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PRINCIPAL INVESTIGATOR: Weizhou Zhang

CONTRACTING ORGANIZATION: The University of Florida

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6. AUTHOR(S)

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5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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12. DISTRIBUTION / AVAILABILITY STATEMENT

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In contrast to its important function in DNA repair, overexpression of human MSH2/6 mismatch recognition protein is driving the progression of basal like breast cancer and is associated with poor prognosis. This project will establish the molecular and biological function of MSH2/6. The first funding period has established several models to test this hypothesis. Our preliminary data suggest that this hypothesis is correct and will continue to study as the proposed aims delineate. We also found innovative connection between loss of MSH2 and the activation of Tregs, an immune suppressive T cell subtypes. During the next funding period we will complete the biological and immunological studies that test this hypothesis.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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1. INTRODUCTION:

Current treatment options for basal like breast cancer (BLBC) rely on cytotoxic dose-dense chemotherapy and surgery. The work under this award builds on our preliminary data that suggested that MSH2/6 may be a viable molecular target for BLBC therapy. Our goal is to define the role of MSH2/6 at different stages of BLBC pathogenesis and to establish MSH2/6 as a molecular target for BLBC therapy. As the lead PD/PI of this award, the Zhang lab will focus on the biology of MSH2 in BLBC progression and its influence on cancer immunity. We use mouse models and flow cytometry, including different drug treatment regimens, for this purpose.

2. KEYWORDS:

Basal like breast cancer, BLBC; DNA mismatch repair, MMR; Human MutS homolog MSH2/6, MutSα; cancer progression; cancer immunology and immunotherapy

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals are the same as originally proposed. Here are the following:

Specific Aim 1: Determine the role of MSH2/6 in BLBC pathogenesis and immunotherapy

Major Task 1: Developing mouse models for proposed research: Zhang lab, ongoing.
Major Task 2: Tumor Study: Zhang lab, ongoing

Specific Aim 2: Determine the mechanism how MSH2/6 contributes to epi/genetics and immune modulation. Below are the tasks relevant to this reporting period
Major Task 3: Determine the epigenetic alterations by MSH2: Spies lab, ongoing;
Major Task 4: Determine DNMT1 dependency: Spies lab, ongoing;
Major Task 5: Site-directed DNA methylation and de-methylation: both labs, ongoing;
Major Task 6: Impact of epigenetics on T cells and tumors: both labs, ongoing

What was accomplished under these goals?

We have made the following progress towards the major goals of this project:

For Aim 1, we have successfully transferred all required animals from the University of Iowa to the University of Florida without delay. Now that all the required genotypes have been undergoing active breeding to perform proposed tumor studies. We have accumulated and will continue to accumulate tumor data using the proposed mouse models, as well as using additional models proposed under alternative approaches. In addition, we have found that MSH2 and MLH1, two proteins involving mismatch DNA repair pathways, exhibited distinct tumor phenotypes in BLBC. We used CRISPR/Cas9 protein/guide RNA complex to construct genetic knockout clones for MSH2 and MLH1 within two widely used murine breast cancer models – 4T1 and PY8119

(**Fig.1A-B**). MSH2 knockout leads to no change in tumor growth but decreased lung metastasis in two syngeneic mouse models including 4T1 and Py8119 BLBC models (**Fig. 2A-D**), but MLH1 knockout results in increased tumorigenesis/metastasis (**Fig. 2A-D**). This suggests that MSH2 is a real tumor/metastasis promoter in BLBC, but MLH1 is still a tumor-suppressor as similar in BLBC as in colorectal cancers. At immunological levels, genetic knockout of MSH2 not only induces the accumulation of activated and exhausted T cells as proposed, but also induces an expected activation of regulatory T cells; whereas knockout of MLH1 only leads to T cell chronic activation and exhaustion (**Fig. 3**). The distinct difference between MSH2 and MLH1 warrants further investigation.

For Aim 2, we have also made substantial progress. We have found that interferons induce significant increase in immune checkpoints PD-L1 and PVR from BLBC cell lines only with MSH2-difiency, but not the wild type parental cells (**Fig. 4A-B**). We further found that removing epigenetic marks by treating BLBC cells with 5-azacytidine induces PVR gene expression only within parental cells, but not MSH2-KO cells (**Fig. 4C**); while PD-L1 induction occurred in parental cells, but further enhanced in MSH2-KO cells (**Fig. 4C**). We did not find that MSH2-KO has any influence on the two predicted methylation sites in BLBC cells as predicted by bisulfite sequencing (data not shown), but instead using a more robust tool to screen the epigenetic alterations between parental cells and MSH2-KO cells to find out the epigenetic sites mediated by MSH2-KO (**Table 1**). We have several potential targets with ongoing analysis and will try to target these sites for CRISPR-mediated site-directed mutagenesis. At genetic levels, we found that MSH2-KO cells express increased levels of BMP2 and BMP4 in 4T1 tumors, as well as human BLBC cells (**Fig. 5**). We are also testing if these BMPs have direct influence on the regulatory T cell infiltration into tumors of MSH2-KO cells.

We will continue to explore the proposed aims and no delays are expected.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

We will continue to accumulate mice that are required for finishing Aim 1, treating these mice with tamoxifen at different proposed time points for tumor initiation and progression studies.
We will explore the roles of DNA methylation sites as identified by 850K array and perform site-directed mutagenesis and other studies proposed in Aim 2.
We are studying the immune suppression by Tregs from MSH2-KO and T cell activation.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

When completed, our work will characterize a previously unknown, pathological function of the MSH2/6 protein, which is primarily thought of as a guardian of genome integrity through its function in DNA mismatch repair. This will differentiate the biological functions of MSH2 from MLH1 as indicated

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Nothing to report

Journal publications.

With partial support from this funding, two manuscript were published (see References). A publication directly related to this funding is currently under preparation.

1. Sajid Khan, Xuan Zhang, Dongwen Lv, Qi Zhang, Yonghan He, Peiyi Zhang, Yaxia Yuan, Xingui Liu, Dinesh Thummuri, Janet S Wiegand, Jing Pei, Weizhou Zhang, Abhisheak Sharma, Christopher R. McCurdy, Vinitha M. Kuruvilla, Natalia Baran, Adolfo A. Ferrando, Anna Rogojina, Peter J Houghton, Guangcun Huang, Robert Hromas, Marina Konopleva, Guangrong Zheng, and Daohong Zhou. DT2216, a BCL-XL proteolysis targeting chimera, is a safer and more potent antitumor agent than ABT263. Nature Medicine. 2019 Dec;25(12):1938-1947. PMID: 31792461.
2. Gaurav Pandey, Nicholas Borcherding, Ryan Kolb, Wei Li, Sonia Sugg, Jun Zhang, Dazhi A. Lai, Weizhou Zhang. ROR1 potentiates FGFR signaling in basal-like breast cancer. Cancers (Basel). 2019 May 24;11(5). pii: E718. PMID: 31137681; PMCID: PMC6562526.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Weizhou Zhang
Project Role:	Principal Investigator
Research Identifier (e.g. ORCID ID):	0000-0002-8236-0346
Nearest person month worked:	1
Contribution to Project:	Dr. Zhang has supervised work outlined in Specific Aim 1-2, Major Tasks outlined under SOW
Funding Support:	No change.
Name:	Jiao Mo
Project Role:	Postdoctoral Fellow
Research Identifier (e.g. ORCID ID):	
Nearest person month worked:	10
Contribution to Project:	Dr. Mo has performed work outlined in Specific Aim 1-2, Major Tasks outlined under SOW
Funding Support:	No change.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No change.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: This report reflects the progress of Weizhou Zhang, PI.

QUAD CHARTS: N/A

9. APPENDICES: None

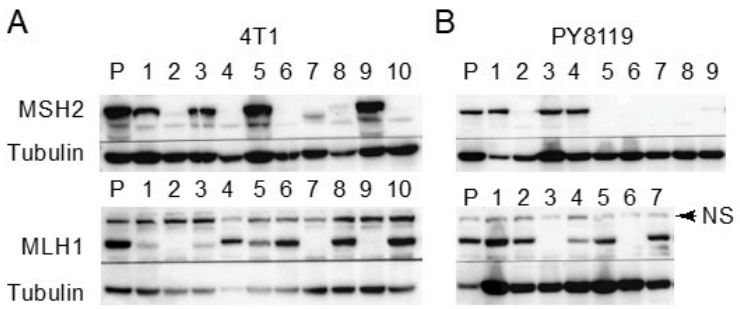

Figure 1. Generation of genetic knockout (KO) clones for MSH2 and MLH1. Protein/guide RNA complex was transfected into 4T1 (**A**) or PY8119 (**B**) murine breast cancer cells per instruction (Synthego). Single clones were chosen without any drug selection and combined to avoid clonal difference for tumor studies.

Pooled clones (Figure 1) were used for tumor/metastasis studies in the following figures:

- 4T1 control (Ctrl, combination of clone 1, 3, 5, 9 from A-upper panel and 4, 6, 8, 10 from A-lower panel)
- 4T1 MSH2-KO (combination of clone 2,4,6,8,10 from A-upper panel)
- 4T1 MLH1-KO (combination of clone 2,7,9 from A-lower panel)
- PY8119 control (Ctrl, combination of clone 1, 3, 4 from B-upper panel and 1, 2, 5, 7 from B-lower panel)
- PY8119 MSH2-KO (combination of clone 2,5,6, 7,8 from B-upper panel)
- PY8119 MLH1-KO (combination of clone 3,6 from B-Lower panel)

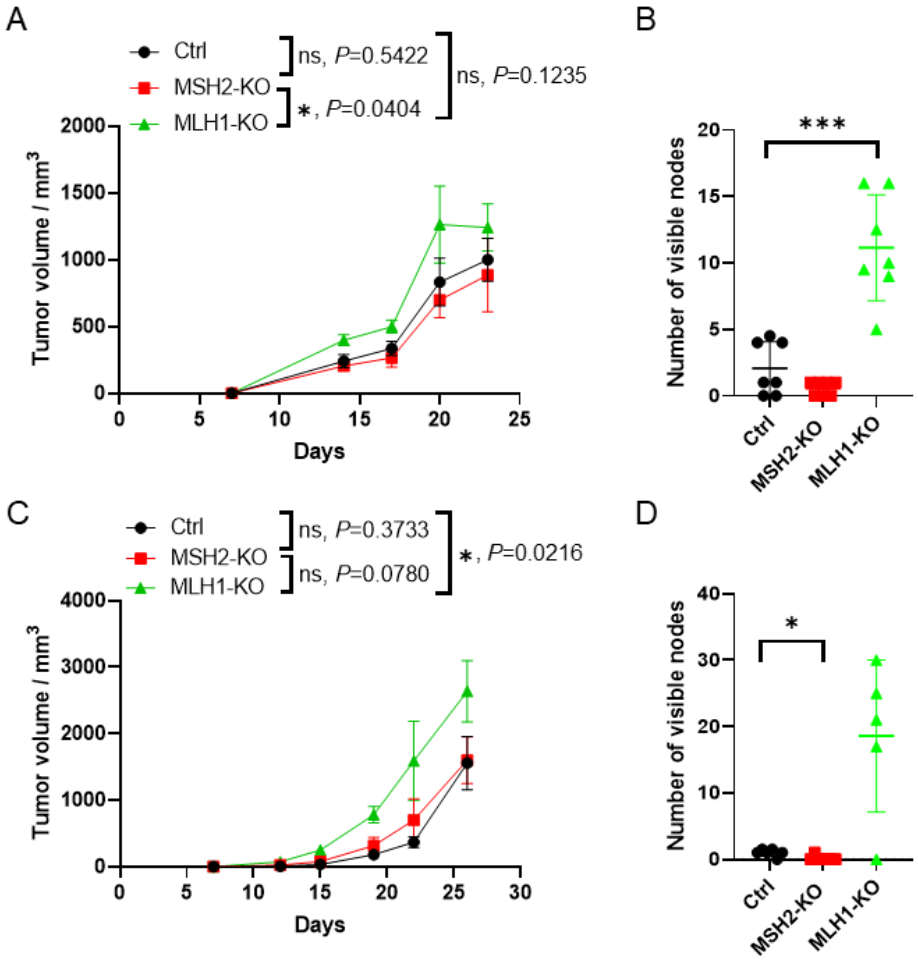

Figure 2. Distinct properties of MSH2 and MLH1 during tumor growth and metastasis. A-B. 1000 of 4T1 Ctrl, MSH2-KO, or MLH1-KO cells from Figure 1 were injected into #2 mammary fatpads of 7-week old Balb/C female mice, following with tumor growth monitoring with caliper (A) and metastasis study by immunohistochemistry of lung sections (B). **C-D.** 500 of PY8119 Ctrl, MSH2-KO, or MLH1-KO cells from Figure 1 were injected into #2 mammary fatpads of 7-week old C57BL/6 female mice, following with tumor growth monitoring with caliper (C) and metastasis study by immunohistochemistry of lung sections (D). n=6-7. * $P < 0.05$; *** $P < 0.001$.

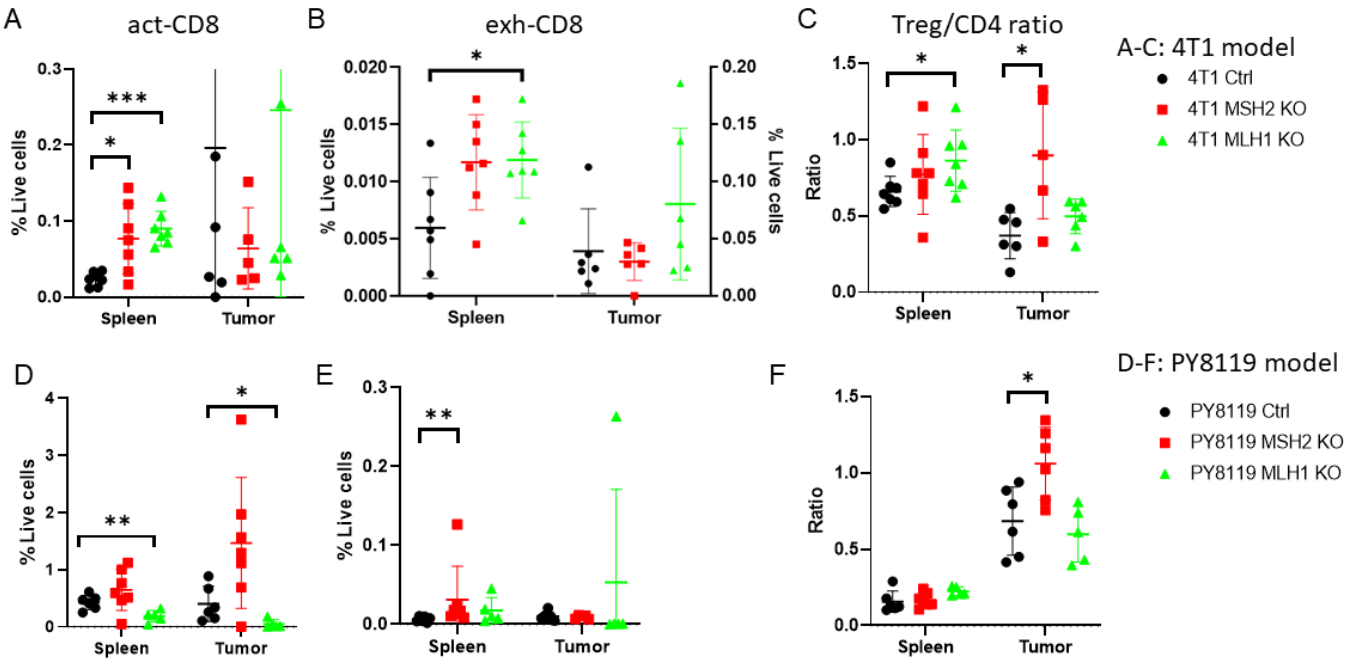

Figure 3. Differential influences of MSH2 and MLH1 on tumor-infiltrating lymphocytes. Tumors or Spleens from tumor-bearing mice from Figure 2 were dissociated into single cells for immune cell profiling. **A-C.** 4T1 model from Figure 2A. **D-F.** PY8119 model from Figure 2C. A and D: activated CD8 T (act-CD8) cells are defined as CD3+CD8+GranzymeB+). B and E: exhausted CD8 T (exh-CD8) are defined as CD3+CD8+PD-1+TIM-3+; C and F: regulatory T cells are defined as CD3+CD4+FoxP3+. * P<0.05; ** P<0.01; ***P<0.001.

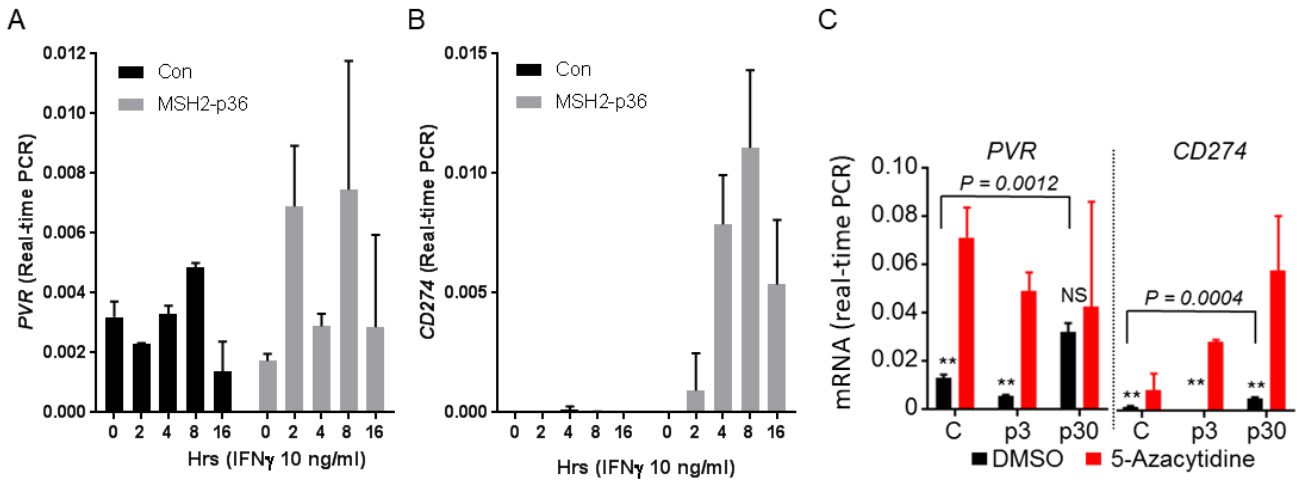

Figure 4. Influences of MSH2-KO on Induction of exhaustion markers by interferon (IFN) or demethylation. A-B. Control or MSH2-KO cells from MDA-MB-231 were treated with IFN-γ for different hrs. Realtime PCR was performed to determine the mRNA expression of PVR or CD274. **C.** Control or MSH2-KO cells from MDA-MB-231 were treated by 5-Azacytidine for 3 days. Realtime PCR was performed to determine the mRNA expression of PVR or CD274.

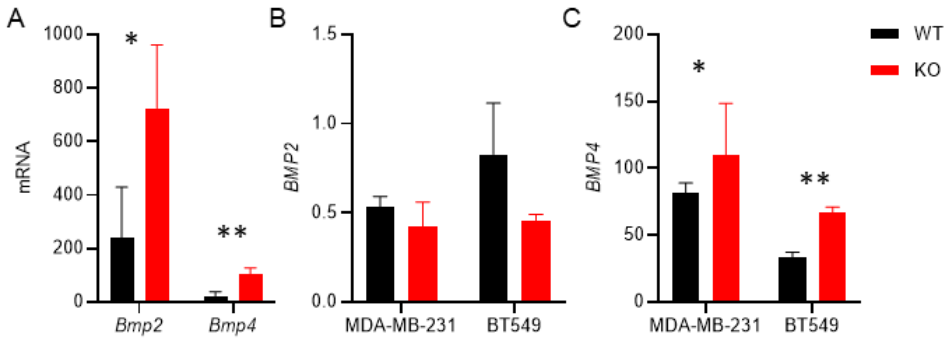

Figure 5. Influences of MSH2-KO on Induction of BMP2 and BMP4. A. mRNA were purified from 4T1 tumors (Figure 2A) formed by Ctrl or MSH2-KO cells. RNAseq was performed to determine transcriptomic changes with the influence of MSH2-KO. **B-C**. BMP2 and BMP4 expression in human breast cancer cells MDA-MB-231 and BT549, determined by RNAseq used for preliminary data of the grant application. * P<0.05; ** P<0.01.

Column ID	Gene Symbol	UCSC_Island	p-value	Δ (C1-P35 vs. P11)	Δ (Parental vs. MSH2-KO)
cg08130500	CD274		0.0017	C1-P35 up vs P11	0.0540377
cg00883027	CD274		0.5981	C1-P35 up vs P11	0.0254746
cg11493635	CD274		0.0194	C1-P35 up vs P11	0.00378188
cg16427113	CD274	Island	0.1595	C1-P35 up vs P11	3.98E-05
cg27643579	CD274	Island	0.0799	C1-P35 down vs P11	-0.0015116
cg02823866	CD274	Island	0.111	C1-P35 down vs P11	-0.00348941
cg15833535	CD274		0.5725	C1-P35 down vs P11	-0.00447458
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cg03379064	CD274		0.2547	C1-P35 down vs P11	-0.00656363
cg00714675	CD274		0.1688	C1-P35 down vs P11	-0.00945655
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cg00586206	CD274	Island	0.1204	C1-P35 down vs P11	-0.0149823
cg04478497	CD274		0.0011	C1-P35 down vs P11	-0.0384197
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cg01396723	PVR	N_Shore	0.0289	C1-P35 up vs P11	0.0415674
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cg05012825	PVR	Island	0.0162	C1-P35 up vs P11	0.000955013
cg15481638	PVR	N_Shore	0.7557	C1-P35 up vs P11	0.000725359
cg07917289	PVR	N_Shore	0.9907	C1-P35 up vs P11	0.000171001
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