Consequently, the reactivation of NDRG1 by Dp44mT restored membrane expression of β-catenin followed by suppression of migration and invasion of the tumour cells (Fig 7B, Supporting Information Fig S6B and C). We then evaluated the efficacy of Dp44mT in mice by intracardially injecting human bone metastatic breast cancer cells MDA-MB231-BoM, followed by a short- and long-period of Dp44mT treatment. We found that, at day 9, mice that received only four treatments of Dp44mT showed dramatically reduced circulating tumour cell burden by 85% compared with those that received saline (Fig 7C). Extended treatment of Dp44mT for a period of 4 weeks significantly blocked the incidence and growth of distant bone metastasis without apparent toxicity to the mice (Fig 7D left panel). Importantly, as shown in Fig 7D (middle and right panels), the knock-down of NDRG1 expression in MDA-MB231-BoM cells significantly blocked Dp44mT-induced metastasis suppression, suggesting the high selectivity of Dp44mT Taken together, our results suggest the potential utility of Dp44mT as an agent to prevent and inhibit metastasis disease.

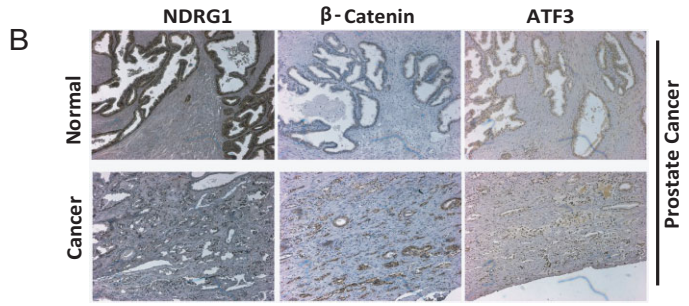
DISCUSSION

Aberrant activation of Wnt pathway is known to be linked to both tumorigenesis and progression of various types of human cancers (Clevers, 2006; Klaus and Birchmeier, 2008; Logan and Nusse, 2004; Moon et al, 2004); however, the role of Wnt signalling in a clinical setting is rather complex and appears to be dependent on types and stages of tumours. Atypical expression of Wnt signalling has been observed at relatively early stages and plays oncogenic roles in colorectal, liver and pancreatic cancers (Doucas et al, 2005; Hoshida et al, 2009; Whittaker *et al*, 2010), while it has been found to be significantly correlated with poor prognosis of metastatic tumours in prostate, breast and lung cancers (Bismar et al, 2004; De la Taille et al, 2003; Dolled-Filhart et al, 2006; López-Knowles et al, 2010). Of note is that the nuclear expression of β-catenin at an early stage is a relatively rare event in prostate and breast cancers (Arenas et al, 2000; Bismar et al, 2004; De la



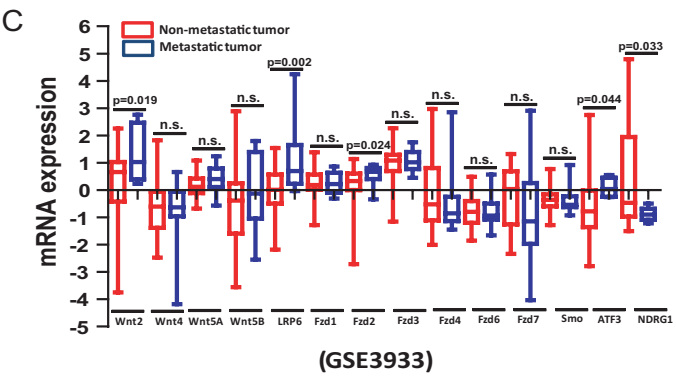
Relationship between NDRG1 and β-catenin

NDRG1	β-catenin expression (membrane) positive	reduced	P-value
Prostate (29)			
positive	11	1	
reduced	8	9	0.036
Breast (33)			
positive	13	5	
reduced	4	11	0.024



Relationship between β-catenin, NDRG1 and ATF3

N=29		β-catenin +	β-catenin -	NDRG1 +	NDRG1 -	Metastasis +	Metastasis -
ATF3	+	9	2	1	10	9	3
	-	5	13	16	2	3	14
P valule		0.015		0.0001		0.007	



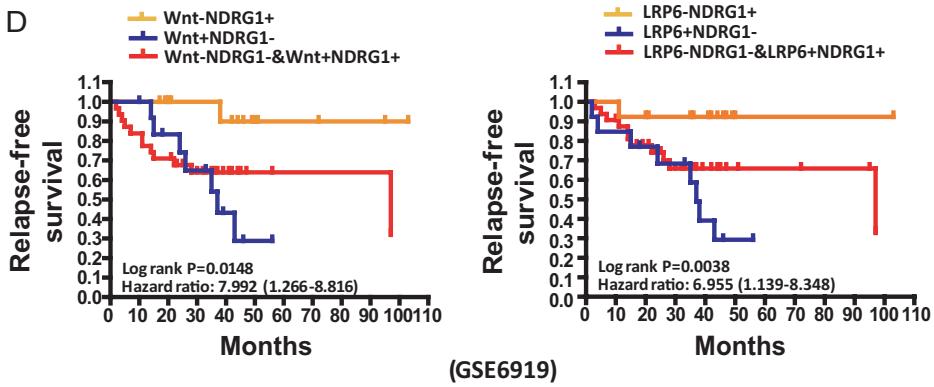


Figure 6. Clinical relevance of NDRG1, β-catenin and ATF3 in prostate and breast cancers.

A. Paraffin block sections of prostate ($n = 29$) and breast ($n = 33$) cancer specimens from patients were subjected to IHC analysis using antibodies to NDRG1 and β-catenin. Upper and lower panels show normal grade and high-grade tumours, respectively. Right panel; correlations of NDRG1 and β-catenin expression were calculated by the χ^2 test.

B. IHC analysis was performed for the same set of prostate cancer specimens to determine the association of ATF3 with NDRG1and β-catenin as well as the patient metastatic status.

C. Expression of NDRG1 and Wnt-related factors in prostate cancer patients were determined using the Lapointe cohort (GSE3933). *p* values were calculated by Mann–Whitney *t*-test.

D. Kaplan–Meier survival analysis to determine the diagnostic value of the combined Wnt, LRP6 and NDRG1 in the database of Yu cohort (GSE6919). The relevance to relapse-free survival of patients was assessed by calculating relative hazard ratio and log rank *p*-value.

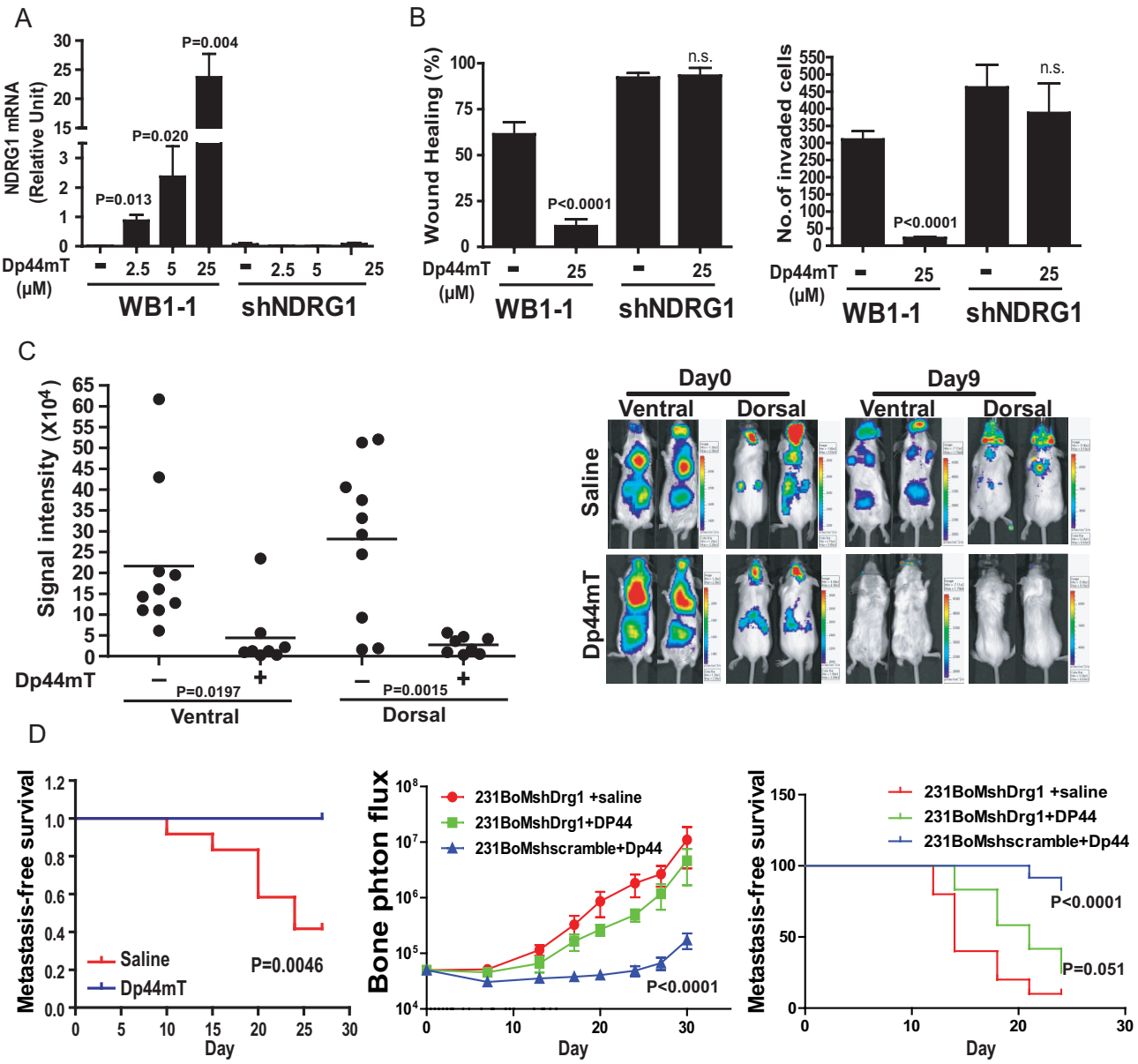


Figure 7. Reactivation of NDRG1 expression by Dp44mT suppresses bone metastasis of human breast cancer cells *in vivo*.

A-B. WB1-1 cells with or without shRNA for NDRG1 were treated with Dp44mT or vehicle alone. NDRG1 mRNA expression was measured by qRT-PCR (**A**), and the motility and invasive abilities of tumour cells were also examined (**B**). Values are from three independent experiments in triplicate. *p* values were calculated by two-sided Student's *t*-test.

C. 231-BoM cells (5×10^4) were inoculated into SCID mice by *i.c.* injection, followed by *i.v.* administration of Dp44mT (0.4 mg/kg) ($n = 8$) or saline ($n = 10$) for four consecutive days. The degree of clearance of the tumour cells in the mice was measured by BLI at day 9. Right panels show representative images of BLI at day 0 and 9. *p* values were calculated by two-sided Student's *t*-test.

D. 231BoM cells (1×10^5) were inoculated into nude mice through intracardiac injection followed by the treatment with Dp44mT (0.4 mg/kg) ($n = 10$) or saline ($n = 12$) for five consecutive days, and the treatment was repeated 4 times with 2 days interval. The left panel indicates metastasis-free survival of the hind limb of mice. *p* values are based on Log-rank test. Middle and right panels: 231BoM/shNDRG1 and 231BoM/shScramble cells (1×10^5) were inoculated into nude mice through intracardiac injection followed by the Dp44mT treatment (0.4 mg/kg, $n = 12$) as above. The middle panel shows the strength of BLI signal of bone metastasis. Data are presented as the mean photon flux, error bars represent the SEM. *p* values were calculated by two-sided Student's *t*-test. The right panel indicates metastasis-free survival of mice. *p* values were calculated by Log-rank test.

Taille et al, 2003; Dolled-Filhart et al, 2006; Khramtsov et al, 2010; López-Knowles et al, 2010). Recently, Nguyen et al have shown that Wnt-β-catenin signalling specifically promoted brain and bone metastasis of human lung adenocarcinoma while it did not affect primary tumour growth (Nguyen et al, 2009). Their results are consistent with the clinical observation that aberration of Wnt signalling in lung adenocarcinoma is relatively infrequent (Ding et al, 2008; Sharma et al, 2007), and therefore, Wnt likely contributes to metastatic progression rather than initial tumorigenesis. In another study, DiMeo et al (2009) have identified Wnt signalling as a lung metastasis signature to predict the outcome of breast cancer patients. Both studies showed that Wnt signalling had little effect on proliferation and viability of cancer cells, while it significantly promoted metastatic phenotypes of cancer cells including self-renewal, EMT and chemotactic invasion. In our model of WB1-1 cell, blocking Wnt signalling by NDRG1 led to drastic suppression of metastatic phenotypes of this cell line both *in vitro* and *in vivo*, while it did not affect cell growth in culture or in animals. Previous studies showed that strong activation of Wnt signalling, besides its well-known role in carcinogenesis, was significantly correlated with tumour stages and metastasis in colorectal cancer (Fodde et al, 2001; Inomata et al, 1996). In particular, Wnt-β-catenin downstream factors, BAMBI (bone morphogenetic protein and activin membrane-bound inhibitor) and S100A4/metastasin, was found to promote spontaneous metastasis of colorectal cancer cells *in vivo* (Fritzmann et al, 2009; Stein et al, 2006), and the expression of these two effector genes have been shown to predicts metastasis potential of colon cancer (Fritzmann et al, 2009; Sherbet, 2009; Togo et al, 2008). On the other hand, the proto-oncogene, Bcl9 promotes nuclear translocation of β-catenin followed by increased transcriptional activation of BAMBI (Fritzmann et al, 2009; Mani et al, 2009). These results suggest that activation of Wnt signalling leads to two distinct pathways to induce initial tumorigenesis or metastatic progression depending on the different transcriptional output of Wnt signal in a cell context dependent manner. It is considered that different Wnt downstream targets display differential sensitivity to the upstream β-catenin/TCF, which may tilt the balance of Wnt signalling in controlling tumour growth and metastasis. Therefore, our results that NDRG1 suppressed metastasis without affecting primary tumour growth may reflect the intrinsic regulatory mechanism by which Wnt signalling controls tumorigenesis and metastasis that are two distinct processes during tumour progression.

In prostate and breast cancers, loss of NDRG1 expression has been consistently linked to tumour progression and metastasis status as well as poor survival of cancer patients (Ellen et al, 2008; Kovacevic and Richardson, 2006). We previously showed that NDRG1 blocked metastasis in an animal model and that this suppressor effect is mediated by modulating ATF3, which functions as a strong pro-metastatic transcription factor (Bandyopadhyay et al, 2006a,b). Here, we found that ATF3 is the direct target of β-catenin and that activation of Wnt signalling significantly augmented its expression. Notably, ATF3 is known to activate EMT-inducing factors, Slug and Twist (Yin et al, 2008), and we found that they were indeed activated by Wnt signalling; however, this activation was significantly blocked by NDRG1. Interestingly, Yan et al (2011) recently showed that ATF3 acts as an oncogene to drive the mouse mammary tumorigenesis by activating Wnt-β-catenin signalling, suggesting a positive feedback regulation of Wnt-β-catenin-ATF3 axis. On the other hand, the NDRG1-mediated Wnt suppression restored epithelial cell–cell adhesion complex (E-cadherin-β-catenin), whose integrity is essential for epithelial cell morphology, polarity and motility (Heuberger and Birchmeier, 2010). NDRG1 expression was indeed found to be significantly associated with more differentiated and epithelial phenotype of prostate and breast cancer cells. Therefore, NDRG1 exerts its metastasis suppressor ability by blocking Wnt signalling, followed by down-regulation of ATF3, as well as restoring intracellular adhesion of tumour cells, which blocks the EMT process and abrogates subsequent acquisition of metastatic capabilities by tumour cells.

Consistent with the role of Wnt signalling in prostate and breast cancer progression (Bismar et al, 2004; Dolled-Filhart et al, 2006; López-Knowles et al, 2010), we observed significantly enhanced expressions of Wnt components in these cancer patients at advanced stages, which further strongly linked Wnt with tumour progression and poor clinical outcomes. Importantly, we found that a combination of NDRG1, Wnt ligands and LRP6 can be used to predict relapse-free survival in prostate cancer patients, and that a combination of Wnt ligands, LRP6 and ATF3 had a significant inverse correlation with metastasis-free survival of breast cancer patients. Therefore, these signatures would be useful diagnostic markers to determine the treatment course of patients with metastatic disease. Finally, because blocking any step of the metastasis cascade is believed to effectively prevent metastasis, restoring the expression of negative regulators of metastatic process such as NDRG1 is an attractive option for therapeutic intervention. We have shown that Dp44mT was capable of reactivating NDRG1 expression in metastatic tumour cells and blocking their invasive ability. Importantly, administration of Dp44mT significantly suppressed the systemic spread of tumour cells and the incidence of distant metastasis in our animal models without showing notable toxicity. Therefore, Dp44mT in combination with other Wnt pathway inhibitors may serve as an effective regimen for prevention and therapy for metastatic disease.

MATERIALS AND METHODS

Yeast two-hybrid screening

A full-length cDNA of NDRG1 was cloned into the yeast vector pEG202-NLS (Origene Technologies) as a bait, the yeast two-hybrid screening was carried out using a cDNA library of normal human prostate tissue followed by performing a mating assay according to the manufacturer's protocol.

Quantitative β-Galactosidase assay

β-Galactosidase assay (Miller test) was performed as previously described (Bandyopadhyay et al, 2006a,b).

Cell culture

Human prostate cancer cell line PC3mm was kindly provided by Dr. I. J. Fidler (The University of Texas M. D. Anderson Cancer Center. Houston. TX). Human breast cancer cell line MCF7 was obtained from American Type Culture Collection (Manassas, VA). The PC3mm/Tet cell line was established previously as a derivative of PC3mm and contains a tetracycline-inducible suppressor. MCF7/Tet cell line was established by a similar method. Mouse mammary tumour cells were isolated from the mammary adenocarcinoma of MMTV-Wnt1 mice using a similar procedure as previously described (Orimo et al, 2000). L/Wnt3a cells and mouse lung microvasculature endothelial cells (mLMVECs) were obtained from ATCC. HEK293/TOP Flash cells carrying a TOPFlash reporter were established in our lab. All cells were maintained in RPMI 1640 medium supplemented with 10% foetal calf serum, streptomycin (100 μg/ml), penicillin (100 U/ml) and 250 nM dexamethasone (Sigma Chemical Co., St. Louis, MO) and grown at 37°C in a 5% CO_2 atmosphere. For all transfection experiments, LipofectAMINE 2000 (Invitrogen) was used according to the manufacturer's protocol.

Wnt conditioned medium (WNT-CM)

Wnt-CM was prepared by collecting the supernatants from L/Wnt3a cells stably expressing WNT3a. The collected WNT-CM was applied to 293/TOPFlash cells, which was then subjected to luciferase assay to determine the Wnt activity.

Reporter assay

The chloramphenicol acetyl transferase (CAT) reporter plasmid of the ATF3 promoter was described previously (Bandyopadhyay et al, 2006a). Briefly, 48 h after transfection of the CAT reporter plasmids DNAs, cells were collected and then subjected to CAT assay. The reaction was done and acetylated [^{14}C] chloramphenicol was quantified with a Phosphor Imager (Packard Instrument, Meriden, CT). For TOPflash assay, the TOPflash reporter plasmid was transfected into cells. The luciferase activities were then measured by using Dual-Luciferase Reporter Assay System (Promega, Madison, MI) and a Luminometer (Berthold Detection Systems, Huntsville, Alabama). For each transformation experiment, the Renilla expression plasmid phRG-TK (Promega) was co-transfected as an internal control and the promoter activities were normalized accordingly. Because WB1-1 series are luciferase expressing cell lines, we constructed TOPRenilla plasmid, in which firefly luciferase reporter was replaced by Renilla luciferase, and TOPRenilla assay was performed. When appropriate, a GFP-expressing plasmid was co-transfected into cells as an internal control.

Chromatin immunoprecipitation assay

PC3mm/Tet/Flag-NDRG1 cells were cultured in T75 flasks and fixed with 37% formaldehyde for 10 min at room temperature. To stop the reaction, 125 mM glycine was added to the culture medium, and the cells were washed with phosphate buffered saline (PBS) and harvested. Cells were then suspended in cell lysis buffer (5 mM PIPE, 85 mM KCl, 0.5% Nonidet P-40) and homogenized with a type A Dounce homogenizer. The cell nuclei were collected and lysed with Nuclei lysis buffer (50 mM Tris–HCl, 10 mM EDTA (ethylenediamine-tetraacetic acid), 1% SDS (sodium dodecyl sulfate)). The chromatin was sonicated on ice to an average length of 400 bp. The sample was then centrifuged at 4°C and the precipitates were resuspended in ChIP dilution buffer (16.7 mM Tris–HCl, 167 mM NaCl, 1.1% Triton X-100, 0.01% SDS). After pre-clearing the sample with Protein G agarose beads (Dynal Biotech, Invitrogen, Carlsbad, California) followed by brief centrifugation, the supernatant was transferred to a new tube and anti-β-catenin (Santa Cruz) antibody was added. After overnight incubation at 4°C, Protein G agarose beads were added, and the sample was incubated for 3 h at 4°C. Beads were then washed with washing buffer (100 mM Tris–HCl, 500 mM LiCl, 1% NP-40, 1% deoxycholic acid), and DNA-protein complexes were eluted with Elution buffer (100 mM $NaHCO_3$, 1% SDS). DNA-protein was de-crosslinked followed by phenol extraction, and the purified DNA was subjected to qPCR using both specific F1R1 (5′-CCCTTCCTCCGCTCCG-TTCGG-3′ and 5′-CCTGGCTGCGTGCGACTGTGGC-3′) and non-specific primers F2R2 (5′-TACCACTCGCCCTAGTTTC-3′ and 5′-GTGTCCAAAGT-CACCCTTCG-3′) for the β-catenin/TCF binding sequence of the ATF3 promoter. The qPCR products were also visualized after electrophoresis on 8% acrylamide gel followed by staining with ethidium bromide.

Adhesion assay

Mouse lung microvascular endothelial cells were grown to confluence in 24-well plates. Before seeding the tumour cells, mLMVECs monolayers were washed three times with PBS. Tumour cells were stained by CellTracker Green CMFDA (Invitrogen) for 30 min and briefly trypsinized and counted. 2×10^5 cells tumour cells were then seeded in each well, and allowed to adhere to the monolayer for 30 min. Plates were washed three times, and subjected to a fluorescent microscopy to count the number of cells. Alternatively, if cells expressed luciferase, they were trypsinized and lysates were subjected to luciferase activity assay using the Dual-Luciferase Reporter Assay System.

Invasion and wound healing assays

For invasion assays, 1×10^5 cells were seeded in a Matrigel-coated upper chamber (BD Biosciences) in serum-free media, and 20% foetal bovine serum was added to the bottom chamber followed by incubation for 24 h at 37°C. The upper chamber was removed, and the cells that invaded through the membrane were stained with tetrazolium dye and counted under microscope. Triplicate tests were performed in each case. For wound healing assays, tumour cells were pulsed with 5 μM CellTracker Green CMFDA (Invitrogen) for 30 min, and seeded in a 6-well plate and cultured to a confluent monolayer. The 'scratch' was introduced by scraping the monolayer with a p200 pipette tip and marked for orientation and photographed by a fluorescent microscope immediately and after 24-, 48- and 72 h.

Lung extravasation assay

Tumour cells were pulse labelled by incubating with 5 μM CellTracker Green CMFDA (Invitrogen) for 30 min and inoculated into the circulation of mice through tail vein. Mice were sacrificed 2- or 48 h later, and lungs were inflated with PBS, soaked with 30% sucrose in PBS +2 mM $MgCl_2$ at 4°C overnight, they were then embedded in OCT for frozen sectioning. Lung vasculature was visualised by rhodamine-conjugated lectin and tumour cells in the lung were visualised under fluorescent and confocal microscope.

The paper explained

PROBLEM:

Majority of cancer deaths is attributed to metastatic disease; however, the exact molecular mechanism of this disease is largely unknown due to its intricate pathological process. A potential loss of metastasis suppressor has been implicated to contribute to this pathway. Previously, we showed that NDRG1 is a metastasis suppressor in prostate and breast cancers. Herein, we addressed the question how NDRG1 gene exerts its metastasis suppressor function. During the course of our study to address this question, we serendipitously found that NDRG1 is capable of modulating Wnt-β-catenin signalling, which is an evolutionally well-conserved signalling pathway in normal development.

RESULTS:

Herein, we reported that the tumour metastasis suppressor gene, NDRG1, pleiotropically suppresses tumour metastasis through modulation of the canonic Wnt-β-catenin signalling. Importantly, we found that Wnt/NDRG1 signature has a strong predictable value for recurrence-free survival of cancer patients. Restoring NDRG1 expression by a small molecule compound significantly suppressed the capability of otherwise highly metastatic tumour cells to thrive in the circulation and distant sites *in vivo*.

IMPACT:

We have elucidated the molecular mechanism by which NDRG1 functions as a metastasis suppressor. Our findings not only revealed an intriguing cross-talk mechanism between the critical developmental signalling (Wnt) and a metastasis suppressor gene (NDRG1), but also provided the molecular basis by which Wnt signalling modulates tumour progression. We also found a unique signature of Wnt/NDRG1 pathway which has strong predictable value for recurrence-free survival of cancer patients. We believe that our newly discovered mechanism of metastasis suppression by NDRG1 opened a window of opportunity to identify a novel therapeutic target for metastatic disease.

Bioinformatics and statistical analysis

Results are reported as mean ± SD (or mean ± SEM) as indicated in figure legends. Statistical significance was determined by two-sided Student's *t*-test. Oncomine and GEO database were used to evaluate the clinical relevance of NDRG1, Wnt ligand (Wnt1, Wnt2 and Wnt3), LRP6 and ATF3 in prostate and breast cancers. For clinical cohort dataset analysis, we downloaded five publicly available prostate and breast cancer data sets including Taylor (GSE21034, $n = 218$), Lapointe (GSE3933, $n = 112$), Yu (GSE6919, $n = 60$) and MSK (GSE2603, $n = 82$, GSE14020, $n = 57$). The data was log-2-transformed, with the median set as 0 and SD set as 1. Each patient was assigned to have positive or negative expression of each gene and was matched with relapse-free survival data (GSE6919) and metastasis-free survival data (GSE21034 and GSE2603). The gene expressions in primary tumour (GSE3933 and GSE 2603) or metastatic sites (GSE14020) in patients who have either metastatic or non-metastatic disease were compared by Box-and-whisker plot analysis and evaluated by Mann–Whitney test. The association between genes and clinical outcomes was calculated by Pearson χ^2 test. The Kaplan–Meier method was used to calculate the overall survival rate, and prognostic significance was evaluated by the Log-rank test. Univariate or multivariate analysis for the prognostic value of gene signatures was performed by the Cox proportional hazard-regression model. For all statistical analysis, Prism and SPSS software were used.

Immunohistochemistry

Immunohistochemical analysis on paraffin-embedded, surgically resected specimens of prostate and breast was carried out using anti-NDRG1 rabbit polyclonal antibody (1:100), anti-ATF3 rabbit polyclonal antibody (1:500, Santa Cruz, CA) and anti-β-catenin rabbit polyclonal antibody (1:500, Santa Cruz, CA). Briefly, sections were deparaffinized, rehydrated and heated at 80°C for 20 min in 25 mM sodium citrate buffer (pH 9) for antigen exposure. They were then treated with 3% H_2O_2 to block endogenous peroxidase activity and further incubated with primary antibody for 1 h at 24°C. After washing with Tris-buffered saline/0.1% Tween-20, the sections were incubated with horseradish peroxidase-conjugated rabbit-specific IgG (Dako Corp.). The sections were washed extensively and DAB substrate chromogen solution was applied followed by counterstaining with hematoxylin.

Animal studies

Four to six weeks old SCID mice (Harlan Sprague-Dawley, Indianapolis, IN) were used for the spontaneous metastasis studies. Viable tumour cells were resuspended in 50:50 solutions of PBS and Matrigel, and 5×10^5 cells were injected into the thoracic mammary fat pad. The primary tumour and metastasis outgrowth were monitored twice a week using bioluminescent imaging (BLI) with IVIS® Imaging System (Xenogen, Hopkinton, MA). After 6 weeks, mice were sacrificed and lungs and bones were extracted, the metastatic lesions on the individual lungs and bones were evaluated by *ex vivo* imaging and histologic analysis. Tumour growth was also monitored by measuring the tumour length (L) and Width (W). Tumour volume was calculated as (width + length)/2 × width × length × 0.5236. Dp44mT (Sigma, Mo) was dissolved in 15% propylene glycol (Fisher) in 0.9% saline, and injected intravenously into mice over five consecutive days. All protocols were approved by the Southern Illinois Universtiy Institutional Review Board. For histological analysis, tumour and/or lungs were excised, fixed in 10% neutral-buffered formalin and embedded in paraffin. They were then subjected to immunocytofluorescence staining with specific antibodies or staining with hematoxylin and eosin (H&E, American Histolabs Inc.).

For more detailed Materials and Methods see the Supporting Information.

Author contributions

WL and KW developed the hypotheses, designed the experiments and wrote the manuscript. WL performed most experiments and analyzed data. MIG conducted experiments including design and generation of expression constructs, yeast mating assay, Western blotting and qRT-PCR. PRP assisted with generation of deletion constructs and yeast mating assay. HO helped in isolation of WB1-1 cells and clinical cohort data analysis. MW provided technical support in tissue culture. SKP performed immunohistochemistry and provided pathological review. SH provided prostate and breast cancer patients' specimens and pathological review. FX, AK and KF helped in mouse work. YYM provided key experimental reagents and experimental advice. YL provided MMTV-Wnt mice. KW directed this study.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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