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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 eukaryotes. In this funding cycle, we show that Orc2 RNAi activates several mechanisms to arrest the cell cycle in G_1 phase, preventing entry into S-phase without Orc2. We then studied a stable cell line expressing low levels of Orc2. As these cells are stable, they have escaped the requirement for Orc2, making them useful for examining the effects of low Orc2 on replication. We find that several ORC members are decreased in the absence of Orc2, and that other Orcs, as well as the pre-RC, are not properly loaded on chromatin. Despite this, the Orc2 hypomorph cells (which are p53-) don't show any additional origin firing defect when compared to p53-/- cells. The hypomorph cells replicate at the same rate, and even grow at the same rate as the p53 -/- cells, indicating that the absence of Orc2 has little, if any, effect on replication, despite extremely reduced pre-RC loading.

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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 (ORC), 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 (mini-chromosome maintenance). 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 (cyclin dependent kinase 2) 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 (EBV) 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.

We have previously reported that Orc2 RNA interference (RNAi), in addition to a decrease in chromatin loading of the pre-Replication complex (pre-RC) and overall replication, also causes a strong G1 arrest, and cyclin/cdk kinase inhibition. We believed this to be due to an increase in levels of the cdk inhibitor p27. While pre-RC loading was decreased after Orc1 RNAi treatment, replication was not affected as strongly. Additionally, the kinase inhibition was not observed, nor did we observe the strong cell cycle arrest seen after ORC2 RNAi. This work had interesting implications for breast cancer: loss of this protective mechanism, in which Orc2 is required for cyclin/cdk kinase activity, and thus, cell cycle progression, may allow cells to replicate in the presence of low pre-RC levels, which may lead to genomic instability via a detrimental S-phase.

Body –

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

Previously reported results on ORC and cell cycle were of high interest, so we focused on that work this funding period.

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

We have previously reported RNAi against Orc1 and Orc2 in MCF10A breast cancer cells. We have further studied the phenotype of Orc2 RNAi in an attempt to understand the cell cycle arrest mechanism we observed. Initially, we have found that, although the increase in p27 is an immediate response following Orc2 decrease, it is not sufficient to inhibit the cyclin/cdk activity. We removed p27 from Orc2 RNAi treated cells (thus preventing it from inhibiting cyclin E/cdk2), and found that cyclin E and

cyclin A associated kinase activities were still decreased (Fig. 1). It therefore seems that p27 is not sufficient for the cell cycle arrest in the absence of Orc2. We reexamined the cyclin E co-immunoprecipitated complex from Orc2 RNAi treated cells, and observed that, in addition to increased p27 association with cyclin E, the migration of cdk2 was altered. This time, slightly different gel conditions allowed us to observe an upshift in cdk2 migration. This shift corresponds to a hypophosphorylated state of cdk2. As cdk2 phosphorylation is important for its activity, we studied this further by using phospho-specific antibodies to examine Threonine 160 (Fig. 2a). This site is phosphorylated by cdk2 activating kinase (CAK), and phosphorylation is required for cdk2 activity. We found that phosphorylation at this position is significantly decreased, suggesting that the low levels of Orc2 are preventing cdk2 from being activated. This is a rather surprising result, as activity of CAK, and thus phosphorylation at T160 is thought to be constitutive. We also wondered whether or not any other mechanisms were being used by the cells to stop the cell cycle. We looked at levels of cdc25a, a protein responsible for removing two inhibitory phosphorylations from cdk2. We found that levels of this protein were decreased, which would lead to persistence of cdk2 in its inhibited state (Fig. 2b). These new findings further indicate that the presence of Orc2 is critical for progression into S-phase. This obligate requirement for Orc2 allows cyclin E/cdk2 to be activated, promoting replication initiation. In the absence of Orc2, several critical activations are absent, which presumably serves to protect the cell from a harmful replication cycle.

This strong response to the lack of Orc2 was not observed after Orc1 RNAi. We have previously shown that since we observe pre-RC decrease in both cases, the pre-RC is not being sensed by this cell cycle arrest mechanism. The implications of replication in the presence of fewer pre-RC's is currently unknown, but quite important for cancer biology. To determine any detrimental affects of replication with low pre-RC, we returned to our stable Orc2 hypomorph (Δ/-) cell line [2]. This cell line is able to survive in the presence of low Orc2, which is immediately different from the Orc2 RNAi phenotype. It may be possible that these cells have managed to bypass the requirement of Orc2 for cell cycle progression.

We first reaffirmed earlier results that Orc2 and Orc3 are decreased in the Orc2 Δ/- cells when compared to parental HCT116 cells (colon tumor derived). In addition, we find that Orc5 is slightly decreased, but Orc1, 4 and 6 are unchanged (Fig. 3a). This agrees with published biochemical data from our lab which shows that the ORC is composed of a core subcomplex consisting of Orc2, 3, and 5 [3]. Thus, all members of the subcomplex are destabilized when Orc2 is decreased. The ORC complex is, in lower organisms, important for pre-RC loading, which sets up a foundation for replication initiation. To determine whether the absence of this subcomplex affected pre-RC loading on chromatin, we fractionated cells. The chromatin bound proteins (Fraction s2) were isolated, and levels of the pre-RC component MCM7 part of the MCM2-7 helicase) were determined. The Δ/- cells show a drastic reduction in the amount of MCM7 loaded on chromatin when compared to wild type HCT116 cells (Fig. 3b). Interestingly, the amount of Orc4 loaded on the chromatin is also decreased, suggesting Orc2,3 and 5 play a role in loading Orc4. Thus, the amount of pre-RC loading is significantly decreased, as observed after ORC RNAi. We can now investigate the effect that reduction of pre-RC loading has on the cell cycle.

Since pre-RC loading is decreased, one could reasonably imagine that the number of firing replication forks is decreased. To determine this, we synchronized cells in G1, released them into S-phase in the presence of BrDU for different lengths of time, and harvested the genomic DNA. This DNA was then combed onto slides, and stained with fluorescent anti-BrDU antibody. By examining the slides under a microscope, one can determine lengths of DNA labeled with BrDU (a bi-directional fork) and infer number of firing origins and replication progression. We have found that HCT116 cells show an increased number of forks as S-phase progresses (Fig. 4a). Δ/- cells (labeled here as e83) do not show this increase over time, suggesting there is a defect in origin firing. However, as the Δ/- cells do not express p53, we performed the experiment on p53 -/- HCT116 cells as well. These cells also fail to increase the number of forks during S-phase, similarly to Δ/- cells. Both Δ/- and p53 -/- cells also show a slower replication progression at the examined timepoints (Fig. 4b). This may indicate that the cell cycle effects observed in the Δ/- cells may be caused by the lack of p53, and not Orc2.

As the decrease in origin firing could result from a prolonged G1 phase, we measured both G1 and S phases by labeling cells with brief ^{3}H thymidine pulses after a nocodazole arrest and release. The time point at which cells start incorporating the labeled thymidine marks the end of G1 and beginning of S. As S-phase progresses, one would expect to see an increase in incorporated thymidine, followed by a decrease as S-phase ends. We observe that all three cell lines begin incorporating at the same time, suggesting that the length of G1 phase is unaffected by the reduction in Orc2 (Fig. 5a). However, we observe a lengthened S-phase in both the Δ/- cells and the p53 -/- cells. This indicated that lower fork firing results in a prolonged S-phase, presumably allowing the cells time to complete replication (at their slower rate) before G2. This indicated that both Δ/- and p53 -/- cells are having difficulties in S-phase. It is difficult to deconvolute the effects of Orc2 and p53 absence; however, it appears as if the Δ/- cells do not have any phenotype beyond that of the p53 -/-, indicating that Orc2 loss does not seem to have a measurable effect on the cell cycle or replication dynamics of these cells.

These observations are further supported by the overall growth rates of the cells. We have empirically observed that the Δ/- cells require more time to grow. To test this quantitatively, we used the MTT assay to determine amount of viable cells over three days. We found that Δ/- and p53 -/- cells both grow slower than HCT116, with doubling times of 66.6 hours and 48.8 hours respectively (Fig. 5b). This result agrees with the replication initiation analysis described above; the increased doubling time correlates with an increased time required to finish replication, presumably due to a defect in fork firing. Since the p53 -/- cells again show a similar phenotype to the Δ/- cells, it appears as if the observed effects are due to the absence of p53.

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

I have read many papers and attended many talks related to breast cancer, further increasing my knowledge of the subject. In addition, I attended the "DNA Replication and Genomic Integrity" meeting at the Salk Institute in San Diego. This meeting touched on several interesting new information about breast cancers, and was a valuable learning experience.

Key Research Accomplishments –

- Elimination of p27 is not sufficient to rescue cell cycle arrest after Orc2 RNAi.
- An activating phosphorylation of cyclin E associated cdk2 is absent after Orc2 RNAi.
- Cdc25A, a protein required to activate cdk2, is decreased after Orc2 RNAi.
- Orc3 and Orc5 are reduced in a stable Orc2 hypomorph cell line.
- Low levels of Orc2 prevent loading of the pre-replication complex (pre-RC).
- Origin firing seems to be unaffected by low levels of Orc2.
- In this stable cell line, low Orc2 levels do not affect the replication dynamic, nor the cell cycle.

Reportable Outcomes –

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

1. This research was presented as a poster at the 2004 "DNA Replication and Genomic Integrity" meeting at the Salk Institute in San Diego, California.

Conclusions –

We have followed up on our previously reported work on the MCF10A breast cancer cells. We have shown that the observed G_1 arrest requires other mechanisms in addition to cyclin dependent kinase inhibition by p27. We have found that the cyclin E/cdk2 (the activity of which is significantly inhibited) is being inhibited by two additional mechanisms. First, cyclin E associated cdk2 is lacking the activating phosphorylation at Threonine 160, which has been previously thought to be added constitutively. Second, we see a decrease in cdc25A, a phosphatase required to remove two inhibitory phosphorylations from cdk2. These mechanisms presumably work with the increase in p27 levels to ensure the cell cycle is tightly arrested just before S-phase in cells acutely depleted of Orc2.

Due to these striking results, we further examined a cell line stably expressing very low levels of Orc2. This cell line does not suffer a cell cycle arrest, and proliferates very well, suggesting that it has aquired mutations allowing bypass of the previously described obligate requirement for Orc2. The low levels of Orc2 in this situation still cause a decrease in pre-RC loading. Interestingly, we do not see any defect in origin firing, as one might expect when pre-RC levels are low. We also don't see any overall replication defects due to low levels of Orc2, nor any affect on the growth rate of these cells. We hypothesize that these cells normally have a vast excess of pre-RCs that do not fire in a given cell cycle. Thus, even though we see a drastic decrease, the number remaining is sufficient to proceed with a normal replication event. We plan to study this in the future, as well as study what effects this absence of excess pre-RCs has on genomic stability. It is possible that in this case, some additional DNA damage could occur from a stalled fork, as there would be fewer possible pre-RCs to serve as a backup. More DNA damage could lead to precancerous states. As we have previously observed using RNAi, cells may then, under normal circumstances, avoid this danger by arresting the cell cycle until enough Orc2, and thus pre-RC's, are present.

References

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. Dhar, S.K., Delmolino, L, Dutta, A. Architecture of the human origin recognition complex. *J. Biol. Chem.* **276**, 29067-71 (2001)

Appendix

Figure Legends –

Figure 1: Quantitation of cyclin E associated kinase activity, and western blot for Orc2 and p27 following treatment with indicated RNAi.

Figure 2a: Western blot of input lysate and cyclin E associated proteins.

Figure 2b: Western blot against cdc25a, with UV treatment used as a positive control for degradation.

Figure 3a: Western blot against various proteins in HCT116 wild type cells, HCT116 p53 -/- cells, and Δ/- cells (Orc2 hypomorph of HCT116 wild type.)

Figure 3b: Western blot against fractionated cell extracts. WCE indicates whole cell extract, S1 indicates the soluble fraction, S2 indicates the chromatin fraction, P2 indicates the insoluble fraction.

Figure 4a: Analysis of molecular combing data showing number of forks per length of DNA, ie, fork density.

Figure 4b: Analysis of molecular combing data showing length of labeled DNA versus unlabeled DNA, ie, percent replication completed at a given timepoint.

Figure 5a: Normalized amount of ^{3}H thymidine incorporated by cells at indicated timepoints after nocodazole release.

Figure 5b: Cell number over time as determined by an MTT assay.

Figure 1:

A.

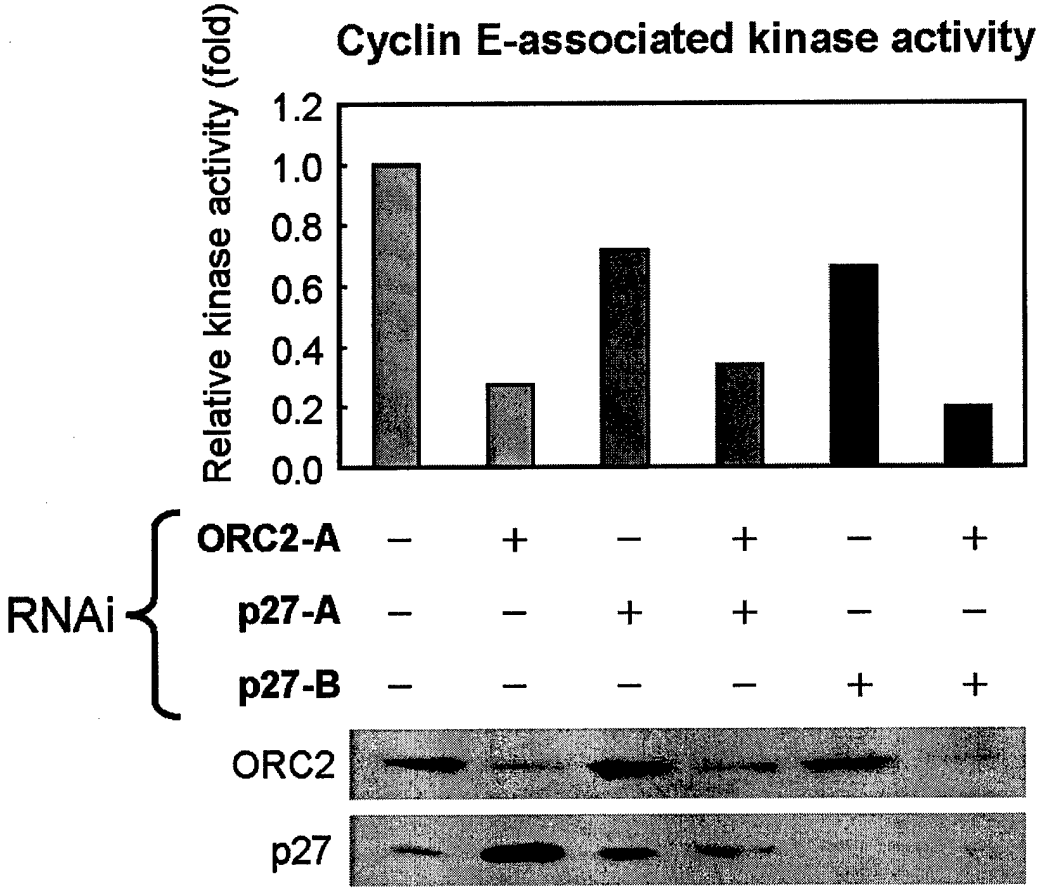
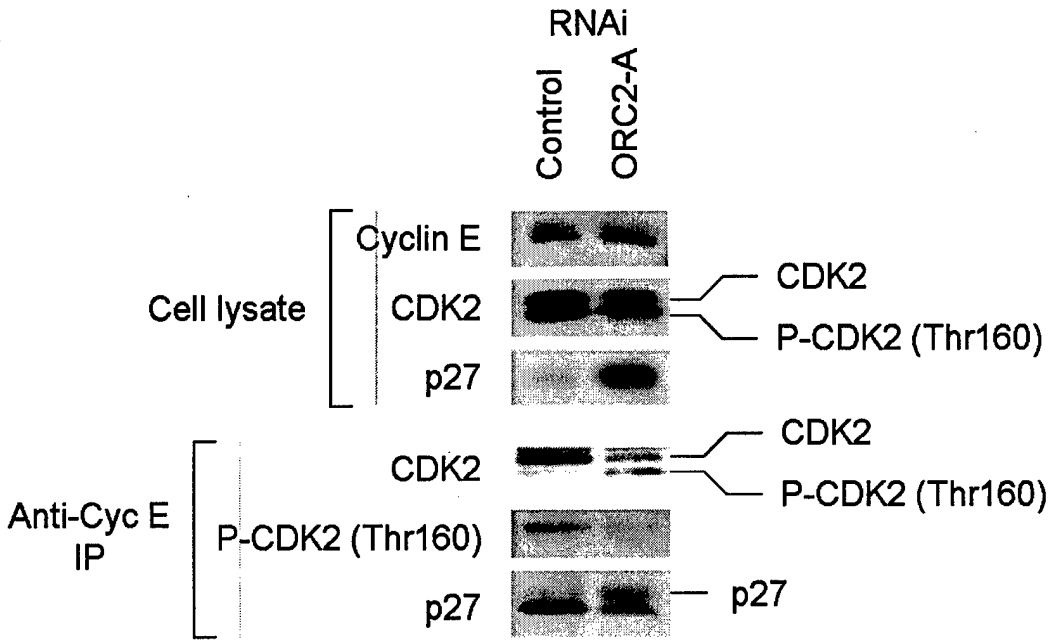

Cyclin E-associated kinase activity
Relative kinase activity (fold)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RNAi
ORC2-A – + – + – +
p27-A – – + + – –
p27-B – – – – + +
ORC2
p27

Figure 2:

A.


RNAi
Control
ORC2-A
Cell lysate
Cyclin E
CDK2
p27
CDK2
P-CDK2 (Thr160)
Anti-Cyc E IP
CDK2
P-CDK2 (Thr160)
p27
CDK2
P-CDK2 (Thr160)
p27

B.

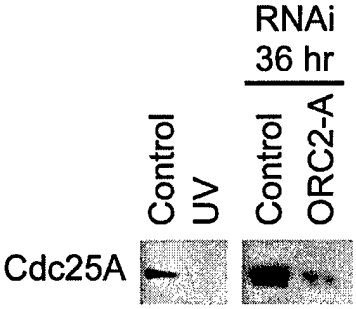

RNAi
36 hr
Control
UV
Control
ORC2-A
Cdc25A

Figure 3

A.

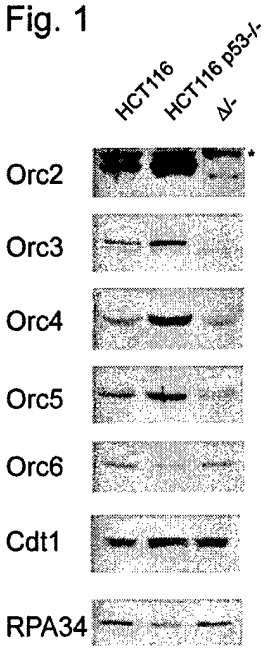


Fig. 1
HCT116
HCT116 p53-/-
Δ/-
Orc2
Orc3
Orc4
Orc5
Orc6
Cdt1
RPA34

B.

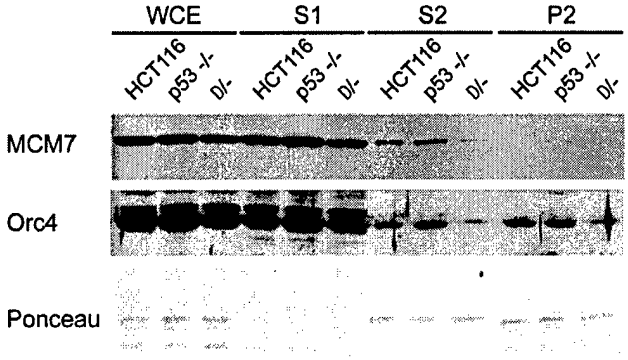
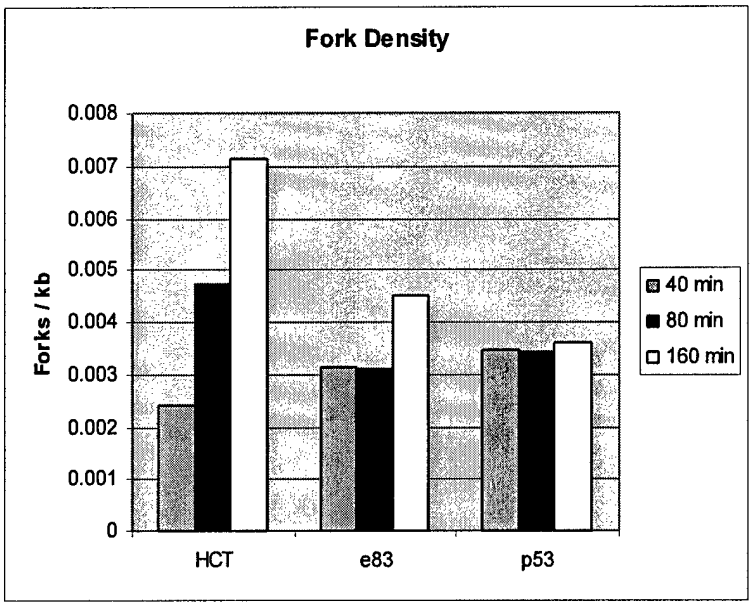

WCE
S1
S2
P2
HCT116
p53 -/-
D/-
MCM7
Orc4
Ponceau

Figure 4

A.


Fork Density
Forks / kb
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
HCT
e83
p53
40 min
80 min
160 min

B.

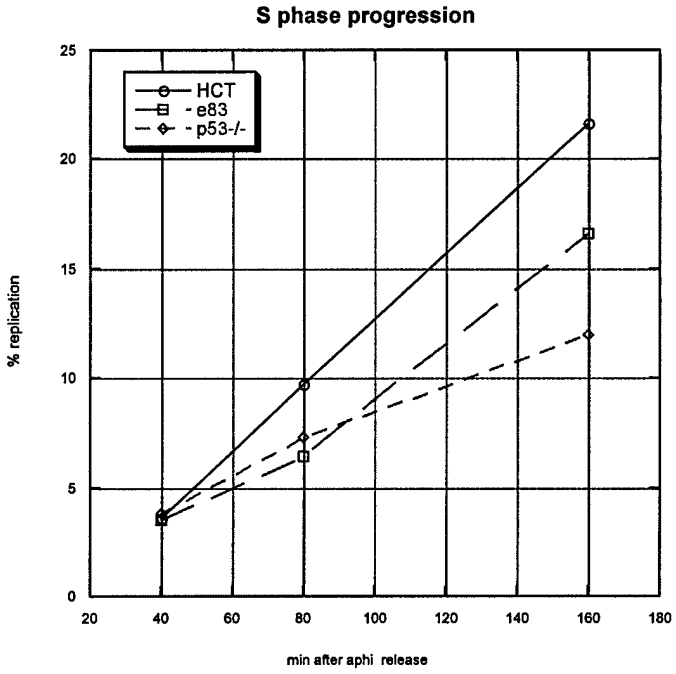
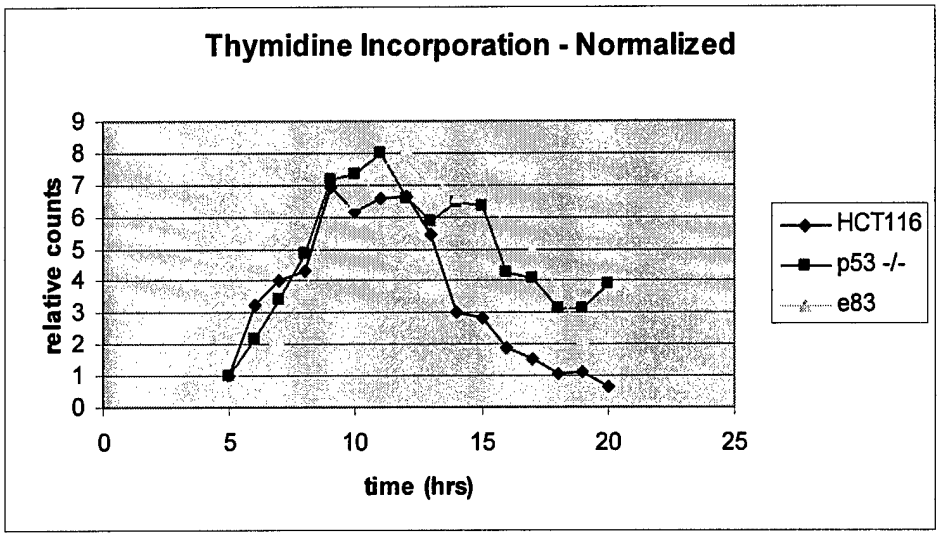
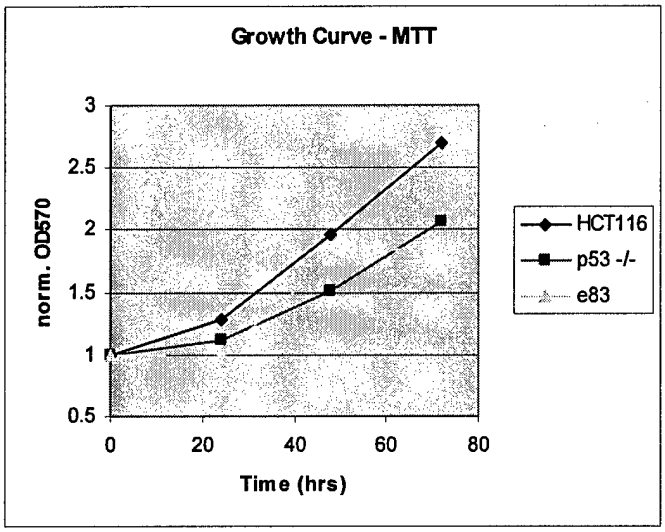

S phase progression
% replication
25
20
15
10
5
0
20
40
60
80
100
120
140
160
180
min after aphi release
HCT
- e83
- p53-/-

Figure 5

A.


Thymidine Incorporation - Normalized
relative counts
0
1
2
3
4
5
6
7
8
9
0
5
10
15
20
25
time (hrs)
HCT116
p53 -/-
e83

B.


Growth Curve - MTT
norm. OD570
0.5
1
1.5
2
2.5
3
0
20
40
60
80
Time (hrs)
HCT116
p53 -/-
e83