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Award Number: DAMD17-01-1-0163

TITLE: Discovery and Development of Inhibitors that Selectively Interfere with Cyclin-Dependent Kinase Substrate Recognition

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REPORT DATE: August 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2003	Annual Summary (30 Jul 02-29 Jul 03)

4. TITLE AND SUBTITLE
Discovery and Development of Inhibitors that Selectively Interfere with Cyclin-Dependent Kinase Substrate Recognition

5. FUNDING NUMBERS
DAMD17-01-1-0163

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The Origin Recognition Complex is thought to recognize Origins of Replication and recruit replication initiation factors in mammalian cells. The loading of this complex on DNA origins is required for replication in lower organisms, and it is thought that these proteins are important for replication control in higher eukaryots. In this funding cycle we show that both ORC1 and ORC2 are important for DNA replication in the breast cancer cell line MCF10A. Furthermore, ORC1 seems to be required for the chromatin loading for not only Pre-replicative complex components such as MCM's, but for the other Orc proteins as well. Interestingly, depletion of ORC2, but not ORC1 results in a cell cycle checkpoint that blocks the cells in G1 phase with low S-phase CDK activity. This suggests that benign breast cancer cells have a mechanism to prevent replication when ORC2 levels are low.

14. SUBJECT TERMS
No subject terms provided.

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction –

One of the hallmarks of human cancer is inappropriate progression through the cell cycle. Under normal circumstances, the cell has numerous checkpoints and mechanisms to ensure that cells do not divide until the genome is fully copied, and that a new cell does not begin copying its DNA until division has finished. DNA replication presents additional challenges, as the process must be tightly coordinated so that the genome is copied accurately, completely, and in a timely fashion.

To this end, cells must recruit a variety of factors to DNA before replication can begin. The first set of proteins to bind the DNA is the Origin Recognition Complex, a 6 member complex that is thought to recognize the actual replication origins, and then recruit necessary downstream factors. These downstream factors include Cdt1 and cdc6, which in turn recruit the MCM2-7 complex. Once MCM2-7 (thought to be the replicative helicase) is recruited to DNA, a pre replicative complex (pre-RC) is formed, and is ready to initiate S-phase pending CyclinE/cdk2 activation[1].

Our lab has been interested in the role the ORC plays in mammalian replication. We have previously reported that an Orc2 hypomorphic cell line has a marginal effect on replication in a colon cancer cell line[2]. While this mutation allows genomic replication, it does not allow replication of an Epstein Barr virus plasmid. We hypothesize that while the reduction in Orc2 level was sufficient for EBV replication inhibition, a further, acute decrease in ORC proteins is required to affect cellular genomic replication.

Body –

1. Characterize the membrane-permeable Cy motif containing peptides that selectively inhibit cyclin/cdk complexes.

We are currently investigating the effectiveness of different "Trojan horse" sequences so that we can introduce these peptides into breast cancer cells. We plan to continue this work in the next funding period.

2. Investigate the Role of Replication Initiation proteins on growth of Breast Cancer.

In order to determine the role replication initiation proteins play in breast cancer growth, we used RNA interference to selectively and specifically deplete proteins critical to replication initiation. Specifically, we generated small interfering RNAs (siRNAs) against two members of the Origin Recognition Complex (ORC), a group of proteins thought to be vital for recruiting factors of the pre-replicative complex (pre-RC) to the chromatin. Using these reagents, we then investigated various aspects of replication initiation.

We generated two different siRNAs against Orc1, and found that Orc1 protein levels decreased to less than ten percent of wild type levels in MCF10A cells (a benign breast cancer cell line). (Interestingly, protein decrease was much less effective in another breast cancer cell line, Hs578T.) We also examined the protein levels of several other replication initiation factors, and found that Orc 2, 3, 5, and MCM7 levels were unchanged after Orc1 RNAi (Fig.1). We found that cyclin A and B protein levels were unchanged, but that cyclin E levels were slightly increased.

In collaboration with a post doctoral fellow in the lab, Yuichi Machida, we generated oligos to Orc2, and were able to decrease the protein levels to less than ten percent wild type in MCF10A cells. Contradictory to the Orc1 RNAi results, Orc2 RNAi reduced protein levels of Orc1, 2, 3, 5, and MCM7. Orc2 RNAi also decreased the levels of cyclin A and B, and drastically increased the levels of cyclin E. (Fig.1)

We then examined the FACS profile of the cells after Orc1 and Orc2 RNAi. Cells treated with Orc1 RNAi showed a slight decrease in the S-phase population, while Orc2 RNAi treated cells showed a strong G1 phase cell cycle block (Fig.2). We were surprised to see such a marked difference between these two complex members. To assay the affect of Orc1 and Orc2 depletion on DNA replication, we examined BrdU incorporation over 24 hours. Approximately 100% of wildtype cells incorporated BrdU, compared to only 50% of the Orc1 depleted cells. Interestingly, only 20% of the Orc2 treated cells incorporated BrdU. (Fig.3) These results not only suggest that Orc1 and Orc2 behave differently in breast cancer cells, but that Orc2 depletion has a stronger effect on mammary cell proliferation.

To investigate the nature of the replication deficiency, we assayed the chromatin loading of MCM7, a member of the MCM2-7 complex. MCM2-7 complex is the putative mammalian DNA helicase, and is the last group of proteins recruited to the DNA to form a pre-RC required for replication initiation. By fractionating the cells, we were able to determine the chromatin loading of various replication initiation factors. Both Orc1 and Orc2 RNAi treated cells showed a decrease in the amount MCM7 loaded onto the chromatin (Fig. 4). This suggests that pre-RC formation is impaired, and that DNA replication is thus affected. However, we noticed a key difference. Orc1 RNAi treatment only decreased the chromatin bound fraction of MCM7, not the soluble fraction. The same result was seen for Orc2 and Orc3 after Orc1 RNAi. This suggests that Orc1 is not only required for the chromatin loading of Mcm7, but other ORC proteins as well. As Orc2 RNAi decreased total protein levels of Orc 2, 3, and MCM7, we were unable to determine its affect on chromatin loading of these factors. While both proteins are important for pre-RC formation, low levels of each protein affect pre-RC formation via different mechanisms.

We noticed that the replication defect following Orc2 RNAi was more severe than that following Orc1 RNAi. The replication defect following Orc2 RNAi, therefore, may result from more than the pre-RC formation defect. The stronger G1 block following Orc2 depletion also supports this. In order to determine the nature of the G1 block, we examined the activity of S-phase cyclin-CDK complexes. We found that Orc1 RNAi treatment did not greatly affect cyclin E associated kinase activity. Cyclin A associated kinase activity was decreased by 50%. Interestingly, Orc2 RNAi decreased cyclin E associated kinase activity by 80%, and cyclin A associated kinase activity by 90% (Fig.5). These results indicate that Orc2 depletion prevents cell cycle progression by inhibiting cyclin E associated kinase activity (which in turn prevents activation of cyclin A.) Orc1 depletion does not affect cell cycle progression in this manner. These findings agree with the FACS data, and help to explain the differing severity of the replication defect.

Additionally, cyclin E associated kinase activity is thought to be responsible for phosphorylation of pRb. This phosphorylation event releases E2F, which can then activate transcription of a variety of replication factors, including Orc1, and MCM7. This

explains the low levels of these proteins after Orc2 RNAi treatment. The inhibition of cyclin E/CDK2 activity leads to decreased phosphorylation of Rb, which leads to decreased amounts of many mRNAs required for replication initiation.(Fig 6.) This would also explain the greater effect of Orc2 RNAi: its total effect on the cell includes the effects of Orc1 RNAi in addition to its own unique properties.

To explain the kinase inhibition following Orc2 depletion, we examined levels of different kinase inhibitors. We found that p27 was upregulated after Orc2 RNAi, but not Orc1 RNAi. In addition, we determined that more p27 is bound to the cyclin E/cdk2 complex after Orc2 RNAi. (Fig. 7) This supports the hypothesis that Orc2 depletion results not only in pre-RC formation, but also in cell cycle arrest via upregulation of cdk inhibitors. Thus, in the breast cancer cell line MCF10A, an unknown mechanism senses Orc2 but not Orc1 levels, and causes a subsequent cell cycle arrest via p27.

In the next funding period we plan to examine the role of Orc3 and Orc5 in breast cancer cells using RNA interference. We are interested to see if these proteins behave more like Orc1, Orc2, or potentially in an altogether different fashion. We will also examine whether the Orc2 checkpoint is retained in breast cancer cell lines obtained from more advanced stages of the disease.

3. Increase my knowledge about the biology of breast cancer.

In addition to reading a wide variety of papers pertaining to breast cancer, I have attended various research talks describing a wide variety of breast cancer related research. In addition, I attended a symposium at the University of Virginia entitled "Cellular Consequences of Genome Instability". I also plan on attending the Cold Spring Harbor meeting on Eukaryotic Replication, where I expect to learn about cutting edge work on replication in breast cancer cells. In the next funding period I will continue to expand my knowledge of the molecular basis, and potential treatment of breast cancer.

Key Research Accomplishments –

We have completed an analysis of the role played by two members of the Origin Recognition Complex in breast cancer cells, and have discovered several novel aspects of these proteins.

- Orc1 and Orc2 depletion using RNAi causes replication defects in breast cancer cells.
- Orc1 is required for the loading of MCM7, Orc2 and Orc3 onto chromatin.
- Orc2 depletion results in the inhibition of CyclinE associated kinase activity, causing a G1 block and failure to begin S-phase.
- The kinase inhibition resulting from Orc2 RNAi is caused by upregulation of the kinase inhibitor p27.

Reportable Outcomes –

Investigate the Role of Replication Initiation proteins on growth of Breast Cancer

1. The results and conclusions of this research have been submitted for publication.[3]
2. This research will be presented as a poster at the 2003 Eukaryotic DNA Replication meeting at Cold Spring Harbor.

Conclusions –

We have demonstrated that depletion of two proteins, Orc1 and Orc2, can inhibit replication in breast cancer cells. We have shown that depletion of both proteins can inhibit pre-RC formation, and that Orc1 depletion not only prevents loading of MCM7, but other ORC proteins as well, which suggests it may be the first component to recognize the DNA origin of replication. We also found that Orc2 inhibits replication by several different mechanisms, including prevention of pre-RC formation, cell cycle arrest following S-phase kinase inhibition, and inhibition of Rb phosphorylation resulting in transcriptional downregulation of several replication factors. We were surprised to see such a pronounced difference between two members of the ORC complex. Previous work has led to the assumption that the two proteins worked as part of the same functional complex. While our results indicate that both proteins inhibit pre-RC formation, Orc2 is part of a unique and previously undescribed mechanism of cell cycle control. This mechanism seems to allow the cell an additional method of control to prevent inappropriate replication initiation when levels of Orc2 protein are depleted, which might otherwise lead to cancer. Even in early forms of breast cancer, loss of such a mechanism may lead to a more serious form of cancer. It will be interesting to further investigate this mechanism in other, more advanced, types of breast cancer, as it may well be an important "hit" required to progress from a benign tumor to a cancer with uncontrolled cell proliferation that is much more difficult, if not impossible, to treat.

We have shown that reduction in both Orc1 and Orc2 can decrease replication, and ultimately, cell number (unpublished data) in MCF10A breast cancer cells. RNA interference has become a potential therapy to reduce the amount of inappropriately expressed proteins, which can potentially treat a wide variety of diseases. The technique also seems promising to reduce normal amounts of proteins required for cell growth, which would make it useful for treating a key disease of inappropriate cell growth: breast cancer.

Works Cited

1. Dutta, A. & Bell, S. P. Initiation of DNA replication in eukaryotic cells. *Ann. Rev. Cell Dev. Biol.* **13**, 293-332 (1997).
2. Dhar, S. K., Yoshida, K., Machida, Y., Khaira, P., Chaudhuri, B., Wohlschlegel, J. A., Leffak, M., Yates, J. & Dutta, A. Replication from oriP of Epstein-Barr virus requires human ORC and is inhibited by geminin. *Cell* **106**, 287-296 (2001).
3. Machida, Y., Teer, J. K. & Dutta, A. Acute reduction of ORC subunits in human cancer cells reveals a cellular checkpoint pathway in G1. *[submitted]* (2003).

Appendix

Figure Legends

Figure 1: Western blot of MCF10A cells following RNAi treatment with Orc1 or Orc2 siRNAs.

Figure 2: Propidium Iodide FACS following RNAi treatment on MCF10A cells.

Figure 3: A. MCF10A cells were treated with BrDU for the last 24 hrs of RNAi, and stained with anti-BrDU or DAPI. B. Percentage of BrDU labeled cells after RNAi treatment.

Figure 4: A. Chromatin fractionation scheme. B. Western blot of fractionated cell extracts following Orc2 RNAi and C. following Orc1 RNAi. (Chromatin Fraction is S2.)

Figure 5: In vitro kinase assay using RB-C as a substrate for Cylin E associated kinase and Histone H1 for Cylin A associated kinase activity.

Figure 6: A. Western blot and in vitro kinase assay using MCF10A extracts following Orc2 RNAi. B. Northern blot following Orc2 RNAi.

Figure 7: A. Western blot of MCF10A lysate. B. Western blot following immunoprecipitation of equal amounts of Cyclin E from Orc2 RNAi treated cells.

Fig 1.

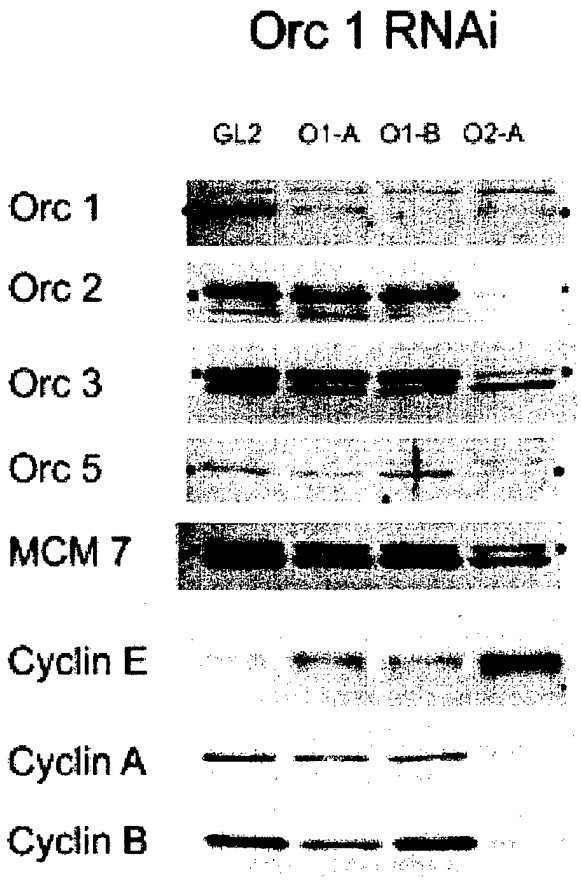
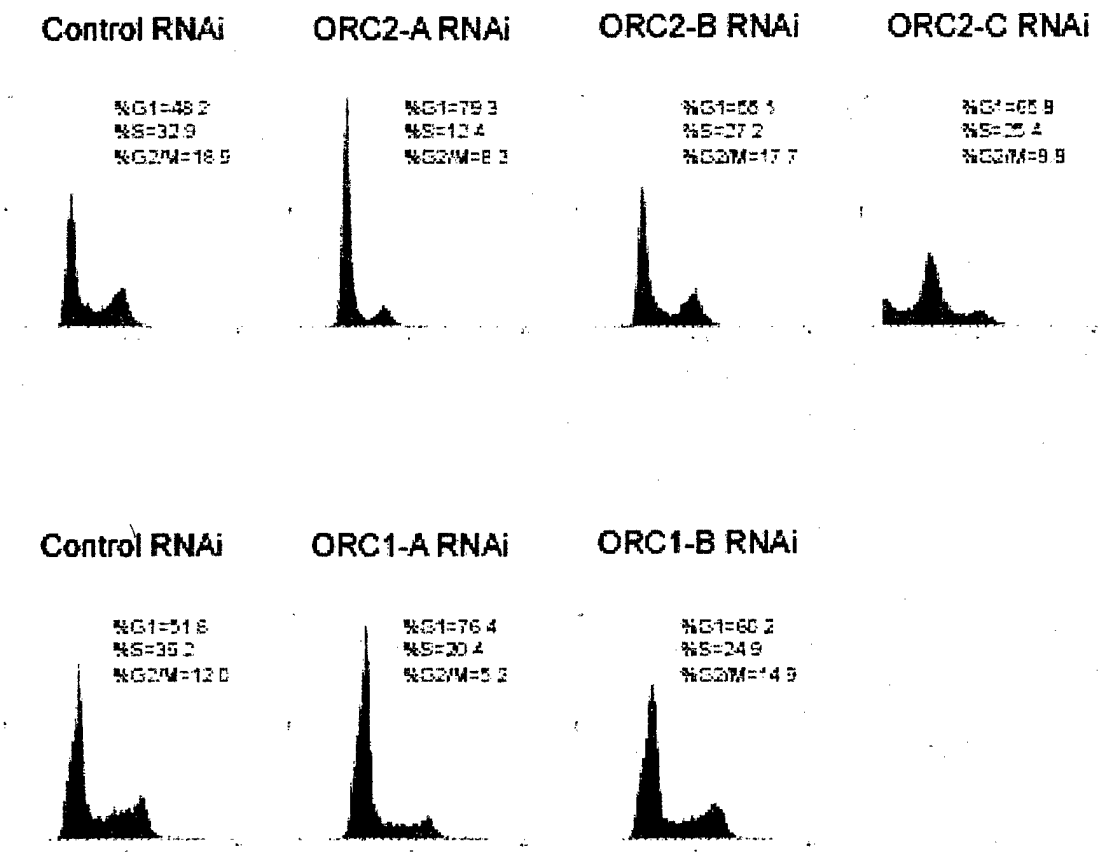

Orc 1 RNAi
GL2
O1-A
O1-B
O2-A
Orc 1
Orc 2
Orc 3
Orc 5
MCM 7
Cyclin E
Cyclin A
Cyclin B

Fig 2.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%G2/M=18.9
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%G2/M=8.3
ORC2-B RNAi
%G1=55.1
%S=27.2
%G2/M=17.7
ORC2-C RNAi
%G1=65.9
%S=25.4
%G2/M=9.9
Control RNAi
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%S=35.2
%G2/M=12.0
ORC1-A RNAi
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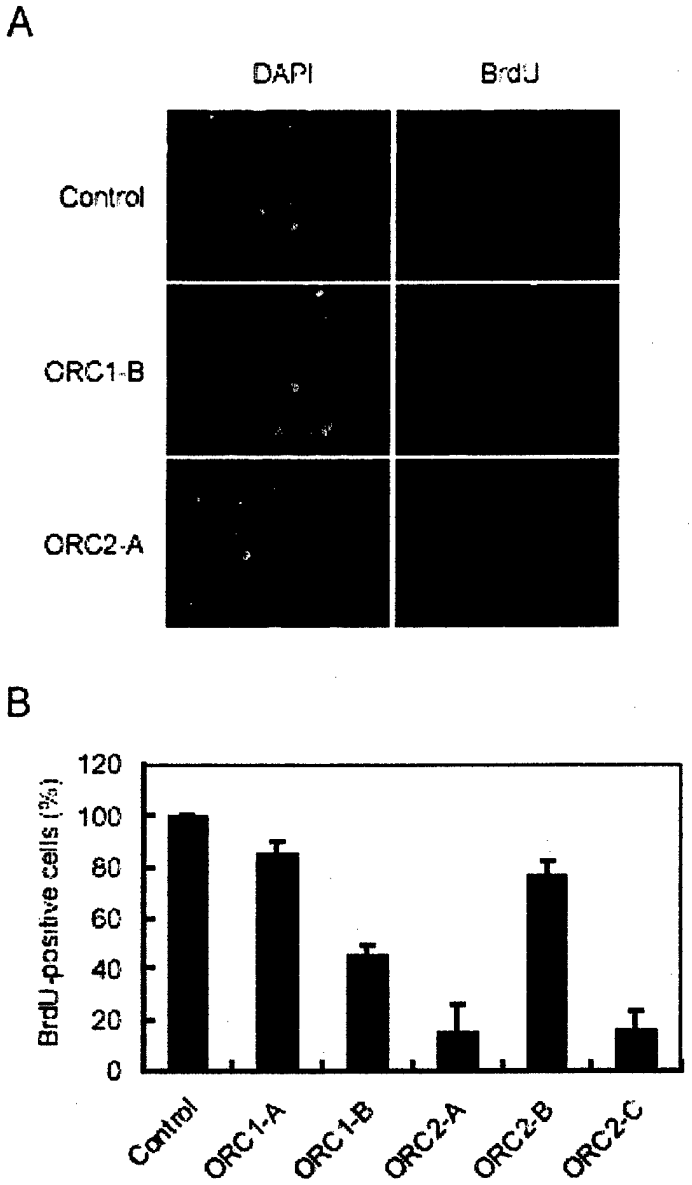
Fig 3.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DAPI
BrdU
Control
ORC1-B
ORC2-A
B
BrdU-positive cells (%)
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100
80
60
40
20
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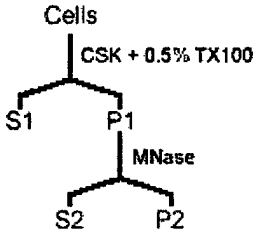
Control
ORC1-A
ORC1-B
ORC2-A
ORC2-B
ORC2-C

Fig 4.

A.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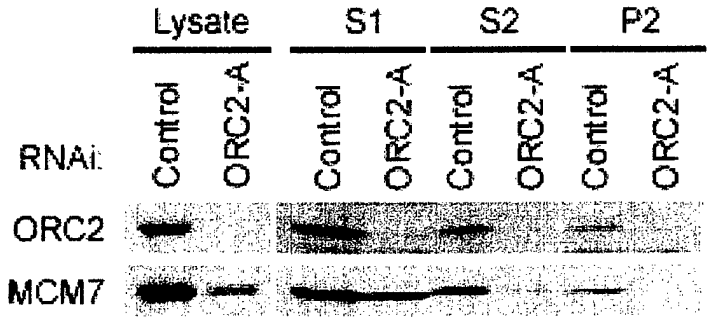
P1
MNase
S2
P2

B.


Lysate
S1
S2
P2
RNAi:
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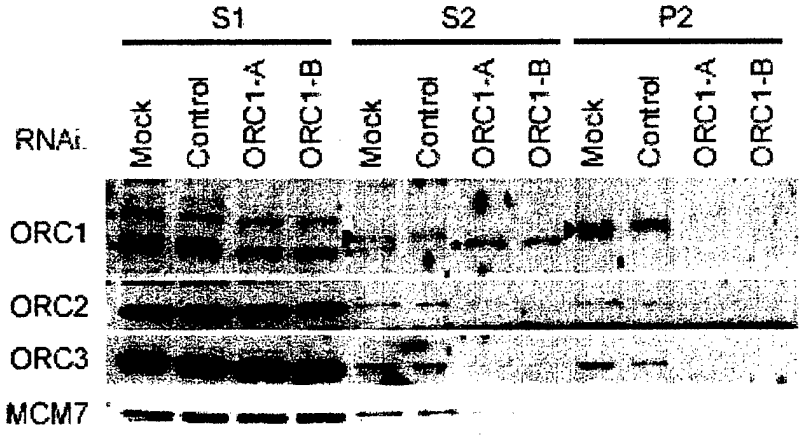
ORC2-A
Control
ORC2-A
Control
ORC2-A
Control
ORC2-A
ORC2
MCM7

C.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S2
P2
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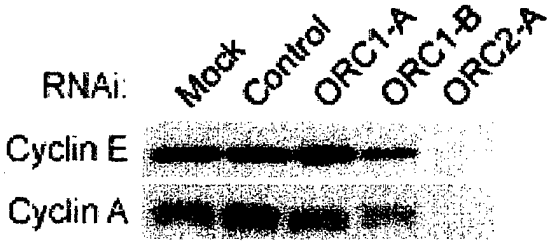
ORC1-B
ORC1
ORC2
ORC3
MCM7

Fig. 5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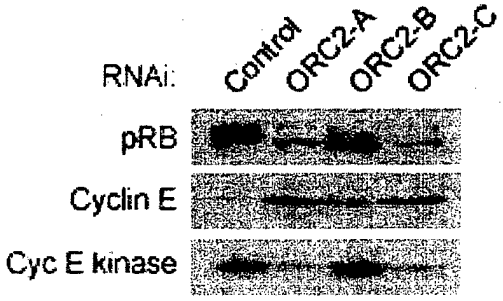
ORC1-A
ORC1-B
ORC2-A
Cyclin E
Cyclin A

Fig. 6

A.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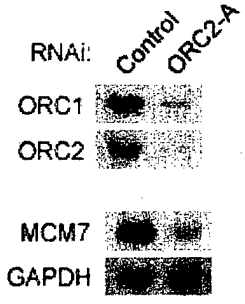
ORC2-B
ORC2-C
pRB
Cyclin E
Cyc E kinase

B.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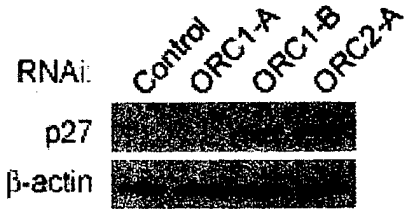
ORC1
ORC2
MCM7
GAPDH

Fig. 7

A.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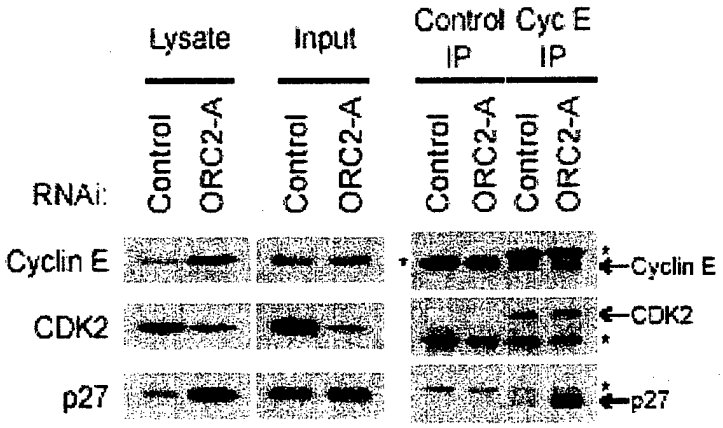
ORC1-B
ORC2-A
p27
β-actin

B.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Control IP
Cyc E IP
RNAi:
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Control
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Control
ORC2-A
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Cyclin E
CDK2
p27
Cyclin E
CDK2
p27