Manuscript No.: #7030
Title: Heat shock protein 90 is involved in the regulation of HMGA2-driven growth and epithelial-to-mesenchymal transition of colorectal cancer cells (#2015:10:7030:0:1:REVIEW)

Dear Editor:

We would like to thank you and the three reviewers for your kind consideration and helpful suggestions regarding our manuscript. We have conscientiously re-examined the manuscript and made certain revisions. We have followed their comments **(in italics)** closely and feel that their suggestions have further strengthened the manuscript.

Editor's comments (in italics)
"After careful consideration, I believe that your study has the potential to be published provided you revise several fundamental aspects of your paper, as listed by reviewer #2 and #3. In particular you should concentrate on the experimental design and provide adequate control and complementary assays. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.".

Response. Thanks for Editor's comments and we have concentrated on the experimental design and provide adequate control and complementary assays in our reversed manuscript. We have made major revisions in this new version according to yours and the reviewers' comments as follows:
1. To make sufficient functional to link between Hsp90 and HMGA2, we performed immunofluorescence analysis to investigate the intracellular localization of both Hsp90 and HMGA2. In addition, we proofed that the suppression of HMGA2 protein expression in response to NVP-AUY922 treatment resulted in ubiquitination and subsequent proteasome-dependant degradation of HMGA2.
2. To point the concrete evidence indicating HMGA2's main role in driving EMT in HCT116 cells, we investigated the effect of siRNA-mediated knockdown of HMGA2 on the expression of EMT effectors in HCT116 cells. In addition, we found that knockdown HMGA2 expression can enhance the effect of NVP-AUY922-mediated suppression of EMT and migratory ability of HCT116 cells.
3. To further investigate whether ERK was indeed involved in regulation of HMGA2, we examined the dose effect of AZD6244, a potent ERK inhibitor, on the ERK activity, CREB and HMGA2 proteins expression of HCT116 cells.

We have substantially revised the manuscript in keeping with the reviewers' comments and we believe that we have answered most of the questions raised in a satisfactory manner. Our response to the reviewer's questions and suggestions are given below:

Reviewer Comments (in italics)

Reviewer 1 (Gennaro Chiappetta)

We appreciate the comments of Dr. Gennaro Chiappetta and believe that our manuscript has been improved by attention to him or her. The followings are our responses to the specific issues raised by Dr. Gennaro Chiappetta:

Comments#1. *" The authors should further describe and discuss the IP experiment presented in Figure 3C ".*

Response. We are sorry for the misleading and thank Dr. Gennaro Chiappetta's valuable suggestion. We have added method of IP experiment and the description of IP experiment presented in revised Fig. 3, and have included to following description of the associated results.

Materials and Methods section

Immunoprecipitation

The interaction between Hsp90 protein and HMGA2 was studied by immunoprecipitation analysis of extracts prepared from HCT116 or DLD1 cell lines. Cells were lysed, incubated in IP lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, protease inhibitor mixture) for 30 min on ice, and then sonicated (3 times for 10 sec each). After centrifugation at 1400 g for 5 min at 4°C, the supernatants were collected from each sample and then pre-cleared by incubation with 50% protein A/G agarose beads in the IP lysis buffer at 4°C for 1 h with rocking. After removal of the protein A/G beads by centrifugation, protein content in each sample was measured and aliquots containing 500 μg of protein were incubated with primary antibodies overnight at 4°C. The immunoprecipitates bound to the protein A/G–Sepharose beads were washed, boiled and analyzed by immunoblotting.

Section 3 of Results

To evaluate a potential Hsp90-HMGA2 interaction, we performed an immunoprecipitation assay to determine the effect of NVP-AUY922 on the physical interactions between Hsp90 and HMGA2. After NVP-AUY922 treatment, the HMGA2

protein was immunoprecipitated with an anti-HMGA2 antibody and analyzed through Western blotting with an anti-Hsp90 antibody. As shown in Fig. 3D, a single band was detected using anti-Hsp90 antibody in immunoprecipitates or input lysate from NVP-AUY922-treated HCT116 cells.

Comments#2. *" The authors should explain why the concentration of NVP-AUY922 used in Figure 3C is 0-20 nM, while in Figure 3B the effects of the inhibitor are shown at higher concentrations ".*

Response. Thanks for Dr. Dr. Gennaro Chiappetta's comments. In the original Fig. 3B, HMGA2 protein expression was significantly reduced on NVP-AUY922 treatment in both the 40 and 80 nM of HCT116 cells. However, in the original Fig. 3C, to evaluate the potential Hsp90-HMGA2 interaction, we performed an immunoprecipitation assay to determine the low dose effect of NVP-AUY922 on the physical interactions between Hsp90 and HMGA2 to make sure HMGA2 can be immunoprecipitated.

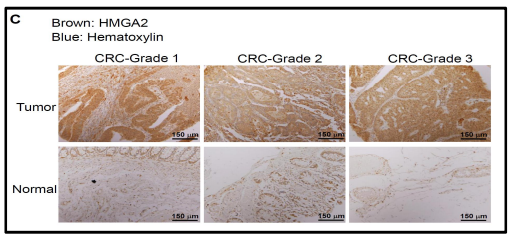
Reviewer 2 (Anonymous)

We appreciate the comments **(in italics)** of this reviewer and believe that our manuscript has been improved by attention to him or her. The followings are our responses to the specific issues raised by this reviewer:

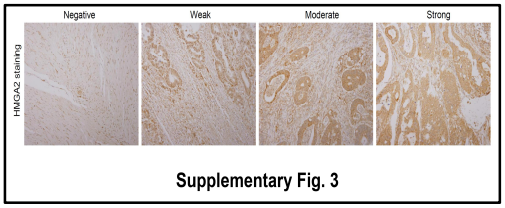
" Basic reporting"
The resolution and magnification of Fig. 1C and Fig. S2 is not fit for publication. More clear images should be supplied by the authors and the pathological structure should be recognized in the images.

Response. We would like to thank reviewer for the comments and constructive suggestions. The resolution and magnification of Fig. 1C and revised Fig. S3 have been improved in revised manuscript and shown below:

Fig. 1C



Revised Fig. S3



Supplementary Fig. 3

" Experimental design 1"

As shown in Fig. 4, although the effects of gene-specific inhibition of HMGA2 or Hsp90 and pharmaceutical inhibition of Hsp90 were similar, we still cannot get the conclusion that there is some relationship between HMGA2 and Hsp90. More solid evidence is required.

Response. We would like to thank reviewer for the comments and constructive suggestions. To make sufficient functional to link between Hsp90 and HMGA2, we performed immunofluorescence analysis to investigate the intracellular localization of both Hsp90 and HMGA2. In addition, we proofed that the suppression of HMGA2 protein expression in response to NVP-AUY922 treatment resulted in ubiquitination and subsequent proteasome-dependant degradation of HMGA2. The associated results and revised its legend accordingly, and have shown below:

Section 3 of Results

Hsp90 regulates and interacts with HMGA2

Hsp90 is critical in regulating cell growth (Cheung et al., 2010; Ko et al., 2012; Miyata, 2003; Nagaraju et al., 2014), and HMGA2 has a well-documented role in this process (Di Cello et al., 2008; Malek et al., 2008; Sun et al., 2013; Wend et al., 2013); therefore, we examined whether the interaction between the Hsp90 and HMGA2 is existence. Thus, we first performed RNA interference to deplete Hsp90 in the HCT116 cells and examined the effect of its depletion in the intracellular localization of both Hsp90 and HMGA2 by immunofluorescence analysis. As shown in Fig. 3A, Hsp90 siRNA-mediated endogenous Hsp90 knockdown significantly reduced CDK4 (Hsp90 client protein) and HMGA2 expression, as well as induced Hsp70 (Hsp90 client protein) expression in the siHsp90-transfected HCT116 cells. Further, immunofluorescence result revealed that Hsp90 (red color) co-localized with HMGA2 (green color) in the nucleus of control siRNA-transfected HCT116 cells (Fig. 3B, top panel: merged image show colocalization of Hsp90 and HMGA2; overlap of red and green: yellow). However, this phenomena cannot observe in siHsp90 transfected HCT116 cells (Fig. 3B, bottom panel of merged image). These results indicated that these is existence the interaction between Hsp90 and HMGA2. Next, we investigated whether the inhibition of HMGA2 through Hsp90-mediated inhibition by using Hsp90 inhibitor. As shown in Fig. 3C, HMGA2 protein expression was significantly reduced on NVP-AUY922-treated HCT116 cells in both the 40 and 80 nM. To evaluate a potential Hsp90-HMGA2 interaction, we performed an immunoprecipitation assay to determine the effect of NVP-AUY922 on the physical interactions between Hsp90 and HMGA2. After NVP-AUY922 treatment, the HMGA2 protein was immunoprecipitated

with an anti-HMGA2 antibody and analyzed through Western blotting with anti-Hsp90 or anti-HMGA2 antibodies. As shown in Fig. 3D, a single band was detected using anti-Hsp90 antibody in immunoprecipitates or input lysate from NVP-AUY922-treated HCT116 cells. In addition, the protein interaction between Hsp90 and HMGA2 was not affected by treatment with NVP-AUY922 at 20 nM. Hsp90 inhibitors cause degradation of Hsp90 client proteins via a proteasome-dependent pathway (Basso et al., 2002). Therefore, we examined whether proteasomal degradation mediates the loss of HMGA2 protein after treatment with NVP-AUY922. As shown in Fig. 3E, decreased levels of CDK4 and HMGA2 by NVP-AUY922 treatment at 40 nM were recovered by treatment with a proteasomal inhibitor, MG132, indicating the involvement of proteasomal degradation in this loss of HMGA2 protein. Previous study have demonstrated that Hsp90 inhibitor-mediated proteasomal degradation of Hsp90 client proteins was preceded by their ubiquitination (Grbovic et al., 2006); therefore, we then tested whether HMGA2 was ubiquitinated prior to its degradation in NVP-AUY922-treated cells. Immunoprecipitation of HMGA2 followed by Western blot analysis with an anti-ubiquitin antibody detected significantly higher levels of ubiquitinated HMGA2 in the presence of the combination of MG132 and NVP-AUY922, compared with either agent alone (Fig. 3F). Taken together, these data suggest that downregulation of HMGA2 protein was a direct effect of Hsp90 inhibition and also indicate that Hsp90 is necessary for the stability of HMGA2.

Revised Fig. 3

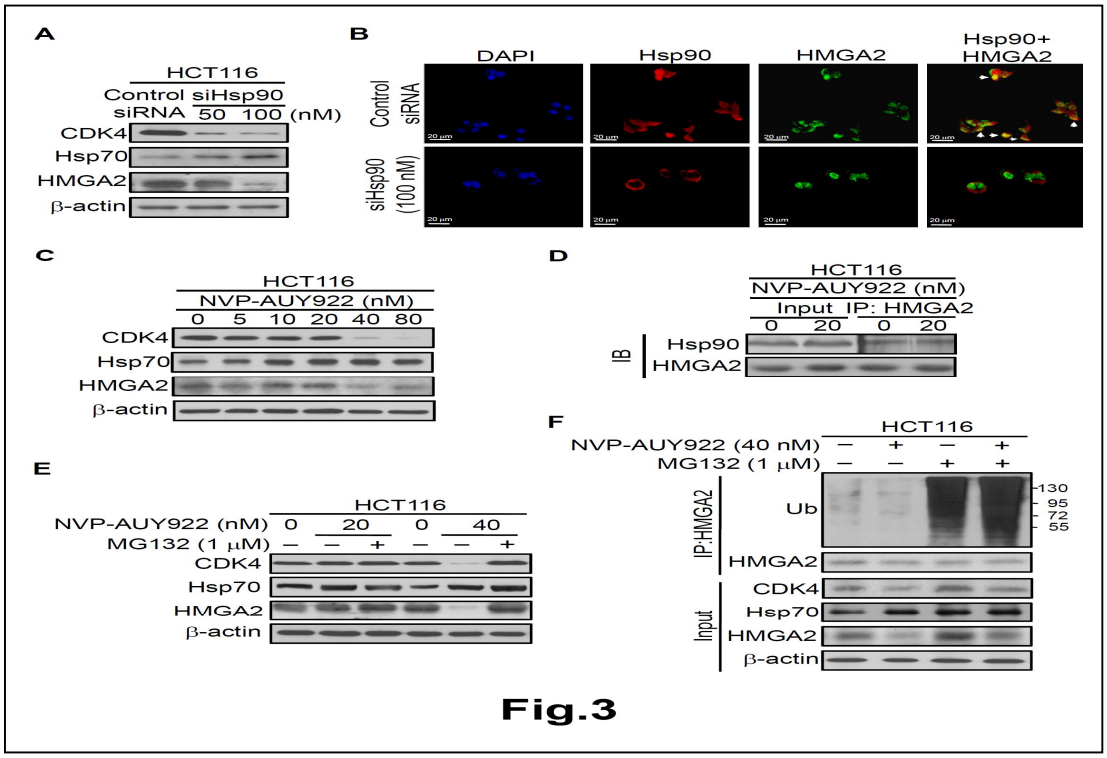


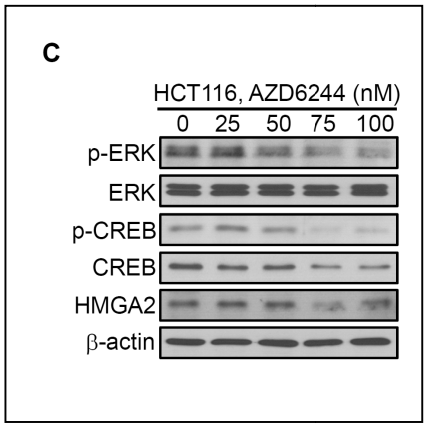
Fig.3

" Experimental design 2"

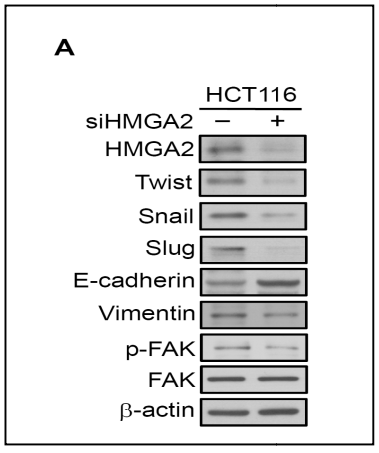
As shown in Fig. 5, the expression and phosphorylation of some kinases were detected in response to different concentration of NVP-AUY922 treatment. Although some kinases were down-regulated after the treatment of NVP-AUY922 in high concentration, we still cannot get the conclusion that there is some kind of relationship among these kinases. The evidence is not enough and more experimental groups is required.

Response. We would like to thank reviewer for the comments and constructive suggestions. To understand whether ERK was indeed involved in regulation of HMGA2, we examined the dose effect of AZD6244, a potent ERK inhibitor, on the ERK activity, CREB and HMGA2 proteins expression of HCT116 cells. As shown in revised Fig. 6C, Western blotting indicated that phosphorylation status of ERK and CREB was significantly inhibited on AZD6244-treated HCT116 cells in both 75 and 100 nM in conjunction with concomitant the decrease expression of HMGA2. FAK has been demonstrated to be regulated by HMGA2 (1). Therefore, downregulation of FAK after the treatment of NVP-AUY922 is through HMGA2-mediated regulation. In revised Fig. 5A, we also shown that siHMGA2 treatment attenuated the phosphorylation of FAK without affecting the total FAK. The associated results shown below:

Revised Fig. 6C



Revised Fig. 5A



" Experimental design 3"

As shown in Fig. S1, the relationship of HMGA2 or Hsp90 mRNA expression with clinical stages was analyzed separately and it was significant in public database, however, it was more important to analyze the relationship of HMGA2 and Hsp90.

Response. We would like to thank reviewer for the comments and constructive

suggestions. To analyze the relationship of HMGA2 and Hsp90, we established the intracellular localization of both HMGA2 and Hsp90 by immunofluorescence in CRC cells. In addition, we demonstrated that Hsp90 inhibitor promoted ubiquitination followed by proteasome-dependent degradation of HMGA2 protein. These data suggest that downregulation of HMGA2 protein was a direct effect of Hsp90 inhibition and also indicate that Hsp90 is necessary for the stability of HMGA2. The results and the description of the results about the relationship of HMGA2 and Hsp90 were shown in Response of *" Experimental design 1"*.

"Validity of the findings 1"
According to the hypothesis of the authors, the Hsp90 inhibitor, NVP-AUY922 was able to down-regulate the expression of HMGA2 through a let-7a dependent way, and subsequently influenced downstream signal pathway. As shown in Fig. 3D, the reactivation effect of NVP-AUY922 on let-7a was significant only when its concentration was more than 40 nm. However, as shown in Fig 2A, the effect of NVP-AUY922 on cell viability was also significant in low concentration. These results did not support the authors' hypothesis.

Response. We would like to thank reviewer for the comments and constructive suggestions. Let-7 reactivation is one of possibility to affect HMGA expression. In addition, as shown in our revised Fig.3, downregulation of HMGA2 protein was also through Hsp90 inhibition. This result indicate that Hsp90 is necessary for the stability of HMGA2. Moreover, Hsp90 inhibitor also can indirectly regulated HMGA2 via inactivation of the ERK signaling pathway (revised Fig.6). Thus, the effect of NVP-AUY922 on cell viability was significant in low concentration would be through Hsp90-mediated direct or indirect regulation of HMGA2.

"Validity of the findings 2"
The IHC images should be evaluated by professional pathologist. As shown in Fig. S2, it only presented the proportion of carcinoma nest, not the expression level of HMGA2.

Response. We would like to thank reviewer for the comments and constructive suggestions. The IHC images have be evaluated by professional pathologist. The staining intensity of HMGA2 expression levels have been redefined as score 0: Negative, score 1: Weak expression, score 2: Moderate expression, score3: Strong expression.

Comments for the author:

The manuscript entitled "Heat shock protein 90 is involved in the regulation of HMGA2-driven growth and epithelial-to-mesenchymal transition of colorectal cancer cells" presents some results about the effect of Hsp90 inhibition on HMGA2 protein expression and subsequent signal pathway. The authors want to demonstrate that the treatment of NVP-AUY922, an Hsp90 inhibitor, was able to regulate the expression of HMGA2, and influence cell growth and migration of CRC cells in a HMGA2 dependent pathway. The effect of NVP-AUY922 on HMGA2 expression occurred through the reactivation of let-7a. However, the results were not enough to support the authors' hypothesis and there are a number of issues with these experiments that need to be addressed.

Response. We would like to thank reviewer for the comments and constructive suggestions. Let-7 reactivation is one of possibility to affect HMGA expression. In addition, as shown in our revised Fig. 3, the suppression of HMGA2 protein expression in response to NVP-AUY922 treatment resulted in ubiquitination and subsequent proteasome-dependant degradation of HMGA2. Moreover, to further investigate whether ERK was indeed involved in regulation of HMGA2, we examined the dose effect of AZD6244, a potent ERK inhibitor, on the ERK activity, CREB and HMGA2 proteins expression of HCT116 cells. Western blotting indicated that phosphorylation status of ERK and CREB was significantly inhibited on AZD6244-treated HCT116 cells in both 75 and 100 nM in conjunction with concomitant the decrease expression of HMGA2 (revised Fig. 6C, which shown in response of *"Experimental design 2"*). Thus, dowenregulation of HMGA2 may be through reactivation of Let7a or Hsp90-mediated direct or indirect regulation of HMGA2.

Reviewer 3 (Anonymous)

We appreciate the comments **(in italics)** of this reviewer and believe that our manuscript has been improved by attention to him or her. The followings are our responses to the specific issues raised by this reviewer:

In terms of format, it would be ideal to revise some parts:

- There are some almost identical sentences used in both abstract and introduction sections.

Response. We would like to thank reviewer for the comments and constructive suggestions. The sentences in abstract have been rephrased and shown below:

Revised abstract

High mobility group AT-hook 2 (HMGA2) is a nonhistone chromatin-binding protein and act as a transcriptional regulating factor involved in gene transcription. In particular, overexpression of HMGA2 has been demonstrated to associate with neoplastic transformation and tumor progression in colorectal cancer (CRC). Thus, HMGA2 is a potential therapeutic target in cancer therapy. Heat shock protein 90 (Hsp90) is a chaperone protein required for the stability and function for a number of proteins that promote the growth, mobility, and survival of cancer cells. Moreover, it has shown strong positive connections were observed between Hsp90 inhibitors and CRC, which indicated their potential for use in CRC treatment by using combination of data mining and experimental designs. However, little is known about the effect of Hsp90 inhibition on HMGA2 protein expression in CRC. In this study, we tested the hypothesis that Hsp90 may regulate HMGA2 expression and investigated the relationship between Hsp90 and HMGA2 signaling. The use of the second-generation Hsp90 inhibitor, NVP-AUY922, considerably knocked down HMGA2 expression, and the effects of Hsp90 and HMGA2 knockdown were similar. In addition, Hsp90 knockdown abrogates colocalization of Hsp90 and HMGA2 in CRC cells. Moreover, the suppression of HMGA2 protein expression in response to NVP-AUY922 treatment resulted in ubiquitination and subsequent proteasome-dependant degradation of HMGA2. Furthermore, RNAi-mediated silencing of HMGA2 reduced the survival of CRC cells and increased the sensitivity of these cells to chemotherapy. Finally, we found that the NVP-AUY922-dependent mitigation of HMGA2 signaling occurred also through indirect reactivation of the tumor suppressor microRNA (miRNA), let-7a, or the inhibition of ERK-regulated HMGA2 involved in regulating the growth of CRC cells. Collectively, our studies identify the crucial role for the Hsp90-HMGA2 interaction in maintaining CRC cell survival and migration. These findings have significant implications for inhibition HMGA2-dependent tumorigenesis by clinically available Hsp90 inhibitors.

- *The supplementary data should be moved from the introduction section to the results section.*

Response. We would like to thank reviewer for the comments and constructive suggestions. The supplementary Fig. 1 have be moved from the introduction section to the results section and shown following:

Section 1 of Results

Elevated expression of HMGA2 mRNA and protein in CRC cell lines and tissues

To determine the HMGA2 expression levels in these CRC patients, we first analyzed the gene expression of *HMGA2* in 132 CRC tumor samples: 67 primary CRC tissues, 65 metastatic tissues, and nine normal colon controls. As expected, HMGA2 expression was significantly upregulated in metastatic and primary CRC tissues compared with that in the normal colon controls (Fig. S1A). Similarly, the Hsp90 expression levels were analyzed in the same metastatic and primary CRC tissues, and the mRNA expression levels of Hsp90 were similar to those of HMGA2 (Fig. S1B).

- *There are paragraphs in the manuscript that are ambiguous and not clearly written.*

Response. We would like to thank reviewer for the comments and constructive suggestions. The paragraphs in the manuscript have been rephrased and highlighted in yellow color of reversed manuscript.

The method section is inadequate, and therefore, additional details should be added. It is also unclear how many times and replicates for each experiment had been performed.

Response. We would like to thank reviewer for the comments and constructive suggestions. The times or replicates for each experiment had been performed which have been added to method section or figure legends in revised manuscript. The additional details of method section have been added in revised manuscript and the responses of the requested questions shown following:

1. Include the names of institution affiliated with the scientists providing the cell lines.

Response. We would like to thank reviewer for the comments. The names of institution affiliated with the scientists providing the cell lines has included in the Materials and Method section of revised manuscript.

2. List the type of antibiotics used in the cell culture media.

Response. We are sorry for the misleading and thank reviewer for the comments. The type of antibiotics used in the cell culture media was penicillin and streptomycin

(P/S) which has been listed in revised manuscript.

3. Indicate if TaqMan probes or SybrGreen is used. Primer sequences are missing.

Response. We are sorry for the misleading and thank reviewer for the comments. The amplification and detection of specific products were performed using the cycle profile of the Qiagen miScript SYBR green PCR starter kit. The sequences of the primers used in this study were listed in the revised manuscript.

4. Immunoprecipitation protocol is missing.

Response. We are sorry for the misleading and thank reviewer for the comments. The immunoprecipitation protocol have been added in the section of Materials and Methods of revised manuscript and shown below:

Immunoprecipitation

The interaction between Hsp90 protein and HMGA2 was studied by immunoprecipitation analysis of extracts prepared from HCT116 or DLD1 cell lines. Cells were lysed, incubated in IP lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, protease inhibitor mixture) for 30 min on ice, and then sonicated (3 times for 10 sec each). After centrifugation at 1400 g for 5 min at 4°C, the supernatants were collected from each sample and then pre-cleared by incubation with 50% protein A/G agarose beads in the IP lysis buffer at 4°C for 1 h with rocking. After removal of the protein A/G beads by centrifugation, protein content in each sample was measured and aliquots containing 500 μg of protein were incubated with primary antibodies overnight at 4°C. The immunoprecipitates bound to the protein A/G–Sepharose beads were washed, boiled and analyzed by immunoblotting.

5. Transfection protocol is missing; how long was the transfection? List oligo sequences for siHsp90 and siHmga2.

Response. We are sorry for the misleading and thank reviewer for the comments. The transfection protocol have been added in the section of Materials and Methods of revised manuscript and the time for transfection and sequences for siRNAs shown below:

Transfection

Cells were transfected with siRNAs for 48 h using Lipofectamine 2000 (Life

Technologies) according to the manufacturers' instructions. The siRNA used in this study from Life Technologies and their sequences were as follows: Hsp90 (siRNA ID: s6994): sense 5'-CUAUGGGUCGGUGGAACAAAtt-3' and antisense 5'-UUUGUUCCACGACCCAUAGgt-3'; HMGA2 (siRNA ID: s224869): sense 5'-GGAGAAAAACGGCAAGAGtt-3' and antisense 5'-CUCUUGGCCGUUUUUCUCCag-3'.

6. It is written 'standard error' in the method section but elsewhere in the manuscript, values are often reflected as standard deviation. Which one of them is used?

Response. We are sorry for the misleading and thank reviewer for the comments and constructive suggestions. We have corrected "standard error" to "standard deviation" in the method section of revised manuscript.

Validity of the findings

The authors have shown that Hsp90 has a new role in regulating HMGA2 expression. However, the data remains incomplete due to lack of adequate controls and complementary assays. There was no concrete evidence indicating HMGA2's main role in driving EMT in HCT116 cells, bearing in mind that NVP-AUY922 have multiple effects on other oncogenic proteins, of which does not represent the title of the manuscript. Therefore, it is still early to draw conclusions from the data.

Response. We would like to thank reviewer for the comments and constructive suggestions. To make the complete of results can strongly support the conclusion, we make the changes of two parts and show below:

First, to make the link between Hsp90 and HMGA2, we performed immunofluorescence analysis to investigate the intracellular localization of both Hsp90 and HMGA2. In addition, we proofed that the suppression of HMGA2 protein expression in response to NVP-AUY922 treatment resulted in ubiquitination and subsequent proteasome-dependant degradation of HMGA2. These data suggest that downregulation of HMGA2 protein was a direct effect of Hsp90 inhibition and also indicate that Hsp90 is necessary for the stability of HMGA2. The revised its legend accordingly, and have included to following description of the associated results:

Section 3 of Results

Hsp90 regulates and interacts with HMGA2

Hsp90 is critical in regulating cell growth (Cheung et al., 2010; Ko et al., 2012; Miyata, 2003; Nagaraju et al., 2014), and HMGA2 has a well-documented role in this process

(Di Cello et al., 2008; Malek et al., 2008; Sun et al., 2013; Wend et al., 2013); therefore, we examined whether the interaction between the Hsp90 and HMGA2 is existence. Thus, we first performed RNA interference to deplete Hsp90 in the HCT116 cells and examined the effect of its depletion in the intracellular localization of both Hsp90 and HMGA2 by immunofluorescence analysis. As shown in Fig. 3A, Hsp90 siRNA-mediated endogenous Hsp90 knockdown significantly reduced CDK4 (Hsp90 client protein) and HMGA2 expression, as well as induced Hsp70 (Hsp90 client protein) expression in the siHsp90-transfected HCT116 cells. Further, immunofluorescence result revealed that Hsp90 (red color) co-localized with HMGA2 (green color) in the nucleus of control siRNA-transfected HCT116 cells (Fig. 3B, top panel: merged image show colocalization of Hsp90 and HMGA2; overlap of red and green: yellow). However, this phenomena cannot observe in siHsp90 transfected HCT116 cells (Fig. 3B, bottom panel of merged image). These results indicated that these is existence the interaction between Hsp90 and HMGA2. Next, we investigated whether the inhibition of HMGA2 through Hsp90-mediated inhibition by using Hsp90 inhibitor. As shown in Fig. 3C, HMGA2 protein expression was significantly reduced on NVP-AUY922-treated HCT116 cells in both the 40 and 80 nM. To evaluate a potential Hsp90-HMGA2 interaction, we performed an immunoprecipitation assay to determine the effect of NVP-AUY922 on the physical interactions between Hsp90 and HMGA2. After NVP-AUY922 treatment, the HMGA2 protein was immunoprecipitated with an anti-HMGA2 antibody and analyzed through Western blotting with anti-Hsp90 or anti-HMGA2 antibodies. As shown in Fig. 3D, a single band was detected using anti-Hsp90 antibody in immunoprecipitates or input lysate from NVP-AUY922-treated HCT116 cells. In addition, the protein interaction between Hsp90 and HMGA2 was not affected by treatment with NVP-AUY922 at 20 nM. Hsp90 inhibitors cause degradation of Hsp90 client proteins via a proteasome-dependent pathway (Basso et al., 2002). Therefore, we examined whether proteasomal degradation mediates the loss of HMGA2 protein after treatment with NVP-AUY922. As shown in Fig. 3E, decreased levels of CDK4 and HMGA2 by NVP-AUY922 treatment at 40 nM were recovered by treatment with a proteasomal inhibitor, MG132, indicating the involvement of proteasomal degradation in this loss of HMGA2 protein. Previous study have demonstrated that Hsp90 inhibitor-mediated proteasomal degradation of Hsp90 client proteins was preceded by their ubiquitination (Grbovic et al., 2006); therefore, we then tested whether HMGA2 was ubiquitinated prior to its degradation in NVP-AUY922-treated cells. Immunoprecipitation of HMGA2 followed by Western blot analysis with an anti-ubiquitin antibody detected significantly higher levels of ubiquitinated HMGA2 in the presence of the combination of MG132 and NVP-AUY922, compared with

either agent alone (Fig. 3F). Taken together, these data suggest that downregulation of HMGA2 protein was a direct effect of Hsp90 inhibition and also indicate that Hsp90 is necessary for the stability of HMGA2.

Revised Fig. 3

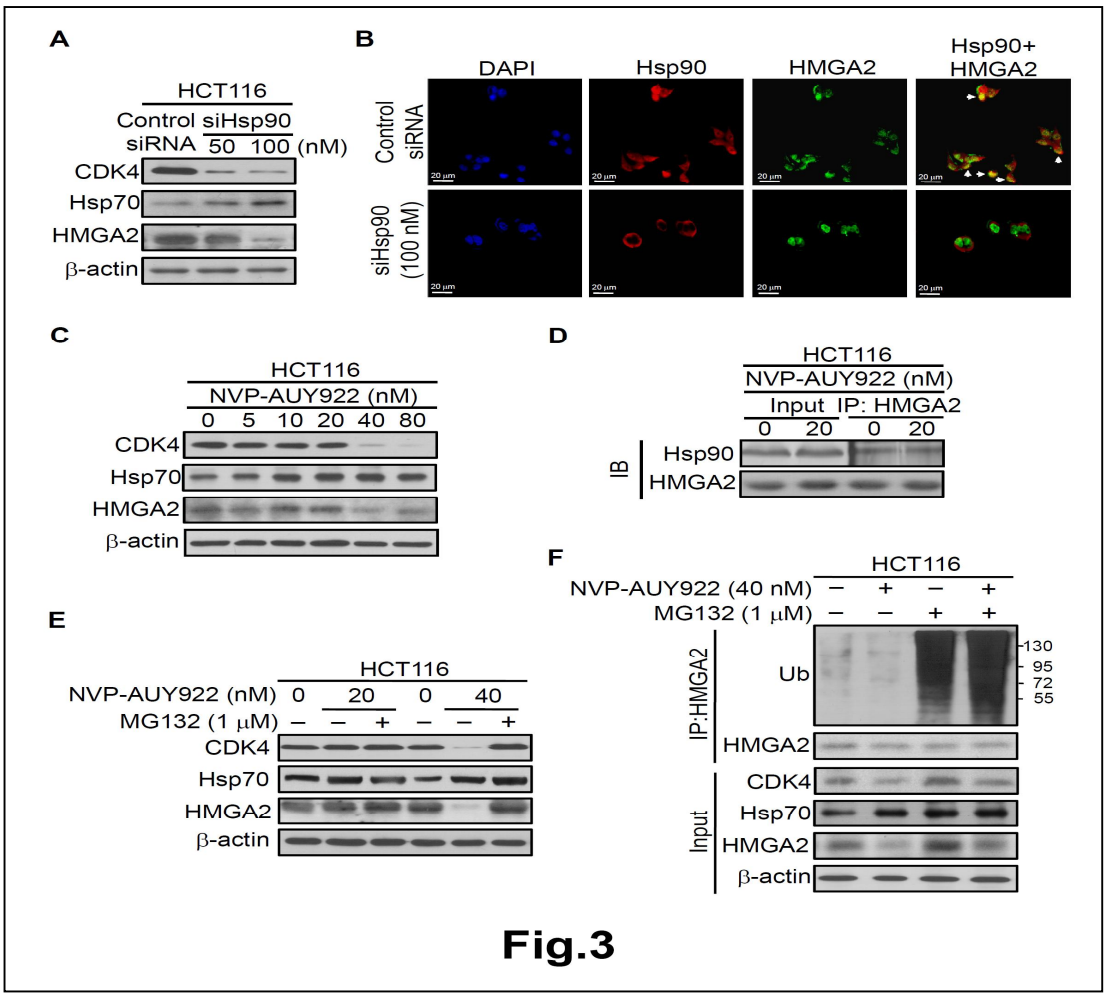


Fig.3

Second, to point the HMGA2's main role in driving EMT in HCT116 cells, we have investigated the effect of siRNA-mediated knockdown of HMGA2 on the expression of EMT effectors and FAK in HCT116 cells. In addition, we found that knockdown HMGA2 expression can enhance the effect of NVP-AUY922-mediated suppression of EMT and migratory ability of HCT116 cells. The revised its legend accordingly, and have included to following description of the associated results:

HMGA2 as a master regulator of epithelial–mesenchymal transition (EMT) and involved in NVP-AUY922-mediated suppression of EMT

To investigate the role of HMGA2 in regulating EMT of CRC cells, we investigated the effect of siRNA-mediated knockdown of HMGA2 on the expression of EMT effectors

in HCT116 cells. As shown in Fig. 5A, HMGA2 knockdown inhibited EMT in HCT116 cells, which was evidenced by reduced HMGA2-regulated mesenchymal markers (Twist, Snail, and Slug) as well as Vimentin expression in conjunction with concomitant increases in the expression of the E-cadherin. In addition, Focal adhesion kinase (FAK) activation is important for cancer motility. It has demonstrated that FAK is regulated by HMGA2 in melanoma cells (*Zhang et al., 2015*). siHMGA2 treatment attenuated the phosphorylation of FAK without affecting the total FAK in HCT116 cells (Fig. 5A). Pursuant to these findings, we used the siRNA-mediated knockdown of HMGA2 to verify its effect in the NVP-AUY922-mediated suppression of EMT in HCT116 cells. As shown in Fig. 5B, knockdown HMGA2 expression can enhance the effect of NVP-AUY922-mediated suppression of EMT in HCT116 cells to compare with control siRNA group. Next, the *in vitro* efficacy of NVP-AUY922 in suppressing cancer cell mobility was illustrated by its dose-dependent inhibition of the migration of HCT116 cells-transfected with control siRNA or siHMGA2 after 24 hours of treatment in transwell assays. As shown in Fig. 5C and D, the migratory abilities significantly reduced about 50% of siHMGA2-transfected HCT116 cells compared with those in the control cells and the number of migrating cells was significantly reduced in siHMGA2-transfected HCT116 cells-treated with NVP-AUY922. Together, these findings suggest that NVP-AUY922 can enhance the reduction of EMT in siHMGA2-transfected HCT116 cells.

Revised Fig. 5

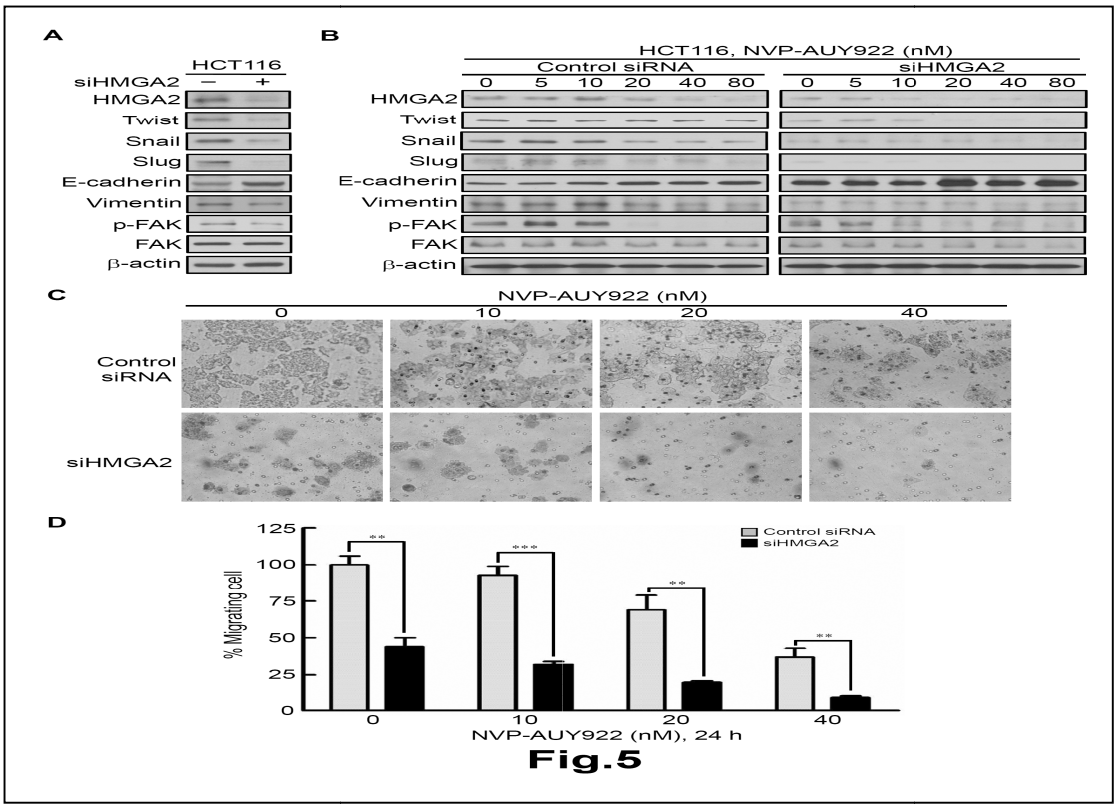


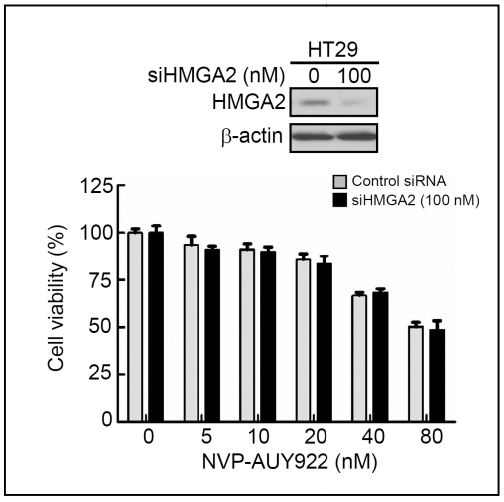
Fig.5

Major points:

- To strengthen the notion that HMGA2 confers resistance in CRC, include another cell line that is more resistant to NVP-AUY922, e.g. HT29 (based on their previous work); examine HMGA2 levels and then knockdown HMGA2 to see if the cell line becomes sensitized towards NVP-AUY922. HMGA2 WBs should be included for both HCT116 and DLD1-GFP-HMGA2 cells treated with/without NVP-AUY922 (Fig. 2).

Response. We would like to thank reviewer for the comments. We have investigated the requested data about knockdown HMGA2 in HT29 cells to see if the cell line becomes sensitized towards NVP-AUY922. The associated results shown below to indicate that NVP-AUY922 treatment was not significant reduction the cell viability of siHMGA2-transfected HT29 cells compared with the control siRNA-transfected HT29 cells. HMGA2 WBs have be included for both HCT116 and DLD1-GFP-HMGA2 cells treated with/without NVP-AUY922 in the revised Fig. 4. The associated results shown below:

The sensitivity of NVP-AUY922 in HT29 cells with HMGA2 knockdown



HMGA2 WBs in both HCT116 and DLD1-GFP-HMGA2 cells treated with/without NVP-AUY922 (Revised Fig. 4)

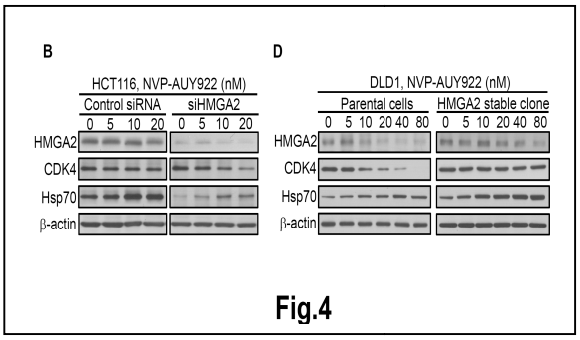


Fig.4

- CDK4 appears to be a poor readout for the efficacy of NVP-AUY922 treatments in this study. Authors should consider to include additional surrogate marker of Hsp90 inhibition, e.g. Hsp70 (Dakappogari, et al., Biomarker, 2010). Fig. 3A & 3B: Hsp90 WB to show knockdown efficiency and also protein levels after treatment with NVP-AUY922. How long was the siRNA transfection? CDK4 WB quality is poor and can be improved.

Response. We would like to thank reviewer for the comments and constructive suggestions. The point by point responses to reviewers' comments shown below:

1. We have added the Hsp70 protein expression data to examine the efficacy of NVP-AUY922 treatments in our study in the revised Fig. 3 and 4.
2. We are sorry for the misleading and the time for siRNA transfection is 48 h which has added in the figure legend of Fig. 3 of revised manuscript.
3. We have improved the quality of CDK4 WB in the revised Fig. 3 and 4.

- *The interaction between Hsp90 and HMGA2 is not convincing, as there are no IgG controls or WB for HMGA2. In the text (page 8, line 8), how is HMGA2 expression observed if HMGA2 WB is missing? Assuming that the IP experiment worked, authors did not discuss the discrepancies of Hsp90-HMGA2 interaction observed between the two cell lines used, HCT116 and HCT15. Can this interaction be also observed in their overexpression system, i.e. DLD1-GFP-HMGA2 cells?*

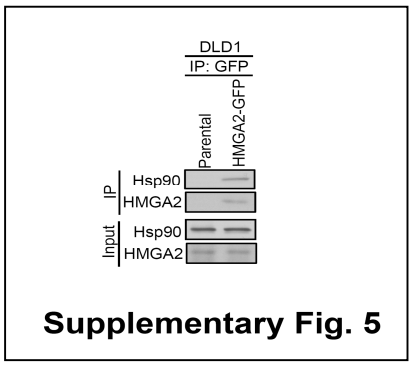
Response. We are sorry for the misleading and thank reviewer for the comments. The point by point responses to reviewers' comments shown below:

1. We have added the data for investigation the interaction between Hsp90 and HMGA2 to reversed Fig. 3 and shown in response of "*Validity of the findings*".
2. HMGA2 WB has been added in revised Fig. 3D and shown in response of "*Validity of the findings*".
3. In our revised manuscript, we have deleted the result of HCT15 cells.
4. This interaction can be also observed in DLD1-GFP-HMGA2 cells. The associated results and description of the associated results shown below:

Section 3 of Results

Hsp90 regulates and interacts with HMGA2

The interaction between Hsp90 and HMGA2 was also observed in DLD1 HMGA2-GFP cells to show that Hsp90 was coimmunoprecipitated by the GFP antibody in DLD1 HMGA2-GFP cells (Fig. S5).

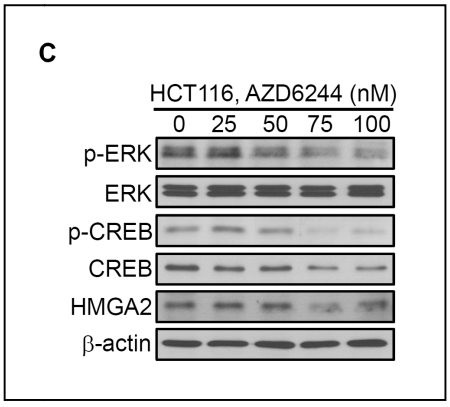


Supplementary Fig. 5

- Hsp90 activity is required for many kinases, e.g. ERK, FAK, and Hsp90 negatively regulates let-7a expression (in this study). Therefore, pharmacological inhibition of Hsp90 could be an indirect effect on HMGA2 regulation. Is HMGA2 a bona fide client protein of Hsp90? This is not shown or discussed. The interaction study between Hsp90 and HMGA2 is not sufficient to prove this.

Response. We would like to thank reviewer for the comments and constructive suggestions. Let-7 reactivation is one of possibility to affect HMGA expression. In addition, the interaction between Hsp90 and HMGA2 have been demonstrated in our revised Fig. 3 and shown in response of "*Validity of the findings*". These data suggest that downregulation of HMGA2 protein was a direct effect of Hsp90 inhibition and also indicate that Hsp90 is necessary for the stability of HMGA2. Moreover, to further investigate whether ERK was indeed involved in regulation of HMGA2, we examined the dose effect of AZD6244, a potent ERK inhibitor, on the ERK activity, CREB and HMGA2 proteins expression of HCT116 cells. As shown in Fig. 6C, Western blotting indicated that phosphorylation status of ERK and CREB was significantly inhibited on AZD6244-treated HCT116 cells in both 75 and 100 nM in conjunction with concomitant the decrease expression of HMGA2. Thus, dowenregulation of HMGA2 may be through reactivation of Let7a or Hsp90-mediated direct or indirect regulation of HMGA2.

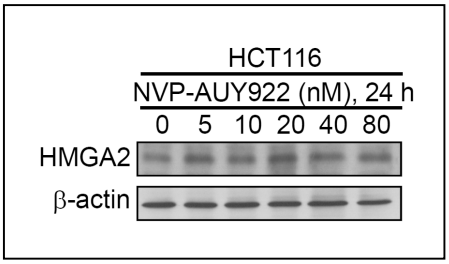
Revised Fig. 6C



- Fig. 3D: NVP-AUY922 treatments were for 24 h, in contrast to 48 h in other experiments, and the increment of let-7a expression was small, 1.5 fold at 40 nM. Is there a reason for the shorter treatment and how many times have the experiment been done? Since let-7a affects HMGA2 at the post-transcriptional level, what are the protein levels of HMGA2 at the 24-h time point?

Response. We would like to thank reviewer for the comments. Let-7 reactivation is

one of possibility to affect HMGA expression. Dowenregulation of HMGA2 may be through Hsp90-mediated direct or indirect regulation of HMGA2. let-7a affects HMGA2 at the post-transcriptional level, thus, the time for induction of let-7a expression must be prior to detect HMGA2 protein expression. According to revised Fig. 3C, decreased level of HMGA2 by NVP-AUY922 treatment at 40 nM for 48 h was observed. Thus, the time for induction of let-7a expression is suitable. The experiment for NVP-AUY922 treatment to induction let-7a expression was perform for three independent experiments. The protein levels of HMGA2 was not significant change by NVP-AUY922 treatment for 24 h. The associated result shown below:



- Fig. 5 comes back to my earlier point on Hsp90 being upstream of many kinases, which are also known to activate the EMT program, independent of HMGA2. Supplementary data should be included to show that HMGA2 is the main driver of EMT in HCT116 cells. Authors attempt to put forth a Hsp90-ERK-CREB-HMGA2 pathway in CRC cells; can they explain the high levels of p-ERK and p-CREB at 10 nM (even at 20 - 80 nM) NVP-AUY922, when HMGA2 is already downregulated? p-FAK and EMT effectors (Twist, Snail and Slug) levels are also high at 10 nM NVP-AUY22. Therefore, the data is not convincing to conclude that NVP-AUY922 could inhibit HMGA2-mediated EMT or HMGA2 regulation by its 'associated kinases' in HCT116 cells. Since HMGA2 confer resistance to NVP-AUY922 in CRC, have the authors check the combinatorial effects of NVP-AUY922 and siHmga2 treatment on cell proliferation, migration and EMT?

Response. We would like to thank reviewer for the comments and constructive suggestions. The point by point responses to reviewers' comments shown below:

1. We have added the data for investigation HMGA2's main role in driving EMT in HCT116 cells to reversed Fig. 5 and shown in response of "*Validity of the findings*".
2. We have deleted the expression of HMGA2 in revised Fig. 6. Indeed, in the original Fig. 5, the data is not convincing to conclude that NVP-AUY922 could inhibit HMGA2-mediated EMT or HMGA2 regulation by its 'associated kinases' in HCT116 cells. The result of revised Fig. 5 can get the summary to indicate NVP-AUY922 could

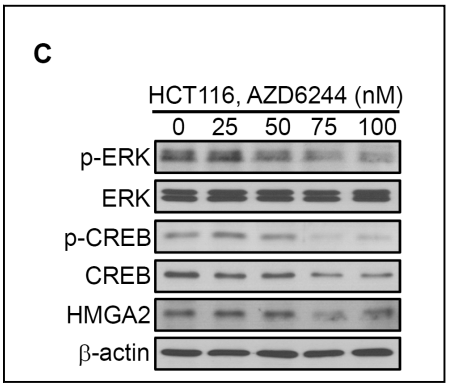
inhibit HMGA2-mediated EMT. To further investigate whether ERK was indeed involved in regulation of HMGA2, we examined the dose effect of AZD6244, a potent ERK inhibitor, on the ERK activity, CREB and HMGA2 proteins expression of HCT116 cells. As shown in Fig. 6C, Western blotting indicated that phosphorylation status of ERK and CREB was significantly inhibited on AZD6244-treated HCT116 cells in both 75 and 100 nM in conjunction with concomitant the decrease expression of HMGA2. The associated results and description of the associated results shown below:

Section 6 of Results

Phospho-kinase array for investigating NVP-AUY922-induced altered activity of HMGA2-associated kinases that regulate growth and mobility of HCT116 cells

To further investigate whether ERK was indeed involved in regulation of HMGA2, we examined the dose effect of AZD6244, a potent ERK inhibitor, on the phosphorylation status of ERK, CREB and HMGA2 proteins expression of HCT116 cells. As shown in Fig. 6C, Western blotting indicated that phosphorylation status of ERK and CREB was significantly inhibited on AZD6244-treated HCT116 cells in both 75 and 100 nM in conjunction with concomitant the decrease expression of HMGA2.

Revised Fig. 6C



3. We have added the data for investigation the combinatorial effects of NVP-AUY922 and siHmga2 treatment on cell migration and EMT to reversed Fig. 5 and shown in response of "*Validity of the findings*". The combinatorial effects of NVP-AUY922 and siHmga2 treatment on cell proliferation have been investigated. The associated results and description of the associated results shown below:

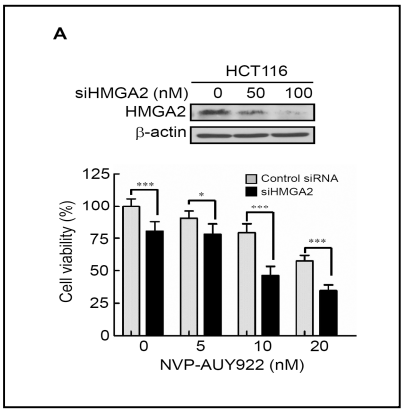
Section 4 of Results

Inhibition of HMGA2 protein increased sensitivity of Hsp90 inhibitor

HMGA2 contributes to resistance against anticancer drugs in various cancer cell lines

(*Gyorffy et al., 2006*). Thus, HMGA2 silencing was hypothesized to increase the sensitivity to anticancer drugs in cancer cells. To test this hypothesis, the HCT116 cell line with elevated HMGA2 expression was selected for transfection with HMGA2 small-interfering RNA oligomer (siHMGA2) or scrambled oligomer (control siRNA). HMGA2 protein expression and cell viability were subsequently examined. As shown in Fig. 4A (upper panel), HMGA2 protein expression was significantly inhibited in siHMGA2-transfected HCT116 cells. To examine the NVP-AUY922 drug sensitivity in siHMGA2-transfected HCT116 cells, a cell viability assay was performed. NVP-AUY922 treatment significantly reduced the cell viability of siHMGA2-transfected HCT116 cells compared with the control siRNA-transfected HCT116 cells (Fig. 4A, bottom panel).

Revised Fig. 4A

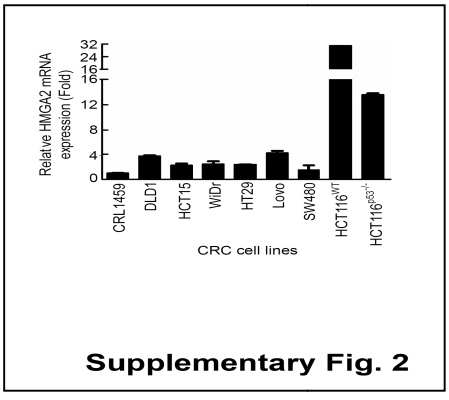


Minor points:

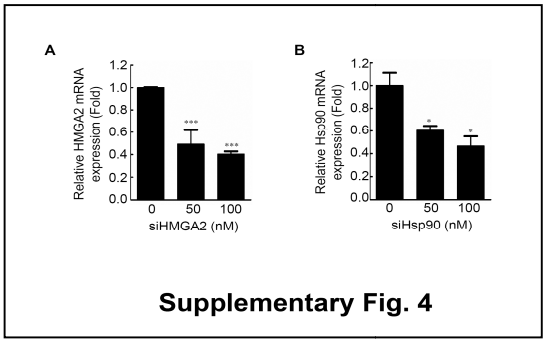
- Fig 1B, 2A: mRNA levels should be validated by qPCR to complement protein expression.

Response. We would like to thank reviewer for the comments. The mRNA levels of HMGA2 have be validated by qPCR in the reversed Fig. S2 and Fig. S4A. The associated results and description of the associated results shown below:

The level of HMGA2 mRNA expression in CRC cell lines



The level of HMGA2 mRNA expression in siHMGA2-transfected HCT116 cells



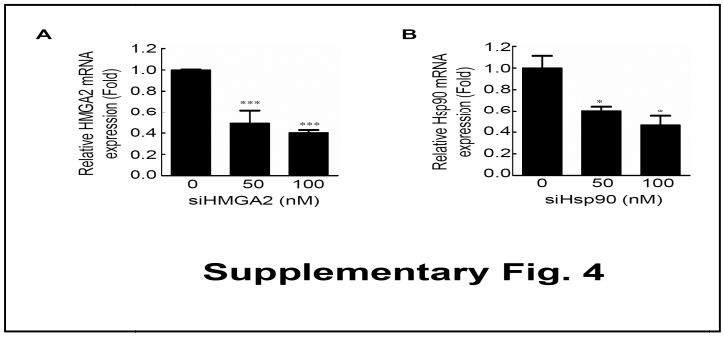
- Fig. 3 did not show any assays that measure HMGA2 activity, please re-phrase the subheading in the figure legend.

Response. We are sorry for the misleading and thank reviewer for the comments. We have rephrased the subheading in the figure legend of revised manuscript.

- Fig. 4A & 4C: How long was the siRNA transfection when the cell viability assay was done and show qPCR or WB to indicate knockdown efficiency.

Response. We are sorry for the misleading and thank reviewer for the comments. The time for siRNA transfection is 48 h which has added in the figure legend of new revised Fig. 2 of revised manuscript. The knockdown efficiency has been investigated and shown in revised Fig. S4. The associated results and description of the associated results shown below:

<u>siHMGA2 knockdown significantly reduced mRNA expression level of HMGA2 about 60% (Fig. S4A). siHsp90 knockdown significantly reduced mRNA expression level of Hsp90 about 50% (Fig. S4B).</u>



- Fig. 4E, 5B & 5C: How long was NVP-AUY922 treatment?

Response. We are sorry for the misleading and thank reviewer for the comments. The time for *NVP-AUY922 treatment* is 48 h which has added in the figure legends of new revised Fig. 2 and revised Fig. 5 of revised manuscript.

- Fig. 5A: This is an array which measures the phosphorylation status of kinases and not correctly described in the figure legend. Indicate what was used for normalisation for the bar graphs.

Response. We are sorry for the misleading and thank reviewer for the comments. We have correctly described in figure legend of Fig. 5A in revised manuscript. The bar

graphs was normalized by using blank spot.

- *Page 6, line 24: should be 9 different types of cancers.*

Response. We are sorry for the misleading and we have corrected the "nine cancer cell lines" to "9 different types of cancers" in revised manuscript.

- *Page 8, line 22: "epithelial-state transition" should be "epithelial-mesenchymal transition".*

Response. We are sorry for the misleading and we have corrected the *"epithelial-state transition"* to "epithelial-mesenchymal transition" in revised manuscript.

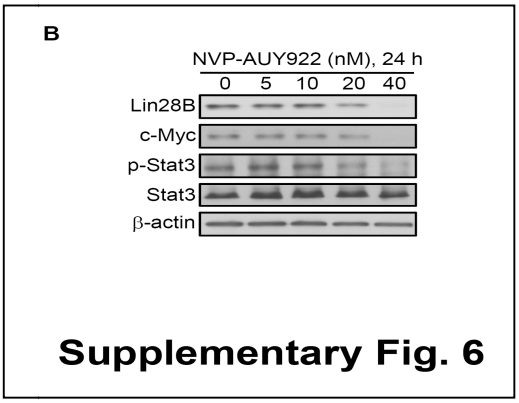
- *Authors should be cautious in their discussion regarding the use of NVP-AUY922 in inhibiting metastasis of CRC cells because they have not done any in vivo experiments, nor referred to other literature (page 11, line 17).*

Response. We are sorry for the misleading and thank reviewer for the comments. We have corrected "inhibition of metastasis" to "inhibition of migration" in revised manuscript.

Comments for the author
The study is interesting, relevant to the field and worth further investigation. Authors could focus on the mechanistic aspect of HMGA2 as a client protein of Hsp90, which is the novelty of this paper. It would be also interesting to know how does Hsp90 regulate let-7a. However, the current finding is still preliminary and requires a major revision before it can be considered for publication. The manuscript can be further improved in terms of clarity and flow

Response. We would like to thank reviewer for the comments and constructive suggestions. We have focused on the mechanistic aspect of HMGA2 as a client protein of Hsp90. The interaction between Hsp90 and HMGA2 have been demonstrated in our revised Fig. 3 and shown in response of "*Validity of the findings*". It has shown that the biogenesis of let-7a was blocked by overexpression of c-Myc/Lin28B axis in cancer cells (2). In addition, it has been demonstrated that Stat3-coordinated Lin28B–let-7–HMGA2 signaling to circuit initiate and maintain oncostatin M-driven EMT (3). To determine whether reactivation of let-7a by

treatment with Hsp90 inhibitor through inhibition of c-Myc/LIN28B axis or Stat3 signaling, these proteins were detected in NVP-AUY922-treated HCT116 cells. As shown in Fig. S6B, the phosphorylation status of Stat3 and protein expression of Lin28B and c-myc were completely inhibited on NVP-AUY922-treated HCT116 cells in 40 nM for 24 h.



References:

1. Zhang P, Bai H, Liu G, Wang H, Chen F, Zhang B, Zeng P, Wu C, Peng C, Huang C, Song Y, Song E. 2015. MicroRNA-33b, upregulated by EF24, a curcumin analog, suppresses the epithelial-to-mesenchymal transition (EMT) and migratory potential of melanoma cells by targeting HMGA2. Toxicol Lett. 234(3):151-61. DOI 10.1016/j.toxlet.2015.02.018.

2. Pang M, Wu G, Hou X, Hou N, Liang L, Jia G, Shuai P, Luo B, Wang K, Li G. 2014. LIN28B promotes colon cancer migration and recurrence. PLoS One. 9(10):e109169. DOI 10.1371/journal.pone.0109169.

3. Guo L, Chen C, Shi M, Wang F, Chen X, Diao D, Hu M, Yu M, Qian L, Guo N. 2013. Stat3-coordinated Lin-28-let-7-HMGA2 and miR-200-ZEB1 circuits initiate and maintain oncostatin M-driven epithelial-mesenchymal transition. Oncogene. 32(45):5272-82. DOI 10.1038/onc.2012.573.