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

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*Corresponding author: Watabe Kounosuke, Southern Illinois University School of Medicine***Review timeline:**

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

18 July 2011

Thank you for the submission of your manuscript "N-myc downstream regulated gene 1 modulates Wnt- β -catenin signaling and pleiotropically suppresses metastasis" to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, Reviewers #1 and #4 highlight that more details regarding Dp44mT specificity and a selectivity profile need to be provided. Importantly, Reviewer #4 feels that additional data to differentiate between effects of NDRG1 on canonical signaling/transcription and E-cadherin-associated beta-catenin are required. In addition, this Reviewer feels that loss of function studies should be carried out.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged differently with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor

EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The manuscript uses state-of-the-art methods to describe a signaling pathway involved in metastasis, coupling the experimental data with clinical linkages. The findings are potentially very important for developing anti-metastatic treatments. The fact that prostate and breast models were employed increases likelihood that the findings can be extrapolated further.

Referee #1 (Other Remarks):

This is a well conceived, logical and experimentally well done manuscript. The conclusions and interpretation are consistent with the findings. The authors have been careful to word their findings in such a way as to ensure clarity.

There is one aspect of the manuscript that needs to be corrected throughout -- fold change refers to INCREASES and cannot be properly used to describe decreases from a baseline (as the wording is ambiguous). When values decrease, they should be referred to as percentage decrease from control or percent of control.

Also, more details regarding Dp44mT need to be provided -- is it a small molecule or peptidomimetic? Some validating data showing selectivity (since no agent is 'specific') should be provided in the supplemental data section.

Referee #2 (Comments on Novelty/Model System):

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Referee #2 (Other Remarks):

This manuscript shows that the metastasis suppressor gene NDRG1 interacts with the Wnt receptor LRP6 and inhibits this receptor and blocks Wnt signaling. Remarkably, a small NDRG1-interfering molecule suppressed metastasis formation. Moreover, it was shown that ATF3 is a Wnt/ β -catenin target in these processors.

Minor points to be corrected:

- 1) Page 5: Colony formation does also reflect growth and expansion of tumor cells in primary tumors. Not only invasiveness into distant organs. The colony formation assay has been used by the oncogene researchers for 30 years.
- 2) Page 12: Say that ATF3 is a direct target, not the target.
- 3) In General: A lot of work has been performed on, for instance, colon cancer metastasis. And several of the involved molecules have been discovered that promote not growth but in particular metastasis depending on Wnt. These are Bambi, S100/A4-metastasin, BCL9-2. In particular, Bambi has been shown to be a predictable marker for metastasis formation and induces experimental metastasis formation, as shown any two groups. These publications need to be cited.

Referee #3 (Other Remarks):

This is a very well conducted study, with tight experimental design and controls, encompassing not only overexpression studies, but also knockdown and agonist studies, both in vitro and in vivo. I have only very minor comments.

- 1) The authors mention that they looked at Wnts 1,2 and 3A. Did any other Wnts come up on the microarray? Wnt5A for example, while not a known activator of beta-catenin, has been implicated in prostate cancer metastasis. What about frizzled receptors?
- 2) On page 8, the authors state that "Strikingly, however, WB1-1 cells with constitutively active β -

catenin ($\Delta N90$ and $\Delta N90/NDRG1$) exhibited similar, if not less aggressive abilities of migration, anoikis resistance, adhesion as well as survival in the lungs despite over-expression of NDRG1." I think this should read "...exhibited similar if not MORE aggressive...." based on the data.

3) The same is true for the sentence: "a combination of Wnt ligands, LRP6 and ATF3 was significantly correlated with metastasis-free survival of breast cancer patients." Again, I think that should read was inversely correlated with metastasis-free survival.

Referee #4 (Comments on Novelty/Model System):

Please see remarks to author

Referee #4 (Other Remarks):

The key finding detailed in this manuscript is that NDRG1 can bind to Lrp6 and inhibit Wnt signalling. The expression of ATF3, a Wnt target is attenuated and this results in the inhibition of a number of cellular outputs related to metastasis. This study presents an intriguing explanation of how NDRG1 exerts its anti-metastatic effects, namely by inhibition of Wnt signalling, a contributor to this process. This manuscript however does require further clarification on the following points:

NDRG1 was originally identified as a factor upregulated by N-Myc expression. C-Myc is a validated, functionally relevant target gene of the Wnt pathway and indeed, is the key effector of mutant Wnt-pathway-induced intestinal tumorigenesis in the mouse. Both N- and C-Myc are negative regulators of NDRG1 expression. Are either C- or N-Myc Wnt pathway target genes in PC3mm and MCF7 cells? Does this restrict NDRG1 expression? Does the expression of NDRG1 change with Wnt signalling?

In Figure 1F and G, the authors show that the majority of endogenous and overexpressed β -catenin is reduced with NDRG1 overexpression. The major pool of β -catenin in epithelial cells is cytoskeletal-associated β -catenin and a very minor amount of Wnt-signalling associated, transcriptionally active β -catenin. Can the authors discriminate whether they are affecting both pools or just one of them. This is pertinent because they make a case for effects on metastasis mediated through transcription (ie. of ATF3) however, the results of the metastasis assays (ie. susceptibility to anoikis, attachment to ECs, cellular morphology changes) can be rationalized as effects on the cytoskeletal, E-cadherin-associated pool of β -catenin rather than on canonical signalling (ie. transcriptional effects).

How promiscuous/specific is the effect of NDRG1 expression on phosphorylation in the phospho-site profiling analysis - Were Akt and GSK3 the only two proteins whose phosphorylation state was affected? How does NDRG1 inhibit Akt phosphorylation - is this downstream of NDRG1-Lrp6 binding or is this an additional function of NDRG1?

The authors demonstrate overexpression of NDRG1 inhibits Wnt signalling through LRP6 binding, and this inhibits phosphorylation within its GSK3 pseudo-substrate domain thus maintaining the activation state of this kinase. What about in the absence of Wnt signalling? Is Wnt signalling ectopically active when cells are depleted of NDRG1? Is Wnt signalling potentiated in the absence of NDRG1? Cell-based studies using both Dp44m and the shRNA system used to validate this compound (Figs 7A and B) will allow these loss-of-function studies to be carried out.

Is Wnt-induced expression of Slug and Twist dependant on ATF3 expression fitting in with the hypothesis that Wnt signalling drives ATF3 expression (Fig. 2)? Or is it the reverse (ie. Yan et al. (2011) PlosOne 6(1):e16515 - this paper should be referenced.)?

In the clinical studies in Fig. 6 A and B membrane associated β -catenin is present in normal tissue but absent in cancerous tissue. The image quality is not high enough that the reader can tell whether β -catenin is absent or whether there is increased cytoplasmic/nuclear β -catenin in these cells as is mentioned in the text. Why do the authors use membranous/cytoplasmic vs cytoplasmic/nuclear as their distinction for β -catenin levels and not membranous versus nuclear as is convention? Does nuclear:membranous β -catenin increase in cancer samples? Is this the case, is there a negative

correlation with NDRG1 expression? What is the baseline for this (ie. normal tissue)?

The compound Dp44m is an iron chelator, with anti-tumorigenic properties arising from a number of its properties including its ability to activate NDRG1, VEGF and TfR1 expression (through HIF-1). Accordingly, the statement that Dp44m is a 'specific' agonist of NDRG1 is not correct. The authors do provide some validation it is an agonist of NDRG1 expression in cell culture, however the pleiotropic effects an iron chelator would have in whole organism assays (Fig. 7C) renders data interpretation contentious. These experiments should be complemented with a loss of function approach using the shRNA system (used to validate this compound - Figs 7A and B).

Minor points:

Fig. 1 - MCF7-tet should be mentioned in the text on pg 4 with the PC3mm/tet line

Does Wnt signalling increase 'nuclear localization and expression specifically' or the general expression of ATF3 (pg 5)? This is not clear from Fig S1D.

Pg 6 - Fig 2B should be changed to 3B

Fig.2D - This experiment should be done using real-time PCR (eg. As in Figs S1C, 5g) as the current assay is not quantitative.

Is the scale in Fig. S3E correct?

Pg 9 - Fig. 6C should read Fig. 5C

Pg. 10 - Fig. S6A and B should read Fig. S6A and C and further down the paragraph, Fig. S6B and C.

What are the 2 panels for 6D?

1st Revision - authors' response

25 October 2011

Referee #1

1. *There is one aspect of the manuscript that needs to be corrected throughout -- fold change refers to INCREASES and cannot be properly used to describe decreases from a baseline (as the wording is ambiguous). When values decrease, they should be referred to as percentage decrease from control or percent of control.*

We have corrected the description throughout the text.

2. *Also, more details regarding Dp44mT need to be provided -- is it a small molecule or peptidomimetic? Some validating data showing selectivity (since no agent is 'specific') should be provided in the supplemental data section.*

We appreciate this comment. Dp44mT (di-2-pyridylketone-4,4,-dimethyl-3- thiosemicarbazone) is a small molecule compound (F.W. 285.). Regarding the selectivity of Dp44mT, we performed an additional experiment to provide further information (Fig. 7D). Please also refer to our response to Ref 4, Q7.

Referee #2

1. *Page 5: Colony formation does also reflect growth and expansion of tumor cells in primary tumors. Not only invasiveness into distant organs. The colony formation assay has been used by the oncogene researchers for 30 years.*

We agree with the reviewer that using colony formation assay to assess tumor cells growth in anchorage independent condition can reflect both transformation and invasiveness of tumor cells. Therefore, we stated that “....suggesting that NDRG1 affects invasiveness and outgrowth of tumor cells....” in the text. However, in addition to the already known functions of NDRG1, the results of our *in vitro* functional assays have clearly shown that NDRG1 indeed affected invasiveness, migration and other features of malignant tumor cells for tumor progression. Most importantly, the previous data from our lab and others indicate that NDRG1 expression inhibited spontaneous metastasis but not primary tumor growth in animal models. Taken together, we speculate that the result of our colony formation assay reflected mainly the effect of NDRG1 on the invasiveness.

2. *Page12: Say that ATF3 is a direct target, not the target.*

We have corrected it as suggested.

3. *In General: A lot of work has been performed on, for instance, colon cancer metastasis. And several of the involved molecules have been discovered that promote not growth but in particular metastasis depending on Wnt. These are Bambi, S100/A4-metastasin, BCL9-2. In particular, Bambi has been shown to be a predictable marker for metastasis formation and induces experimental metastasis formation, as shown any two groups. These publications need to be cited.*

Thank you for this suggestion, and we have incorporated this information in the Discussion section (page 12).

Referee #3

1. *The authors mention that they looked at Wnts 1,2 and 3A. Did any other Wnts come up on the microarray? Wnt5A for example, while not a known activator of beta-catenin, has been implicated in prostate cancer metastasis. What about frizzled receptors?*

We examined the expression of Wnt ligands in various cohort data sets (GSE3933, GSE6919, GSE21034). Because available probes for Wnt ligand are different depending on the cohort, it is somewhat difficult to discuss the uniformity; however, Wnt ligands are generally up-regulated in metastatic tumors, particularly Wnt 2 (GSE3933, Fig. 6C), Wnt1,5A and 11 (GSE6919, Fig.S5C) and Wnt 3 (GSE21032, Fig. S5D) are significantly up-regulated. As to Wnt 5A, the expression of this ligand tends to be higher in metastatic tumor compared to non-metastatic tumors, while it is significantly lower in lymph node metastasis. In GSE6919, the expression of Wnt5a is significantly higher in metastatic tumor than primary cancers. The results of these analyses is now included as Fig. 6C and Supplemental Fig. 5C. Regarding Frizzled, because NDRG1 interacts with LRP6 which is the co-receptor of Frizzled and upstream factor of Wnt signaling, we did not expect that the expression of Frizzled would be affected by NDRG1. However, we found that Fzd2 was expressed at significantly higher levels in metastatic tumor compared to non-metastatic tumors in the GSE3933 cohort. Expressions of Fzd4 and Fzd6 were found to be significantly increased in lymph node metastasis compared to that in local tumor. In contrast, several Fzds including Fzd1, Fzd2 and Fzd7 express at significantly lower levels in metastatic tumors compared to non-metastatic tumor in GSE6919. These are interesting data and warrant further studies for the frizzled receptor. However, it is outside the focus of the current study.

2. *On page 8, the authors state that "Strikingly, however, WB1-1 cells with constitutively active β -catenin $\Delta N90$ and $\Delta N90/NDRG1$) exhibited similar, if not less aggressive abilities of migration, anoikis resistance, adhesion as well as survival in the lungs despite over-expression of NDRG1." I think this should read "...exhibited similar if not MORE aggressive...." based on the data.*

We have corrected it as suggested

3. *The same is true for the sentence: "a combination of Wnt ligands, LRP6 and ATF3 was significantly correlated with metastasis-free survival of breast cancer patients." Again, I think that should read was inversely correlated with metastasis-free survival.*

We have corrected it as suggested.

Referee #4

1. *NDRG1 was originally identified as a factor upregulated by N-Myc expression. C-Myc is a validated, functionally relevant target gene of the Wnt pathway and indeed, is the key effector of mutant Wnt-pathway-induced intestinal tumorigenesis in the mouse. Both N- and C-Myc are negative regulator of NDRG1 expression. Are either C- or N-Myc Wnt pathway target genes in PC3mm and MCF7 cells? Does this restrict NDRG1 expression? Does the expression of NDRG1 change with Wnt signalling?*

As the reviewer collectively pointed out, overexpression of Myc genes was indeed found in prostate and breast cancers, and they might be negative factors on NDRG1 expression in both cancers. c-Myc and N-Myc were shown as downstream targets of β -catenin/TCF in colorectal cancer (Science 1998,281:1509-12) and mesenchyme of limbs (Development 2008,135:3247-57), respectively. In addition, NDRG1 was found to be significantly down-regulated in the mammary tumor of MMTV-Wnt1 mice by microarray analysis (J Bio Chem. 2011,286:18949-59). As suggested by the reviewer, to determine whether c-Myc or N-Myc restricts the expression of NDRG1, and whether the expression of NDRG1 changes with Wnt signaling in prostate cancer cells, we silenced N-myc and c-Myc by shRNA followed by examining the expression of NDRG1 in the presence or absence of Wnt in PC3mm/Tet cells by qRT-PCR. We found that knocking-down of either c-Myc or N-myc indeed significantly up-regulated the NDRG1 expression; however, Wnt treatment significantly abrogated this effect. 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 signaling. These results are added as Supplementary Fig. S1G.

2. *In Figure 1F and G, the authors show that the majority of endogenous and overexpressed β -catenin is reduced with NDRG1 overexpression. The major pool of β -catenin in epithelial cells is cytoskeletal-associated β -catenin and a very minor amount of Wnt-signalling associated, transcriptionally active β -catenin. Can the authors discriminate whether they are affecting both pools or just one of them. This is pertinent because they make a case for effects on metastasis mediated through transcription (ie. of ATF3) however, the results of the metastasis assays (ie. susceptibility to anoikis, attachment to ECs, cellular morphology changes) can be rationalized as effects on the cytoskeletal, E-cadherin-associated pool of β -catenin rather than on canonical signalling (ie. transcriptional effects).*

As shown in Fig.1F, G and Fig.3B, we found that NDRG1 affected the expression and the localization of β -catenin. In Fig.3B (right panel), we showed that Wnt treatment enhanced both cytosolic and nuclear β -catenin expression in PC3mm cells. Induction of NDRG1 enhanced membrane β -catenin (cytoskeletal-associated β -catenin) in the absence of Wnt, while it decreased the overall expression of β -catenin in the presence of Wnt. Consistently, Fig.1G showed that NDRG1 reduced the overall amount of Wnt-induced β -catenin expression in the PC3mm cells. This overall down-regulation of β -catenin is likely due to the degradation of this protein because we found that NDRG1 strongly increased phosphorylation of β -catenin at Ser33/Ser37/Thr41 residues (the result is added as Supplementary Fig. S1F). The phosphorylation of these residues is a known target of GSK3 β for the subsequent degradation of β -catenin. We have indeed shown that GSK3 β was strongly activated by NDRG1 (Fig. 1D). Taken together, our results indicate that NDRG1 affects both pools of β -catenin, and that the net result of this NDRG1-mediated overall down-regulation and enhancement of membrane localization of β -catenin suppress the Wnt-induced metastatic traits of cancer cells. In this context, it should also be noted that β -catenin is an essential molecule both in E-cadherin-mediated cell adhesion (the cytoskeletal pool) and in canonical Wnt signaling (the cytosolic and nuclear pool), as the reviewer correctly pointed out. It is known that

there is a crosstalk between these two processes through β -catenin because many target genes of Wnt- β -catenin signaling influence cadherin adhesion. The most well-known of these Wnt target genes are EMT transcription factors, Twist and Slug, that directly inhibit the E-cadherin expression (note that we have shown that Wnt-induced Twist/Slug expression was blocked by NDRG1 (Fig. 3C)). Others include MMPs (Oncogene 1999,18:2883-91; BMC Develop Bio 2003 3:2) and the cell adhesion molecule L1 (Cancer Res 66:11370-80), which promote the degradation of E-cadherin. ADAM10, a recently identified beta-catenin/TCF target gene, cleaves E-cadherin followed by loosening cell adhesion (Cancer Res 2007 67:7703-12). Furthermore, loss of E-cadherin-mediated cell adhesion releases β -catenin and correlates with increased β -catenin-dependent transcription. Our results indeed showed that NDRG1 interferes with Wnt- β -catenin signaling mediated transcriptional activity (Fig.1E, 2A, 3C and Fig.S2C) and cadherin adhesion (Fig.3B, Fig.S2D ,E), which ultimately blocks the pro-metastatic function of this pathway.

3. *How promiscuous/specific is the effect of NDRG1 expression on phosphorylation in the phospho-site profiling analysis - Were Akt and GSK3 the only two proteins whose phosphorylation state was affected? How does NDRG1 inhibit Akt phosphorylation - is this downstream of NDRG1-Lrp6 binding or is this an additional function of NDRG1?*

We initially screened 20 key signaling molecules for their phosphorylation status using phospho-specific antibodies (added as Supplementary Fig. 1C) followed by verification by repeated Western blotting. We found that GSK3 β and Akt were the only two proteins whose phosphorylation status was affected by NDRG1 (Fig. 1D). Although our screening was limited due to availability of phospho-specific antibodies, the effect of NDRG1 appears to be selective. In order to distinguish the two alternative possibilities that NDRG1 affects phosphorylated Akt as suggested by the reviewer, we knocked-down LRP6 expression by shRNA in PC3mm cells and examined the phosphorylation status of Akt by Western blot with or without NDRG1 induction. We found that NDRG1 strongly de-phosphorylated Akt (Thr³⁰⁸) while knockdown of LRP6 did not notably affect the phospho-Akt, suggesting that the de-phosphorylation of Akt by NDRG1 is independent its ability to affect Wnt pathway in our system. This data is added as Supplemental Fig. S1D, left panel. Note that the loss of phosphorylation of Akt at Thr³⁰⁸ results in blocking the inactivation of Wnt suppressor, GSK β , which in turn suppresses the Wnt pathway. Therefore, NDRG1 appears to suppress Wnt signaling by direct inactivation of Wnt co-receptor (LRP6) and by Akt dephosphorylation followed by blocking GSK β inactivation.

4. *The authors demonstrate overexpression of NDRG1 inhibits Wnt signalling through LRP6 binding, and this inhibits phosphorylation within its GSK3 pseudo-substrate domain thus maintaining the activation state of this kinase. What about in the absence of Wnt signalling? Is Wnt signalling ectopically active when cells are depleted of NDRG1? Is Wnt signalling potentiated in the absence of NDRG1? Cell-based studies using both Dp44m and the shRNA system used to validate this compound (Figs 7A and B) will allow these loss-of-function studies to be carried out.*

To answer this question, we used immortalized non-tumorigenic prostate cell line, RWPE1, which has high expression of NDRG1. NDRG1 expression was knocked-down in this cell line followed by infecting lentivirus carrying the TOPFlash reporter gene for assaying Wnt activity. We found that the activity of Wnt signaling was significantly diminished in RWPE1 cells when the expression of NDRG1 was knocked-down. The result strongly indicated that Wnt signaling is indeed negatively controlled by NDRG1. This result is added as Supplementary Fig. S1E.

5. *Is Wnt-induced expression of Slug and Twist dependant on ATF3 expression fitting in with the hypothesis that Wnt signalling drives ATF3 expression (Fig. 2)? Or is it the reverse (ie. Yan et al. (2011) PlosOne 6(1):e16515 - this paper should be referenced.)?*

In our study, we found that ATF3 is a novel downstream target of Wnt- β -catenin. We have previously showed that ATF3 was down-regulated by NDRG1 (Can.Res. 66, 11983). However, we did not conclude that Wnt-induced expression of Slug and Twist is dependent on ATF3 expression. Slug and Twist have been previously identified as direct targets of β -catenin/TCF and also of ATF3

(Oncogene 27:2118-2127). Therefore, we believe that Wnt signaling has multiple mechanisms to drive the expression of Slug and Twist.

As the reviewer pointed out, ATF3 was previously demonstrated as an oncogene in mouse mammary tumorigenesis (BMC Cancer 2008, 8:268), and the same group has recently shown that the canonical Wnt- β -catenin signaling was activated in ATF3-induced mammary tumors (Yan et al. (2011) PlosOne 6(1):e16515). ATF3 indeed showed robust activity to induce activation of the canonical Wnt signaling as it acts as a direct transcriptional regulator of factors involved in Wnt signaling including Wnt ligands and its downstream targets. In their study, however, only parous female of transgenic mice exhibited Wnt activation and developed mammary tumor, indicating that ATF3 alone is not sufficient to induce mouse mammary tumor (while Wnt can). Moreover, it was not demonstrated whether activation of Wnt signaling is indispensable for ATF3-induced mammary tumorigenesis. Nevertheless, a direct transcriptional activation of ATF3, as we also demonstrated, may be important in activating and/or maintaining Wnt signaling in tumor cells through a positive feedback loop. We cited Yan's paper in Discussion as suggested (page 13).

6. *In the clinical studies in Fig. 6 A and B membrane associated β -catenin is present in normal tissue but absent in cancerous tissue. The image quality is not high enough that the reader can tell whether β -catenin is absent or whether there is increased cytoplasmic/nuclear β -catenin in these cells as is mentioned in the text. Why do the authors use membranous/cytoplasmic vs cytoplasmic/nuclear as their distinction for β -catenin levels and not membranous versus nuclear as is convention? Does nuclear:membranous β -catenin increase in cancer samples? Is this is the case, is there a negative correlation with NDRG1 expression? What is the baseline for this (ie. normal tissue)?*

β -catenin expression is localized in the nucleus in tissues like colorectal and hepatocellular cancers (Clin Cancer Res. 2004;10:1401-8); however, most publications report a cytoplasmic or reduced membrane β -catenin expression in prostate and breast cancers (Proc Natl Acad Sci USA 2000; 97:4262-6; J. Pathol 2002;196:145-53; Cancer Res. 2006;66:5487-94), which is consistent with our data. In our study, we found that β -catenin was highly expressed in plasma membranes in normal tissues, but was diffused into the cytoplasm in tumor tissues. Nuclear β -catenin in tumor tissues was evident but it is not as strong as cytoplasmic staining, and it was difficult to be quantified precisely.

7. *The compound Dp44m is an iron chelator, with anti-tumorigenic properties arising from a number of its properties including its ability to activate NDRG1, VEGF and TfR1 expression (through HIF-1). Accordingly, the statement that Dp44m is a 'specific' agonist of NDRG1 is not correct. The authors do provide some validation it is an agonist of NDRG1 expression in cell culture, however the pleiotropic effects an iron chelator would have in whole organism assays (Fig. 7C) renders data interpretation contentious. These experiments should be complemented with a loss of function approach using the shRNA system (used to validate this compound - Figs 7A and B).*

We agree with the reviewer that Dp44mT is more “selective” rather than “specific” to NDRG1, and therefore, we have deleted this word in the text. However, as shown in the original manuscript, we indeed found that Dp44mT failed to induce inhibition of invasion and migration when NDRG1 was knocked-down *in vitro* (Fig.7B). To further validate the result *in vivo*, we tested the effect of Dp44mT using NDRG1 knocked-down cells. We found that the “loss of function” of NDRG1 significantly abrogated the inhibitory effect of Dp44mT on metastasis. This result is added as Fig. 7D (middle and right panels).

8. *Fig. 1 - MCF7-tet should be mentioned in the text on pg 4 with the PC3mm/tet line.*

We added the description for MCF7-Tet cells as suggested.

9. *Does Wnt signalling increase 'nuclear localization and expression specifically' or the general expression of ATF3 (pg 5)? This is not clear from Fig SID.*

When we assessed ATF3 by immunofluorescence staining, we found that the majority of ATF3 was localized in the nucleus under any of the conditions we tested, and the intensity of the nuclear ATF3 signal was strongly enhanced by Wnt while NDRG1 blocked this effect. However, it was

somewhat difficult to distinguish whether NDRG1 affects the nuclear localization of ATF3 or simply alters the overall ATF3 expression. To more directly address this question, we examined the ATF3 expression in the cytosol and nuclear fraction by Western blot. We found that the Wnt treatment indeed remarkably increased the amount of nuclear ATF3 and NDRG1 strongly abrogated this effect, while cytosol ATF3 was almost undetectable and was not altered by these treatments. This result is added as Supplemental Fig. S1H (right panel).

10. Pg 6 - Fig 2B should be changed to 3B

We have corrected it.

11. Fig.2D - This experiment should be done using real-time PCR (eg. As in Figs S1C, 5g) as the current assay is not quantitative.

These experiments were indeed performed by Q-PCR and the ratios of F1R1/F2R2 were calculated based on Ct value.

12. Is the scale in Fig. S3E correct?

We have corrected it.

13. Pg 9 - Fig. 6C should read Fig. 5C

Fig. 6C is correct.

14. Pg. 10 - Fig. S6A and B should read Fig. S6A and C and further down the paragraph, Fig. S6B and C.

We have corrected it.

15. What are the 2 panels for 6D?

As we explained in the Figure legend, we did Kaplan-Meier survival analysis to determine the diagnostic/prognostic value of Wnt-NDRG1 or LRP6-NDRG1 signature in prostate cancer patients using the clinical cohort data, GSE6919.