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The SNAG domain of Snail Functions as a Molecular Hook for Recruiting Lysine-specific Demethylase 1

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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 08 January 2010

Thank you for submitting your manuscript "The SNAG domain of Snail Functions as a Molecular Hook for Recruiting Lysine-specific Demethylase 1" to the EMBO Journal. Your study has now been seen by three referees and their comments to the authors are provided below.

As you can see all three referees find the analysis interesting. However it is also it is also clear that a significant amount of additional work would be needed to consider publication in the EMBO Journal. Important controls are missing and further data in support of the functional significance of the CoREST/LSD1/Snail complex is needed. Also there is need for a better discussion of previous work. Should you be able to extend the analysis and address the concerns raised in full then we would be willing to consider a revised version. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

I recognize that a lot of additional work would be needed and I can extend the revision duration to 6 months if that is needed.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REVIEWS

Referee #1 (Remarks to the Author):

The manuscript describes a potentially important discovery about the cell biology of LSD1 (a histone demethylase) and its role in cell transformation, especially with regard to the expression regulation of E-cadherin, a master player in cancer development. The wealth of data presented in the manuscript indicate that the repressor Snail (known to control E-cadherin expression) exerts its function through interaction with LSD1. Most remarkably, Snail is proposed to bind in LSD1 by means of the specific binding of the Snail N-terminal residues to the histone-tail binding site. In other words, the Snail N-terminal residues are proposed to mimic the H3 tail. This is an interesting and intriguing finding that should appeal the readership of EMBO J.

However, I have two very important concerns. The first one relates to the experiment of page 10: "To test whether the Snail complex contains the LSD1 demethylase activity (Shi et al, 2005), we immunoprecipitated Snail and incubated the complex with purified mono-nucleosome.. ....." (Figure 6C). The fact that Snail complex is active appears to be contradictory with the rest of the paper. The authors data indicate that Snail is a kind of competitive ligand of LSD1 that occupies the H3 binding site. Indeed "Parnate (a LSD1 inhibitor) significantly disrupts the interaction of LSD1 with Snail (Fig. 6A)". Therefore, there cannot be any activity if Snail is bound to LSD1. Furthermore, Snail appears to bind quite tightly; "the SNAG peptide completely abolished the interaction of LSD1 with Snail, suggesting that the SNAG peptide has higher affinity for binding to LSD1 than does the histone H3 peptide". I strongly advice the authors to think about this point and possibly perform some more control experiment. Any general reader would come up with this concern.

A second important point relates to published data by Wang et al (Cell. 2009 Aug 21;138(4):660-72.). Their article states that "We demonstrated that LSD1 inhibits the invasion of breast cancer cells in vitro and suppresses breast cancer metastatic potential in vivo. We found that LSD1 is downregulated in breast carcinomas and that its level of expression is negatively correlated with that of TGFbeta1. Our data provide a molecular basis for the interplay of histone demethylation and deacetylation in chromatin remodeling." This seems to be at odd (actually the opposite) with the data presented in this manuscript (for instance Figures 7B and 7D). Obviously, this point must be addressed

Other minor points:
Page 7: "To test this idea, we immunoprecipitated Snail and subjected it to Western blot analysis using antibodies against the methylation of H3K4, H3K9, H3K27, Pan-Lysine and Pan-Arginine methylation (Supplementary Fig. S5 and data not shown)." What was the result if this experiment?

Figure 2B. Are the authors sure that the reduced repressive activity is not due to protein degradation? It might be worthwhile to carry out a control experiment in the presence of a proteasome inhibitor.

Figure 3F and related text should be deleted. By definition, if the modelling is based on "the LSD1-CoREST-Histone H3 peptide complex structure", the modeled Snail peptide will be in the same conformation as the H3 peptide.

Referee #2 (Remarks to the Author):

Employing a variety of biochemical and cell biological experimentation, the authors demonstrate that Snail via its SNAG domain recruits the lysine-specific demethylase LSD1 together with CoREST into the transcriptional repressor complex, and how this complex exerts its function, for example on E-cadherin gene expression. In a comprehensive approach, the details of Snail-LSD1-

CoREST interactions as well as the functional consequences of such complex formation on E-cadherin gene expression and cell migration have been delineated in great detail. Moreover, the generality of the findings have been demonstrated by the analysis of cell lines derived from different cancer types and by a expression correlation study in human breast cancer samples.
Altogether, the experimental approaches have been adequately designed and controlled, and the data convincingly support the conclusions drawn by the authors. The results certainly provide exciting new insights into an important scientific problem and provide a better understanding of how transcriptional repression relates to epigenetic silencing of target genes.

There are, however, a few specific points that should be adressed:

The failure to detect methylation of Snail by antibodies against specific methylated residues of H3 or against pan-lysin and-arginine may be due to the specificity of the antibodies used and, thus, represents a rather meaningless result. It should be possible to detect Snail methylation by immunoprecipitation/mass spectroscopy experiments? Whether LSD1 affects Snail by demethylation, may be critical for its function.

In Figure S5, the co-immunopreciption of H3 with Snail should be specifically mentioned and discussed in the context of developing the model shown in Figure 8.

In Figure 5E, no correlation is apparent between the expression of Snail, LSD1 and CoREST in MDA 435 cells. Since these cells are suspected to represent melanoma and not breast cancer cells, maybe the (negative) result is meaningful?

In the correlation study of human breast cancer samples presented in Figure 8, no specific information is given about the subtype of breast cancers analyzed. Despite the high statistical significance of the analysis, there are samples where no correlation is apparent. Possibly, these samples fall into a certain category of breast cancer subtype or stage, a possibility that should be considered. In any case, more information about the samples should be provided.

The model presented in Figure 8 is interesting yet poorly presented and described. A detailed legend should be provided, also better explaining the scheme figure (what are the red balls, what do the arrows mean etc.?).

The commonly used nomenclature for Snail and Slug should be used, i.e. Snail1 and 2.

The manuscript requires editing in English style and Grammar.

Referee #3 (Remarks to the Author):

In the present ms. the authors identify the histone H3K4 demethylase LSD1 as a Snail interacting protein by dual chromatography purification and mass spectrometry analysis. Through a careful and well designed study they provide biochemical and molecular evidence for the interaction of LSD1 with Snail depending of the amino oxidase and SNAG domains, respectively. Interestingly, they found that the sequence and structure of the SNAG domain are similar to the H3 histone tail and identify several residues in SNAG (equivalent to those in histone H3 tail) required for the Snail/LSD1 interaction and for Snail repression of E-cadherin promoter. They also analyse the interaction of CoREST with Snail/LSD1 and propose a model whereby the SNAG domain of Snail can recruit a LSD1-CoREST complex critical for the stability and function of Snail. This is an interesting and timely study that provides new information on the molecular mechanisms of Snail-mediated repression, and as such can be of general interest. In particular, identification of Snail/LSD1 interaction is relevant, although Snail has not yet identified as a substrate of LSD1. The evidence for the biochemical and molecular interaction of Snail-LSD1 is strong and convincing and supports this part of the work. However, the evidence presented for the functional/biological implication of the ternary complex and the proposed model is not sufficient and requires further experimental work. In addition, several important controls are lacking as well as discussion of other recent reports highly related to the present data (see below). In its present form, the ms. is considered too preliminary for publication in a wide readership journal.

Main points:

1. The functional implication of the CoREST/LSD1/Snail complex requires further support:
a) The effect of CoREST on LSD1/Snail interaction on E-cadherin expression and, importantly, its biological implication is insufficiently demonstrated. The effect of CoREST silencing on the E-cadherin promoter and, at least, migration ability, needs to be tested to support the proposed model.
b) The ChIP assays on HCT116 cells (Fig. 6E) needs to include proper controls using anti-Snail antibodies (this is indeed indicated in the legend, but the data are missing in the figure). This is mandatory to demonstrate the LSD1/Snail dependence for binding to the E-cadherin promoter, as proposed.
c) Which is the rationale for using BT549 cells in the ChIP assays (Fig. 6D)? This cell line is not included in the analysis for Snail, LSD1 and CoREST (Fig. 5E). The same consideration applies to the use of PC3 cells in subsequent analysis (Fig 7).

2. The effect of siLSD1 on E-cadherin promoter in parental MCF7 cells (Fig. 7A, lane 3) suggests a Snail independent effect, since a strong activation of the promoter was observed in the absence of Snail. It might be that this effect could be explained by an LSD1 effect on the SNAG domain of other Snail factors expressed in those cells (i.e., Slug)? This issue should be addressed to demonstrate whether LSD1 interaction is specific to Snail or can be extended to other SNAG bearing factors.

3. The biological effect of siLSD1 is only studied in vitro on the cell motility behaviour (i.e. wound healing assays) of MCF7-Snail and PC3 cells (Fig. 7B, D). To provide stronger support to the proposed action of LSD1 on the metastatic behaviour, the effect of LSD1 knocking down should be extended to invasion, tumorigenic and/or metastasis assays. In relation to this point, it should be also analysed if E-cadherin expression is modified after LSD1 silencing in the different cell lines, apart from the effect on E-cadherin promoter (Fig. 7 A,C). Western blot for E-cadherin needs to be included in Fig. 7E.

4. Related to the above point, the effect of siSnail should also be included in the motility assays of MCF7 cells (Fig. 7B) and compared to the effect of siLSD1.

5. The study requires several additional controls:
a) Inputs should be included in all IP/pull down experiments, in particular they are lacking in Fig. 4B,C,D and Fig. 5D.
b) IP and pulldown data shown in Fig. 4D,E indicates a very weak interaction of Snail with the AO, AOdeltaTower and AOdeltaC mutants, as compared with the other constructs/experiments (Fig. 4B,C). This needs to be discussed.
c) The effect of siCoREST in expression of LSD1 and Snail in HCT116 and PC3 cells (Fig. 5C) is strongly affected, not slightly as stated. This makes very difficult to interpret the subsequent IP analysis (Fig. 5D), and requires revision/clarification.
d) The efficiency of Snail, LSD1 and CoREST silencing should be shown in all corresponding experiments and for all tested cell lines (i.e., Fig. 6E, Fig. 7A,B,C).
e) The demethylase assay, shown in Fig. 6C, lacks error bars and statistics. The same applies to the kinetics assays shown in Fig. 2D.E. How many times were the experiments done and are the results significant?

6. Analysis of breast tumors (Fig. 8A,B) must indicate at least histological type and grade of the analysed samples. Are all from DIC or also includes lobular carcinomas? This is relevant, since E-cadherin downregulation in lobular carcinomas is mainly due to genetic mutations.

7. In direct relation to the above point, the authors should comment the recent work by Wang et al. (Cell, 138: 660, 2009) showing that LSD1 suppresses breast cancer metastatic protential (MDA-MB231 cells) and that LSD1 is downregulated in breast carcinomas. Are the present data compatible with this previous work?

8. The model presented in Fig. 8C is confusing to me. As depicted, it seems that binding of Snail to E-boxes of E-cadherin promoter (left part) maintains expression ON. Regardless of the recruitment of the proposed complex, this contrast with all present data indicating that binding of Snail to the E-cadherin promoter induces its repression. The model is insufficiently explained in the text or legend

and requires careful revision/clarification.

9. The authors should comment/discuss on previous works in the context of their present findings:
a) Snail stability and functional activity: phosphorylation events on the DB and NES sequences through GSK3beta, previously reported by the authors and other groups (Zhou et al., NCB, 2004; Yook et al., JBC, 280: 11 470, 2005). A previous work from other authors involving a conformational change of Snail, and thus functional activity, upon phosphorylation should also be considered (Dominguez et al., MCB, 23: 5078, 2003).
b) Additional co-repressor complexes for Snail, i.e, Sin3A/HDAC1/2 recruited through the SNAG domain (Peinado et al. MCB, 24: 306, 2004), and thus the role of histone deacetylation in the context of their present findings.
c) It should be interesting if the authors consider and comment previous works describing no effect of conservative mutant K9R of Snail on E-cadherin promoter (Peinado et al., EMBO J., 24: 3446, 2005), in contrast to the results obtained with the K9A mutant (Suppl. Fig. S4).

Minor points:

1. The co-localization images, presented in Suppl Fig. S1, are of insufficient definition to ascertain the nuclear/cytoplasmic localization of Snail. Indeed, it seems that co-localization with DAPI (yellow) is only detected in a few cells in the merge images for Snail WT and no detected in DSNAG cells. This makes difficult to understand the quantification of data presented in the lower panels and needs clarification.
2. Please, refer to Snail as Snail1 and to Slug as Snail2 to follow present nomenclature conventions.
3. Citation of original works showing the requirement of the SNAG domain for Snail repression of E-cadherin should be included (Batlle et al, NCB, 2: 84, 2000; Peinado et al., MCB, 2004).

1st Revision - authors' response 01 March 2010

Point-By-Point Response to the Reviewers' Comments

Major changes in figures

Figure 5E	New data including expression of Snail, LSD1 and CoREST from PC3 and BT549 cells (Reviewer#3)
Figure 7C	New data including E-cadherin promoter luciferase analysis with knockdown of CoREST expression (Reviewer#3)
Supplementary Figure S1	New data of E-cadherin luciferase activity with MG132 treatment (Reviewer#1)
Supplementary Figure S7	New data of input control for Figures 4B & 4C (Reviewer#3)
Supplementary Figure S8	New data showing mutations on the critical residues of LSD1 disrupt the interaction of LSD1 with Snail
Supplementary Figure S9	New data of input control for Figure 5B (Reviewer#3)
Supplementary Figure S10	New data of input control for Figures 7A & 7B (Reviewer#3)
Supplementary Figure S11	New data of input control for Figure 7C (Reviewer#3)
Supplementary Figure S12A	New data of cell motility for HCT116 and MDA-MB231 cells (Reviewer#3)
Supplementary Figure S13	New data of cell invasion assays for PC3 and MDA-MB231 cells (Reviewer#3)

Revisions and clarifications

REVIEWER #1:

(1) The first one relates to the experiment of page 10: "To test whether the Snail complex contains the LSD1 demethylase activity (Shi et al, 2005), we immunoprecipitated Snail and incubated the complex with purified mono-nucleosome....." (Figure 6C). The fact that Snail complex is active appears to be contradictory with the rest of the paper. The authors data indicate that Snail is a kind of competitive ligand of LSD1 that occupies the H3 binding site. Indeed "Parnate (a LSD1 inhibitor) significantly disrupts the interaction of LSD1 with Snail (Fig. 6A)". Therefore, there cannot be any activity if Snail is bound to LSD1. Furthermore, Snail appears to bind quite tightly; "the SNAG peptide completely abolished the interaction of LSD1 with Snail, suggesting that the SNAG peptide has higher affinity for binding to LSD1 than does the histone H3 peptide". I strongly advice the authors to think about this point and possibly perform some more control experiment. Any general reader would come up with this concern.

Response: We greatly appreciate the insightful comment from Reviewer#1. We speculate that the interaction of LSD1 with the SNAG domain of Snail or the histone H3 tail is a competitive, dynamic and reversible process. This is analogous to the interaction of an enzyme (LSD1 in this case) with its substrate (histone H3) or its competitive inhibitor in the Michaelis-Menten equation. Because the interaction of the SNAG domain with LSD1 is tighter than that of histone H3 with LSD1 and because free histone H3 is relatively low in abundance in the nucleoplasm, Snail can interact with LSD1 and bring it to its target gene promoters. Once they reach the chromatin region of the promoters, where the local concentration of histone H3 is dramatically higher, histone H3 out-competes with the SNAG domain for the LSD1 binding. A similar situation applies to the immunoprecipitated Snail that interacts with LSD1 through the SNAG domain. When this complex is incubated with large excess amounts of mono-nucleosomes in the de-methylation assay, the overabundant amount of histone H3 out-competes for the binding with LSD1, and results in LSD1-mediated demethylation of histone H3. Although this issue can be tested by systematic measurement of the Km and Kcat of histone H3 (as a substrate) and the Ki of the SNAG peptide (as a competitive inhibitor) using purified LSD1 in the near future, our model does not exclude the possibility that other LSD1 associated proteins, such as BHC80 (Lan et al, Nature. 448, 718-22, 2007), facilitate the switching between Snail and histone H3 for the binding with LSD1 at the chromatin region. Further investigations will provide new insight about this delicate regulation. We have revised our discussion to clarify this issue in our revised manuscript (please see page 14, line 11).

(2) A second important point relates to published data by Wang et al (Cell. 2009 Aug 21;138(4):660-72.). Their article states that "We demonstrated that LSD1 inhibits the invasion of breast cancer cells in vitro and suppresses breast cancer metastatic potential in vivo. We found that LSD1 is downregulated in breast carcinomas and that its level of expression is negatively correlated with that of TGFbeta1. Our data provide a molecular basis for the interplay of histone demethylation and deacetylation in chromatin remodeling." This seems to be at odd (actually the opposite) with the data presented in this manuscript (for instance Figures 7B and 7D). Obviously, this point must be addressed.

Response: We greatly appreciate the thoughtful comment from Reviewer#1. Overexpression of LSD1 has been correlated with an adverse clinical outcome in neuroblastoma, prostate and colon cancers, suggesting a tumor-promoting role for LSD1 (Kahl et al, Cancer Res, 66, 11341-11347, 2006; Metzger et al, Nature, 437, 436-439). In addition, inhibition of LSD1, using pharmacological inhibitors, suppresses colon cancer and neuroblastoma growth in vitro and in xenograft mouse models (Huang et al, Clin Cancer Res, 15, 7217-7228, 2009; Schulte et al, Cancer Res, 69, 2065-2071, 2009; Huang et al, PNAS, 104, 8023-8028, 2007). A recent study showed that LSD1 is highly expressed in ER-negative breast cancer and correlates with tumor grades and poor clinical outcome for women with breast cancer [Lim et al, Carcinogenesis, 2009 Dec 30 (Epub ahead of print)]. These authors showed that inhibition of LSD1 function by either siRNA or a specific LSD1 inhibitor suppressed the growth of breast cancer cells, including breast cancer MDA-MB231 cell line that mainly used in the study of Wang et al (Cell, 138, 660-72, 2009). We also found that LSD1 expression is highly correlated with tumor grade and a poor 5-year patient survival outcome in breast cancer patients from several publicly available human breast cancer gene expression data sets, such as those described by Van't Veer (Vanít Veer et al, Nature 415, 530-536, 2002) (Reviewers are encouraged to perform similar analyses using multiple human breast cancer expression data sets available from Oncomine, https://www.oncomine.org/resource/login.html). Furthermore, we consistently found that knockdown of LSD1 expression upregulated E-cadherin promoter luciferase activity and inhibited cell migration and invasion in several cancer cell lines. The discrepency

between the uncommon finding from Wang et al and those mentioned above is unclear at this moment. Systematic and thorough investigation using multiple cancer cell lines and human tumor samples by different laboratories will help clarify this issue.

Minor points

(3) Page 7: "To test this idea, we immunoprecipitated Snail and subjected it to Western blot analysis using antibodies against the methylation of H3K4, H3K9, H3K27, Pan-Lysine and Pan-Arginine methylation (Supplementary Fig. S5 and data not shown)." What was the result in this experiment?

Response: The results of these experiments (using either Pan-Lysine or Pan-Arginine methylation antibodies) were negative due to low affinity and low specificity of these antibodies against the SNAG domain of Snail. Thus, we did not include these data in our manuscript. We realize that it is important to determine whether the Lysine and Arginine residues are methylated in vivo in order to fully understand the function and regulation of Snail. Therefore, we are currently developing specific antibodies against these residues and will perform systematic studies once these antibodies become available.

(4) Figure 2B. Are the authors sure that the reduced repressive activity is not due to protein degradation? It might be worthwhile to carry out a control experiment in the presence of a proteosome inhibitor.

Response: We appreciate the insightful comment from Reviewer#1. We have performed the experiment as suggested by the reviewer and the new experimental data is included in Supplementary Figure S1. We found that MG132 treatment did significantly stabilize ΔSNAG-Snail; however, this stabilization did not alter its inability to suppress E-cadherin promoter luciferase activity (please see page 6, line 6 and Supplementary Figure S1).

(5) Figure 3F and related text should be deleted. By definition, if the modeling is based on "the LSD1-CoREST-Histone H3 peptide complex structure", the modeled Snail peptide will be in the same conformation as the H3 peptide.

Response: We thank Reviewer#1 for this critical comment. However, we prefer to keep this panel as part of Figure 3 as it will help the readers to understand the similarity in the association of the histone H3 tail and the SNAG domain of Snail with the LSD1-CoREST complex.

REVIEWER #2:

(1) The failure to detect methylation of Snail by antibodies against specific methylated residues of H3 or against pan-lysine and-arginine may be due to the specificity of the antibodies used and, thus, represents a rather meaningless result. It should be possible to detect Snail methylation by immunoprecipitation/mass spectroscopy experiments? Whether LSD1 affects Snail by demethylation, may be critical for its function.

Response: We appreciate the critical comment from Reviewer#2. We did perform the experiment as suggested by the reviewer using mass spectrometry analysis. However, we were unable to detect the methylation due to the low abundance of endogenous Snail and the intrinsic protein instability of this molecule inside cells. We are currently taking two approaches to re-examine this issue. The first one is to purify Snail from HeLa S3 suspension cells (with 30 liter of cell culture). Once we have large amount of endogenous Snail, we can perform mass spectrometry analysis again as suggested by the Reviewer. The second method is to develop specific antibody against the potential methylation on the Arginine or Lysine residues on the SNAG domain of Snail. We believe that we can address this issue in an unambiguous way by using these two complementary approaches in the near future.

(2) In Figure S5, the co-immunoprecipitation of H3 with Snail should be specifically mentioned and discussed in the context of developing the model shown in Figure 8.

Response: We thank Reviewer#2 for the insightful comment. Although it is interesting that histone H3 co-immunoprecipitates with Snail in Supplementary Figure S5, it is not clear whether Snail interacts directly with histone H3 or indirectly by forming a complex with chromatin modifying enzymes, which bind histone H3 directly. The interaction between Snail and histone H3 requires more thorough and systematic investigation in the near future. We prefer to discuss this observation when we have more convincing and solid experimental data.

(3) In Figure 5E, no correlation is apparent between the expression of Snail, LSD1 and CoREST in MDA 435 cells. Since these cells are suspected to represent melanoma and not breast cancer cells, maybe the (negative) result is meaningful?

Response: We apologized for our mistake. In Figure 5E, this cell line should be MDA-MB453 instead of MDA-MB435 cells. We have corrected this in our revised manuscript. It has been documented that the E-cadherin gene is deleted in MDA-MB453 cells and no E-cadherin protein is detected in this cell line. This may explain why this cell line does not require the Snail, LSD1 and CoREST complex to suppress E-cadherin expression.

(4) In the correlation study of human breast cancer samples presented in Figure 8, no specific information is given about the subtype of breast cancers analyzed. Despite the high statistical significance of the analysis, there are samples where no correlation is apparent. Possibly, these samples fall into a certain category of breast cancer subtype or stage, a possibility that should be considered. In any case, more information about the samples should be provided.

Response: We thank Reviewer#2 for this constructive comment. All the breast tumor samples used in this study are invasive ductal carcinoma. The average age for patients in this group is 56.8 (± 13.4) year with either negative (53.3%) or positive (N1=34.6; N2=12.1%) of lymph node metastasis. The clinical stage of these samples consists of stage I (12.5%), stage II (38.2%), stage III (41.1%) and stage IV (8.2%). We have included this information in the Materials and Methods section of our revised manuscript (please see page 19, line 22).

(5) The model presented in Figure 8 is interesting yet poorly presented and described. A detailed legend should be provided, also better explaining the scheme figure (what are the red balls, what do the arrows mean etc.?).

Response: We thank Reviewer#2 for this excellent comment. We have included a detailed figure legend in our revised manuscript (please see page 24, line 18).

(6) The commonly used nomenclature for Snail and Slug should be used, i.e. Snail1 and 2.

Response: We have changed the nomenclature of Snail throughout the text of the revised manuscript.

(7) The manuscript requires editing in English style and Grammar.

Response: Our manuscript has been carefully edited by an editor at our institution (Markey Cancer Center, University of Kentucky).

REVIEWER #3:

Main points:

(1) The functional implication of the CoREST/LSD1/Snail complex requires further support: a) The effect of CoREST on LSD1/Snail interaction on E-cadherin expression and, importantly, its biological implication is insufficiently demonstrated. The effect of CoREST silencing on the E-cadherin promoter and, at least, migration ability, needs to be tested to support the proposed model.

Response: We thank Reviewer#3 for this excellent comment. We have performed the experiments as suggested by the reviewer and the new data are included in Figure 7C and Supplementary Figure S13. We found that knockdown of CoREST expression partially de-repressed E-cadherin promoter

activity in three cancer cell lines (HCT116, PC3 and MDA-MB231) (Figure 7C). In addition, knocking down the expression of CoREST in PC3 and MDA-MB231 cells partially suppresses the invasion of these cells (Supplementary Figure S13).

b) The ChIP assays on HCT116 cells (Fig. 6E) needs to include proper controls using anti-Snail antibodies (this is indeed indicated in the legend, but the data are missing in the figure). This is mandatory to demonstrate the LSD1/Snail dependence for binding to the E-cadherin promoter, as proposed.

Response: We thank Reviewer#3 for the constructive comment. The new data is included in Figure 6E in our revised manuscript. We showed that knockdown of Snail expression decreased the association of Snail with the E-cadherin promoter; whereas knockdown of LSD1 expression did not alter the binding of Snail with the E-cadherin promoter. However, knockdown of Snail expression significantly reduced the association of LSD1 with the E-cadherin promoter, indicating that the interaction of LSD1 with the E-cadherin promoter requires the association of Snail (please see revised Figure 6E).

c) Which is the rationale for using BT549 cells in the ChIP assays (Fig. 6D)? This cell line is not included in the analysis for Snail, LSD1 and CoREST (Fig. 5E). The same consideration applies to the use of PC3 cells in subsequent analysis (Fig 7).

Response: We thank Reviewer#3 for the thoughtful comment. BT549 is a type B basal-like breast cancer cell line, which has been documented to express many EMT markers (including Snail) and contain the wild-type E-cadherin gene (Neve et al, Cancer Cell, 10, 515-527, 2006; Lombaerts et al, British J of Cancer, 94, 661-671, 2006). We have included the expression analysis of Snail, LSD1 and CoREST in BT549 and PC3 cells in Figure 5E in our revised manuscript.

(2) The effect of siLSD1 on E-cadherin promoter in parental MCF7 cells (Fig. 7A, lane 3) suggests a Snail independent effect, since a strong activation of the promoter was observed in the absence of Snail. It might be that this effect could be explained by an LSD1 effect on the SNAG domain of other Snail factors expressed in those cells (i.e., Slug)? This issue should be addressed to demonstrate whether LSD1 interaction is specific to Snail or can be extended to other SNAG bearing factors.

Response: We thank Reviewer#3 for the insightful comment. MCF7 cells contain an undetectable amount of Snail and Slug (Hajra et al, Cancer Research, 15, 62(6), 1613-1618 and our unpublished data). Thus, it is unlikely that the strong activation of the E-cadherin promoter luciferase construct (after knockdown of LSD1 expression) is due to the expression of Slug in this cell line. Interestingly, LSD1 has been shown to interact with ZNF217 (through an interaction with CtBP1), which also represses the expression E-cadherin in MCF7 cells (Cowger et al, Oncogene, 26, 3378-3386, 2007). We speculate that knockdown of LSD1 expression also disrupts the ZNF217 repressor complex on the E-cadherin promoter in MCF7 cells, and thus, this results in the elevation of E-cadherin promoter luciferase activity. A systematic and thorough study is required to further delineate this issue in the future.

(3) The biological effect of siLSD1 is only studied in vitro on the cell motility behaviour (i.e. wound healing assays) of MCF7-Snail and PC3 cells (Fig. 7B, D). To provide stronger support to the proposed action of LSD1 on the metastatic behaviour, the effect of LSD1 knocking down should be extended to invasion, tumorigenic and/or metastasis assays. In relation to this point, it should be also analyzed if E-cadherin expression is modified after LSD1 silencing in the different cell lines, apart from the effect on E-cadherin promoter (Fig. 7 A, C). Western blot for E-cadherin needs to be included in Fig. 7E.

Response: We appreciate this constructive comment from Reviewer#3. In our previous version, we did measure the cell motility (based on the wound healing assay) of HCT116 and MDA-MB231 cells in addition to Snail/MCF7 and PC3 cells. Due to the space limitation, we only presented the results from Snail/MCF7 and PC3 cells (we mentioned the results from HCT116 and MDA-MB231 cells as ìdata not shownî) in our original submission. We apologize that we did not make this clear. We have now included these data as Supplementary Figure S12A in our revised manuscript. We also knocked down the expression of LSD1 in PC3 and MDA-MB231 cells and measured their

invasiveness using the modified Boyden-chamber invasion assay. Consistent with the finding from the luciferase reporter and cell migration assays, knockdown of LSD1 expression suppresses the invasiveness of PC3 and MDA-MB231 cells. These new data are also presented in Supplementary Figure S13 in our revised manuscript.

We did examine the expression of E-cadherin in HCT116, PC3 and MDA-MB231 cells after knocking down the expression of endogenous Snail, LSD1 or both. Although we observed the downregulation of vimentin and N-cadherin and upregulation of ZO-1 (Figure 7D) in these cells, we did not find the elevation or gain of E-cadherin expression. The reason for this is unclear at this moment. We speculate that this may due to permanent DNA methylation on the E-cadherin promoter in these cancer cells. Re-establishing E-cadherin expression in these cancer cells may require additional suppression of other transcription factors (such as Twist, ZEB1 and ZEB2) or other known transcriptional co-repressors (such as HDAC1/2, sin3A and PRMT5). Alternatively, it is possible that regaining E-cadherin expression requires long-term knockdown of LSD1 expression in these cells, given the relatively long half-life of the E-cadherin molecule. Most of our experimental analyses were performed on the 48-72 hour time point after knocking down LSD1 expression. We are actively testing both of these possibilities and systematic studies will be presented once the underlying mechanism becomes clear.

(4) Related to the above point, the effect of siSnail should also be included in the motility assays of MCF7 cells (Fig. 7B) and compared to the effect of siLSD1.

Response: We thank Reviewer#3 for the insightful comment. We and many other groups have shown that breast cancer MCF7 cells contain undetectable amounts of endogenous Snail (Hajra et al, Cancer Res, 15, 62(6), 1613-1618; Zhou et al, Nature Cell Biology, 6, 931-940), and thus, we have established a stable MCF7 transfectant with exogenous expression of Snail (Snail/MCF7). Expression of Snail induced the migration and invasiveness of these cells (Wu et al, Cancer Cell, 5, 416-428, 2009). Instead of knockdown of exogenous Snail expression, we silenced the expression of LSD1 in these cells (MCF7 vs Snail/MCF7 cells). If the function of Snail requires the association of LSD1, knockdown of LSD1 expression will affect the motility of Snail/MCF7 cells. Indeed, while knockdown of LSD1 expression in MCF7 cells has no significant effect on cell migration, it abolished the enhancement of Snail-mediated cell migration in Snail/MCF7 cells (Fig. 7B), which suggests that the function of Snail requires the association of LSD1. To further establish the causal relationship between Snail and LSD1 and to compare their individual effect on cell motility, we knocked down the expression of endogenous Snail or LSD1 or both in HCT116, PC3 and MDA-MB231 cells (Figure 7D and Supplementary Figure S12A). We found that knockdown of either Snail or LSD1 expression suppressed cell migration and a synergistic inhibitory effect was found upon knockdown of both molecules.

(5) The study requires several additional controls:

a) Inputs should be included in all IP/pull down experiments, in particular they are lacking in Fig. 4B, C, D and Fig. 5D.

Response: We have included all the input controls for these experiments in Supplementary Figure S7 (input for Figure 4B and 4C) and Supplementary Figure S9 (input for Figure 5B) in our revised manuscript. For Figure 5D, the input control is shown in Figure 5C. Figure 4D is a GST pull-down experiment and did not have an input control. Briefly, different deletion mutants of GST-LSD1 were induced in a small-scale bacterial culture (50 ml) by IPTG for 3 hours at room temperature. After lysis, the clear supernatants were incubated with GST beads for 4 hours followed by extensive washing with PBS containing 1% triton X-100. The GST-beads bound with deletion mutants of LSD1 were then incubated with equal amounts of lysate from Snail/HEK293 cells (expressing exogenous Snail) for an additional 3 hours. After extensive washing, the GST-bead-bound complexes were eluted with SDS-PAGE sample buffer. About 1/10 of these elutents were analyzed for the association of Snail by Western blotting (bottom panel) and the rest of elutents were examined for the presence of purified GST-LSD1 by Coomassie staining (top panel). We apologize that we did not make these experimental details clear in our previous version. We have revised our manuscript and included all the detailed experimental procedures in the corresponding figure legends and in the Materials and Methods section (please see page 17, line 2 from bottom).

b) IP and pulldown data shown in Fig. 4D, E indicates a very weak interaction of Snail with the AO, AOdeltaTower and AOdeltaC mutants, as compared with the other constructs/experiments (Fig. 4B, C). This needs to be discussed.

Response: It has been well-documented that the N-terminal SWARM domain plays a critical role in the protein stability and solubility of LSD1. Deletion of the N-terminal SWARM domain significantly compromises the stability and solubility of LSD1 (Stavropoulos et al, Nature Structural & Molecular Biology, 13, 626-632, 2006; Yang et al, Molecular Cell, 23, 377-387, 2006; Chen et al, PNAS, 38, 13956-13961, 2006; Forneris et al, JBC, 282, 20070-20074, 2006). Consistent with this notion, we found that the expression level of the AO domain of LSD1 was much lower than that of full-length LSD1 (please see Supplementary Figure S7). Thus, the IP and pull-down data for AO, AOΔTower and the AOΔC mutants, as shown in Figures 4D and E, are relatively weaker in comparison with that of full-length LSD1 in Figure 4B and 4C.

c) The effect of siCoREST in expression of LSD1 and Snail in HCT116 and PC3 cells (Fig. 5C) is strongly affected, not slightly as stated. This makes very difficult to interpret the subsequent IP analysis (Fig. 5D), and requires revision/clarification.

Response: We thank Reviewer#3 for the critical comment. Knockdown of CoREST expression did decrease the level of Snail and LSD1 in the cell lines tested (Figure 5C). However, the ratio of Snail over LSD1 from either control cells or cells with knockdown of CoREST expression is approximately the same based on densitometry analysis (please see the densitometry analysis on the bottom panel of Figure 5C). In other word, knockdown of CoREST expression in HCT116, PC3 and MDA-MB231 cells induced a proportional decreased expression of Snail and LSD1. However, when an equal amount of LSD1 was immunoprecipitated (Figure 5D, top panel) from these cells, we observed significantly reduced association of Snail with LSD1 from cells with knockdown of CoREST expression, indicating that CoREST is critical in controlling the interaction of LSD1 with Snail.

d) The efficiency of Snail, LSD1 and CoREST silencing should be shown in all corresponding experiments and for all tested cell lines (i.e., Fig. 6E, Fig. 7A, B, and C).

Response: We thank Reviewer#3 for the constructive comment. We have included all the input in Supplementary Figure S10 (for Figure 7A and 7B) and Supplementary Figure S11 (for Figure 7C) in our revised manuscript. Figure 6E is a chromatin immunoprecipitation (CHIP) experiment and we did not have an input lysate control for this experiment, because this experimental procedure involves a paraformaldehye fixation step, in which the protein complexes are cross-linked with chromosomal DNA followed by extensive sonication. Thus, the CHIP procedure renders lysates un-compatible for Western blotting analysis. However, we routinely perform siRNA knockdown experiments and achieve more than 90% knockdown efficiency. In fact, the knockdown efficiency of Snail, LSD1 and CoREST in HCT116 cells has been presented in Supplementary Figure S11 and the same procedure was used for the CHIP experiment in Figure 6E.

e) The demethylase assay, shown in Fig. 6C, lacks error bars and statistics. The same applies to the kinetics assays shown in Fig. 2D. E. How many times were the experiments done and are the results significant?

Response: We have performed the demethylase assay experiment at least three times and the results are significant. We have now included the statistical analysis and standard error for this experiment in Figure 6C of our revised manuscript. We also performed three independent experiments to examine the kinetics of Snail stability. The statistical analysis and standard error for these experiments are now shown in Figures 2D and E in our revised manuscript.

(6) Analysis of breast tumors (Fig. 8A, B) must indicate at least histological type and grade of the analyzed samples. Are all from DIC or also includes lobular carcinomas? This is relevant, since E-cadherin downregulation in lobular carcinomas is mainly due to genetic mutations.

Response: This comment is similar to Comment 4 from Reviewer#2. Please see our response in page 3.

(7) In direct relation to the above point, the authors should comment the recent work by Wang et al. (Cell, 138: 660, 2009) showing that LSD1 suppresses breast cancer metastatic potential (MDA-MB231 cells) and that LSD1 is downregulated in breast carcinomas. Are the present data compatible with this previous work?

Response: This comment is similar to Comment 2 from Reviewer#1. Please see our response in page 1.

(8) The model presented in Fig. 8C is confusing to me. As depicted, it seems that binding of Snail to E-boxes of E-cadherin promoter (left part) maintains expression ON. Regardless of the recruitment of the proposed complex, this contrast with all present data indicating that binding of Snail to the E-cadherin promoter induces its repression. The model is insufficiently explained in the text or legend and requires careful revision/clarification.

Response: We appreciate the constructive comment from Reviewer#3. We apologize that we did not make this clear in our Figure legend. We have modified Figure 8C and included a detailed explanation of our model in the Figure legend (please see page 24, line 18).

(9) The authors should comment/discuss on previous works in the context of their present findings:

a) Snail stability and functional activity: phosphorylation events on the DB and NES sequences through GSK3beta, previously reported by the authors and other groups (Zhou et al., NCB, 2004; Yook et al., JBC, 280: 11 470, 2005). A previous work from other authors involving a conformational change of Snail, and thus functional activity, upon phosphorylation should also be considered (Dominguez et al., MCB, 23: 5078, 2003).

Response: We appreciate the insightful comment from Reviewer#3. Snail is a highly phosphorylated protein (Dominguez et al., MCB, 23: 5078, 2003; Zhou et al., NCB, 6, 931-940, 2004; Yook et al., JBC, 280, 11 470, 2005). The region connecting the SNAG domain and the zinc-fingers of Snail contains a serine-rich motif. We and others previously showed that this serine-rich motif is highly phosphorylated and controls the subcellular localization and stability of Snail. As Snail is a labile protein, we speculate that deletion or mutation of the SNAG domain disrupts the interaction of Snail with the LSD1-CoREST complex, and thus, leads to the accessibility of phosphorylation and consequent proteosome degradation by GSK-3β and β-Trcp. This is supported by the finding that the SNAG domain does not alter the subcellular localization but controls the stability of Snail. We have included this discussion in our revised manuscript (please see page 13, line 27).

b) Additional co-repressor complexes for Snail, i.e., Sin3A/HDAC1/2 recruited through the SNAG domain (Peinado et al. MCB, 24: 306, 2004), and thus the role of histone deacetylation in the context of their present findings.

Response: As we suggested in our manuscript, it is highly likely that the SNAG domain of Snail also acts as a pseudo-substrate of Sin3A/HDAC1/2 for recruiting this de-acetylation complex. Elucidation of a detailed mechanism will require further experimental analysis. We have included this reference and relevant discussion in our revised manuscript (please see page 15, line 21).

c) It should be interesting if the authors consider and comment previous works describing no effect of conservative mutant K9R of Snail on E-cadherin promoter (Peinado et al., EMBO J., 24: 3446, 2005), in contrast to the results obtained with the K9A mutant (Suppl. Fig. S4).

Response: As indicated in our manuscript, the K9A mutant of Snail became unstable and failed to associate with the LSD1 complex (Figure 3). As pointed out from the crystal structure analysis, a positive charge on Lysine 9 of histone H3 is critical for promoting the interaction with LSD1; in the K9R mutant in the study of Peinado et al, although the lysine residue was replaced with arginine, the positive charge at this position remained unaffected, and thus, we speculate that this K9R mutant maintains the ability to interact with the LSD1 complex and is able to suppress the promoter activity of E-cadherin.

Minor points:

(1) The co-localization images, presented in Suppl Fig. S1 are of insufficient definition to ascertain the nuclear/cytoplasmic localization of Snail. Indeed, it seems that co-localization with DAPI (yellow) is only detected in a few cells in the merge images for Snail WT and no detected in DSNAG cells. This makes difficult to understand the quantification of data presented in the lower panels and needs clarification.

Response: This may be due to the strong green color from the GFP tag. We have adjusted the color contrast of our images in Supplementary Figure S2 in our revised manuscript to clarify this point. In addition, the cellular localization of Snail or ΔSNAG-Snail has also been confirmed by cellular fractionation experiment presented in Supplementary Figure S3.

(2) Please, refer to Snail as Snail1 and to Slug as Snail2 to follow present nomenclature conventions.

Response: We have revised our manuscript and changed Snail to Snail1 throughout the text.

(3) Citation of original works showing the requirement of the SNAG domain for Snail repression of E-cadherin should be included (Batlle et al, NCB, 2: 84, 2000; Peinado et al., MCB, 2004).

Response: We thank Reviewer#3 for the excellent suggestion. We apologize that we did not cite the original works in our previous version. We have added these two references into our revised manuscript (please see 5, lines 15 and 28).

Editorial Decision 12 March 2010

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original 3 referees to review the revised version and I have now heard back from them. As you can see below, the referees support publication here and appreciate the added data. Referee #1 has no further comments to the authors. Given these comments, I am therefore very pleased to proceed with the acceptance of the paper for publication in the EMBO Journal. Before doing so, referee #3 has one remaining issue that I would like you to respond to. If you have data on hand to address this issue you can send me a modified manuscript by email. If not, then we will proceed with the acceptance of the present version.

Thank you for submitting your interesting paper to the EMBO Journal

Best wishes

REFEREE REPORTS

Referee #2

The authors have appropriately responded to the reviewers' comments. Notably, they have added important new data and substantially revised the manuscript to meet the reviewers' criticisms.

Referee #3

The authors have successfully addressed most raised queries. The ms. have been greatly improved with important clarifications and additional data. However, a formal issue still remains that should be addressed before publication. This refers to data on human breast tumor samples. Although some information is now provided in Mat & Methods section, the relation between the tumor grade and expression of Snail, LSD1 and/or Co-Rest is still lacking. This analysis should be performed and included in the ms. (Fig. 8B, Table), with the proper statistical evaluation. This analysis can provide relevant information about any preferential action of the ternary complex depending on tumor grade

and should be of great interest to most readers.

Additional Correspondence 15 March 2010

We thank Reviewer#3 for the critical comment. The tumor grade is usually defined by TNM staging. T stands for the tumor size (T1<2 cm; T2=2-5 cm; T3>5cm); N stands for lymph node metastases (N0=0 node; N1=1-3 nodes; N2>5 nodes); and M defines for organ or distant metastases (M0=No and M1=Yes). At stage III and IV, breast tumor commonly has lymph node metastases (N1 and N2). However, distant organ metastasis does not strictly correlated with lymph node metastasis, because tumor cells can penetrate blood vessels for distant metastasis without going through the lymphatic system. In our immunohistochemical analysis, although we found the tendency of a high co-expression of Snail, CoREST and LSD1 in stage III and IV, we also observed the co-expression of these molecules in stage II and late stage I. This can be explained by the notion that EMT is an early essential event for tumor metastasis and it usually occurs at the tumor invasive front (the tumor-stroma boundary). In other words, co-expression of Snail, CoREST and LSD1 can also occur in early stage of tumor if the tumor cells are prone to metastasis. We did perform a statistical analysis to correlate the expression of these molecules with different tumor stages. Unfortunately, it did not reach a significant p-value due to the limited sample size in each tumor grade. Future experiment employing more samples will be able to address this issue.

