## Results

### Part I: Engineering a sequence specific Origin recognition complex

Unspecific DNA binding by human ORC has been a serious bottleneck in the way to elucidate the mechanism of origin selection. I plan to circumvent this shortcoming, by fusing sequence specific DNA binding moiety with ORC. The idea was to fuse one of the six Orc subunits with a well-characterized sequence specific DNA binding protein. Orc part of the chimeric protein thus formed will integrate into the ORC complex which can be recruited to the specific DNA sequences through heterologous DNA binding moiety. Tet transregulators are one of the best-characterized specific DNA binding proteins. Moreover, the binding can be controlled with the doxycyline. This provides a tightly regulated on/off switch. Single chain version of Tet trans regulators (Krueger et al., 2003) with only one regulatory domain was used as a DNA binding fusion partner. In this study, I used Orc4, Orc2 and Orc1 subunits to generate Orc fusions.

ORC is a protein complex of six individual protein subunits. *In S.cerevisiae, these* six subunits form a stable hetero-hexameric complex. In humans, Orc6 is present in traces *in vitro* and so far, its interaction with other ORC subunits has not been detected *in vivo*. Thus, at least five subunits come together both *in vivo* and *in vitro* to form a bulky ORC. Therefore, we tried to minimize the steric hindrances arising during the ORC formation by introducing a flexible linker between TetR and Orc moiety. However, such fusions are often susceptible to proteolysis *in vivo*. So we started out with two linkers (Fig.6) with different amino acid composition. Nucleotide sequence encoding each linker is flanked with suitable restriction enzyme to facilitate the cloning in expression vector. A flag tag sequence (shown in red, Fig. 6) was utilized as an expression indicator.

F Linker → DYKDDDDK$(SG_4)_5$
G Linker → DYKDDDDKAS$(GQ)_3$GPGRGYAYRS

**Fig. 6 Linkers used to fuse Orc subunits to single chain Tet repressor.** A 25 amino acid long F-linker (Krueger et al., 2003) and 18 amino acid long G-linker (Leonhardt et al., 2000) were encoded by synthetic DNA fragments of 134bp and 93 bp respectively. Flag tag (in red) on both the linkers serves as a protein expression marker.

## Cloning and Expression of Orc fusions for mammalian cells

### Consitutively expressing Orc fusions

The plasmid, pWHE120 expressing single chain Tet trans activator (kind gift from Prof. Wolfgang Hillen) was used as a parent plasmid to clone constitutively expressed Orc fusion constructs. Synthesized F and G linkers inserted in a Topo.pcr4 cloning vector were cut out by digestion with compatible restriction sites designed for cloning into the pWHE120 vector. First of all, Orc 4 fusion with F-linker was constructed using double ligation strategy. F-linker and Orc4 inserts were ligated with compatible ends of pWHE120 plasmid in a single a step, resulting in a fusion construct comprising scTet/F-linker/HsOrc4 (Fig. 7). Subsequently referred to as “4F”. G-linker then replaced F-linker using compatible restriction sites resulting in a fusion construct comprising scTet/G-linker/HsOrc4. Subsequently referred to as “4G”. Restriction sites used during the cloning are shown in Fig 8. In the process of cloning, VP16 domain, which is a transcriptional activator and drives the inducible expression of tet promoter driven genes was deleted to avoid any interference due to transcriptional activation.

HOrc2 gene was PCR amplified from TOPO 2.1Orc2 using primer pairs with compatible restriction sites. First of all Orc 2 was fused to the scTet without any linker. Thereafter, using the compatible restriction sites linker F and linker G were

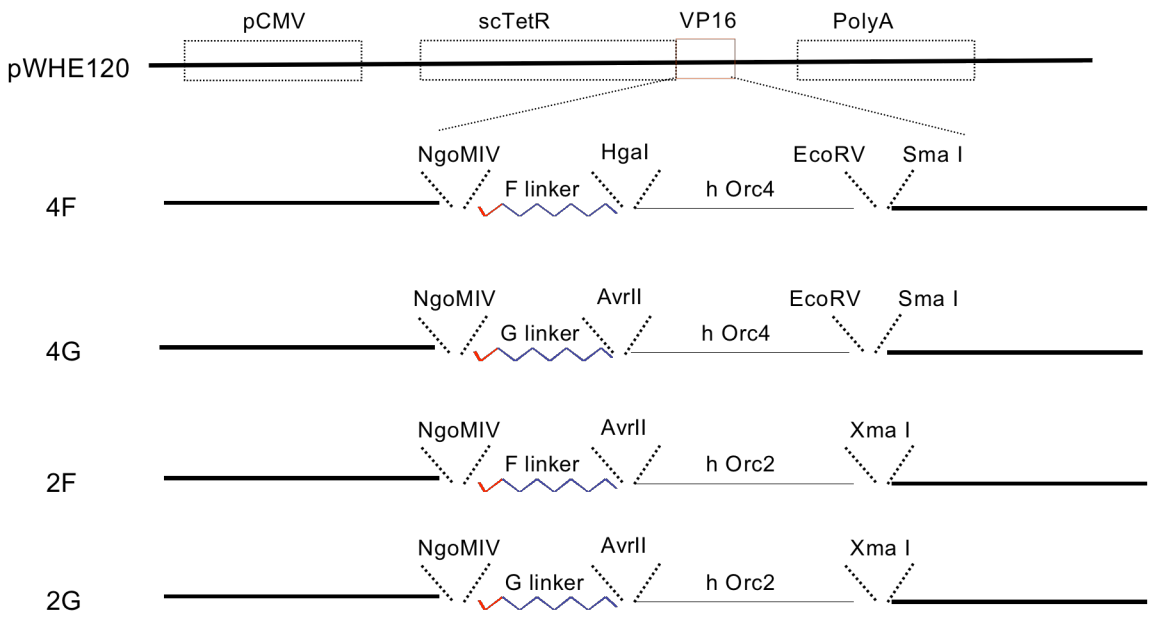


**Fig. 7 Schematic representation of the cloning strategy for consitutively expressed Orc fusions** For Orc 4 fusions, Orc4 open reading frame was derived from plasmid TOPO 2.1Orc4 (Ranjan A., Phd thesis). NgoMIV and AvrII sites were used to generate 4G construct. For Orc2 fusions, Orc2 ORF was PCR amplified using primer pairs with AvrII and Xma I restriction enzyme sites. Digesting 2F with NgoMIV and AvrII and inserting G linker with compatible sites finally produced 2G.

inserted resulting in a fusion construct comprising either scTet/F-linker/HOrc2 or scTet/G-linker/HOrc2. Subsequently referred to as “2F” or “2G”, respectively. Each clone was confirmed by DNA sequencing. The restriction sites used for the cloning are shown in Fig. 7.

Once DNA sequencing confirmed the cloning, I checked the expression of the fusion constructs. HeLa cells were transfected separately with qiagen-purified scTet, 4F, 4G, 2F and 2G plasmids using lipofectamine. Cells were harvested after 3 days and lysates were made for immunoblotting. Protein expression was probed by $\alpha$-HOrc2, $\alpha$-HOrc4, $\alpha$-TetR (Fig.8B) or $\alpha$-Flag antibodies (Fig8A). All proteins migrated at the expected molecular weight on the SDS gel. The theoretical size of the proteins as well as their pI value is shown in Table III.

Where as G constructs (4G, 2G) show a single band of expected molecular weight, F (4F, 2F) constructs show a lower molecular weight band migrating at the size of scTet (or SC) protein. This was visible with α-TetR and α-Flag antibodies for both 4F and 2F constructs. However, no such band was detected with α-HOrc2 and α-HOrc4 antibodies. Most probably in F constructs, proteolysis would have occurred at the linker site giving a fragment containing scTetR and the flag tag.

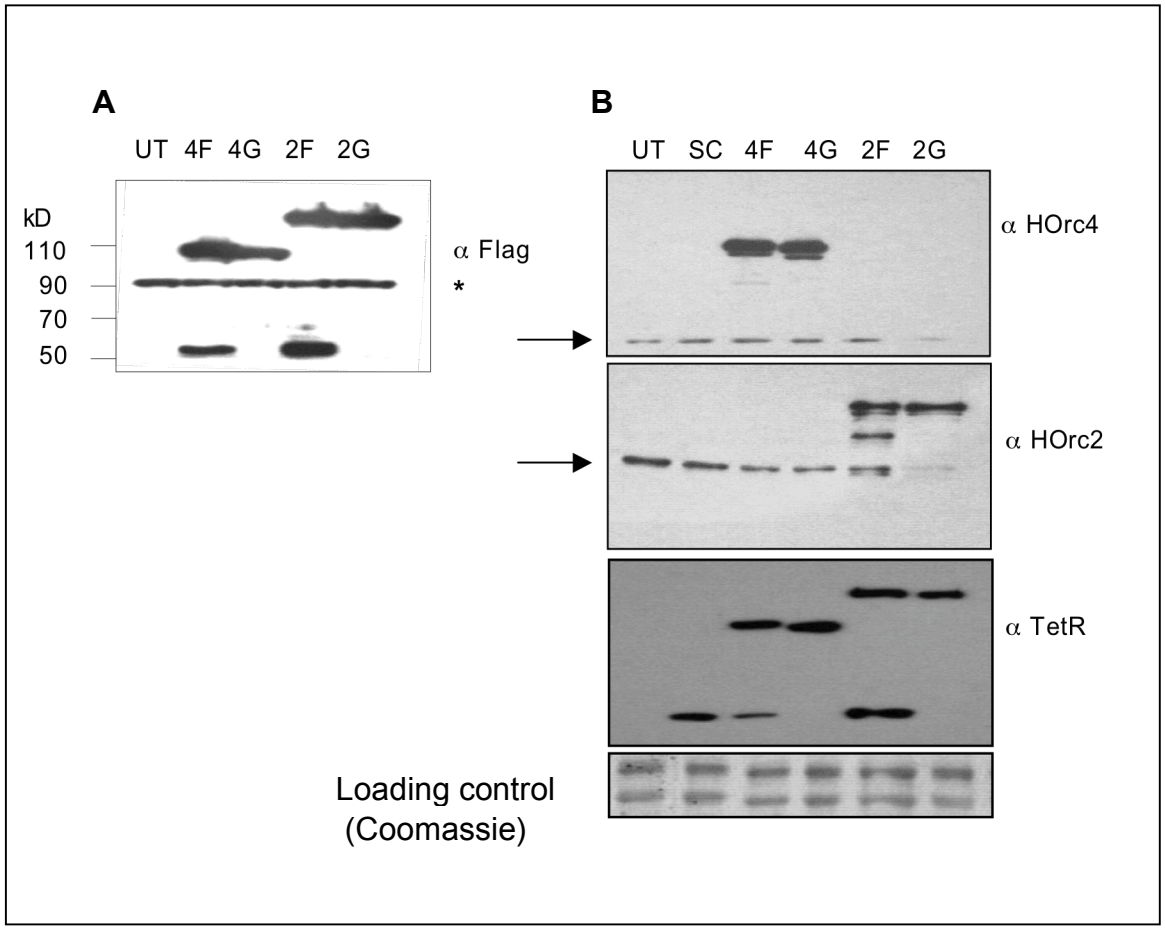


**Fig. 8 Constitutive expression of Orc fusions in mammalian cells.**
A) Immunoblots showing the expression of Orc fusion proteins as probed by anti-Flag antibody. HeLa cells were transfected with different expression vectors. Whole cell lysates are prepared 3 days post transfection. Proteins were resolved on 10% SDS-PAGE. Proteins were transferred on PDVF membrane and immunoblotted with Flag antibody. The cross reacting unspecific band indicated by asterix (∗) served as loading control for the experiment. B) Expression was further confirmed by Orc4, Orc2 and TetR antibody with same procedure as

before. Arrows in top two panels indicate the endogenous Orc subunits. For Tet antibody, coomassie stained PVDF membrane served as a loading control (lowermost panel). UT is untransfected control.

**Inducible expression of Orc1 fusions**

In addition to Orc4 and Orc2 fusions, scTetR moiety was fused at the C terminus of Orc1 subunit. One more construct where EGFP is fused at the C terminus of Orc1 was also made. The plasmid pUHG103-2 Orc1 (Ranjan A.,PhD thesis) was used as a parent plasmid to clone Orc1Tet and Orc1GFP fusions. The expression of the gene can be controlled by Tet promoter, which is sensitive to the presence of doxycycline. In order to test the effect of the flexible linker at the fusion interface, these fusions were made without any linker between the Orc1 and TetR moiety. Both scTetR and EGFP open reading frames were PCR amplified with primer pairs (for primer sequences, see material section) having compatible restriction sites. The Cloning strategy given in Fig. 9 shows the restriction sites used for Orc1 fusions. The PCR product was ligated in the pUHG102-3 plasmid correspondingly digested with these restriction sites. The cloning was confirmed by DNA sequencing.

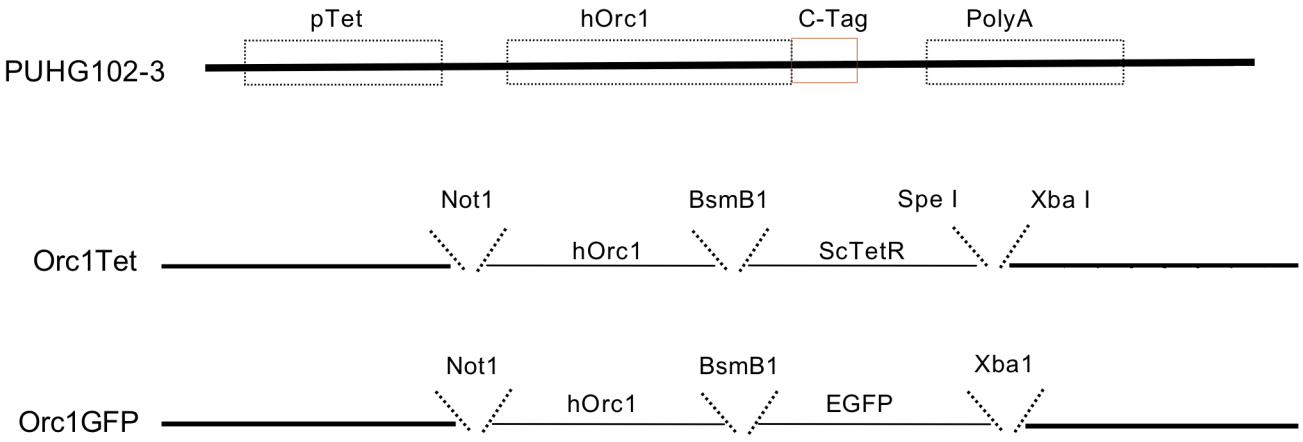


**Fig. 9 Cloning strategy to construct Orc1 fusions**. PUHG102-3 was used as a parent plasmid to clone Orc1fusions. Orc1Tet was made by PCR amplifying scTet moiety with compatible restriction sites. This fragment was then inserted in parent plasmid to have in frame expression of fusion protein. Similarly, Orc1GFP is made by

PCR amplifying EGFP with compatible restriction sites. This fragment is then inserted in the PUHG102-3 plasmid to have in frame expression of Orc1-GFP fusion protein.

The expression of hOrc1 fusions was tested in M2 cells (HeLa derivative) having stable expression of rtTA, reverse tetracycline trans activator (Urlinger et al., 2000). The expression of gene of interest in the pUHG102-3 vector can be induced with addition of doxycycline in the system. M2 cells were transfected with Orc1Tet and Orc1GFP plasmids in cells with or without doxycycline. Cell lysates were subjected to immunoblotting and expression was confirmed either by anti Orc1 antibody (for Orc1GFP, fig. 10B) or TetR antibody (For Orc1Tet, fig. 10A). The protein migrates at the expected molecular weight on a 10% SDS-PAGE.

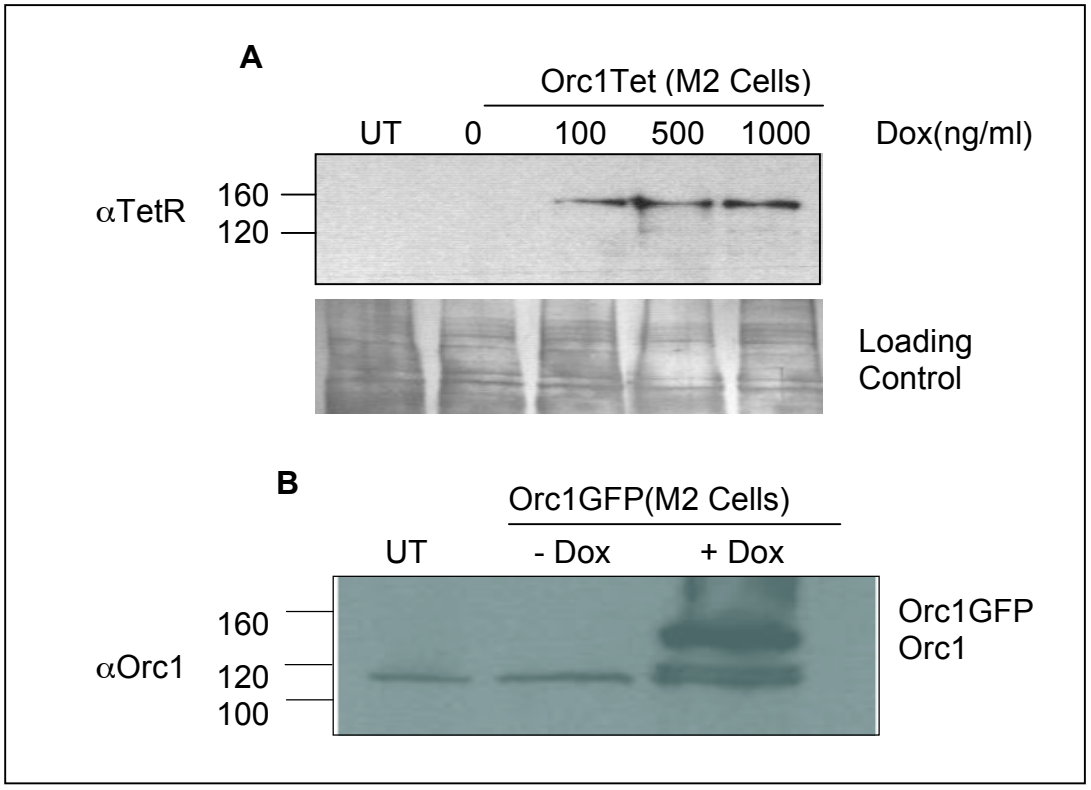


**Fig. 10 Inducible expression of Orc1 fusions in HeLa-M2 cells.** A) ORC1Tet expression in M2 cells. Orc1Tet plasmid was transfected in M2-HeLa cells growing in different concentrations of doxycyline shown above. Intenstiy of Orc1Tet bands as probed by tetR antibody decreases with decreasing Dox concentration and protein is not expressed in absence of Dox (lane with 0 ng/ml). Gold stained PVDF membrane shows the equal loading of protein lysates.B)

Orc1GFP expression in M2 – HeLa cells. Orc1GFP plasmid was transfected in presence or absence of 1ug/ml Dox. As expected, protein expression was doxycyline controlled when probed with orc1 antibody. The endogenous Orc1 band served as equal loading control. A theoretical evaluation of molecular weight and ionic strength is shown for all the Orc fusions. Theatrical and experimental data shows similar results.

| Proteins | Molecular weight | pI |
|---|---|---|
| scTetR | 52.81 kD | 4.99 |
| 4F | 101.70 kD | 6.22 |
| 4G | 101.74 kD | 6.27 |
| 2F | 117.32 kD | 5.83 |
| 2G | 117.36 kD | 5.86 |
| Orc1Tet | 146.13 kD | 8.56 |
| Orc1GFP | 120.38 kD | 8.71 |

**Table III Size and Ionic strength of Orc fusions.** The Open reading frame data was used to calculate theoretical molecular weight and isoelectric point of orc fusions (www.expasy.org). Where as, the scTet was positively charged, fusion with orc subunits resulted in a negatively charged proteins.

**Sequence specific DNA binding by Orc fusions**

After having the expression patterns of Orc fusions as probed by immunoblots, we selected Orc fusions with G-linker to proceed further in our investigation, as fusions with F-linkers appeared to be partially sensitive to proteolysis. Never the less, comparable protein expression was observed in F constructs.

Negative effect on the functionality of scTetR moiety cannot be ruled out as a result of fusion with Orc subunits. So we tested the DNA binding by Orc fusions both in vitro and in vivo. Electrophoretic mobility shift assays (EMSA) with end labeled 34bp tetO was used to assess the ability of Orc fusions to interact with tetO sequences. As there is no data available on the gel shifts by scTetR proteins, I first established the conditions for the EMSAs for scTetR. Protein extracts with tetracycline transactivator (tTA) and scTet were incubated in P-32 labeled tetO sequences in presence or absence of doxycycline for 20 minutes at room temperature. Salmon sperm DNA is used as a non-specific competitor.

scTet and tTA both belong to 'tet-off' class of tetracycline trans regulators, which means that addition of Dox should disrupt the protein-DNA interaction. Hence, a band shift in presence of doxycyline indicated protein-DNA binding. However, the same reaction showed no band shift in absence of Dox (Fig. 11A). Thus, the fusion proteins analyzed bind to tetO specifically and in a Dox dependent manner as expected. Due to overexpression of protein, proteolytic bands also showed up in lanes without Dox. However, they disappear in the presence of Dox.

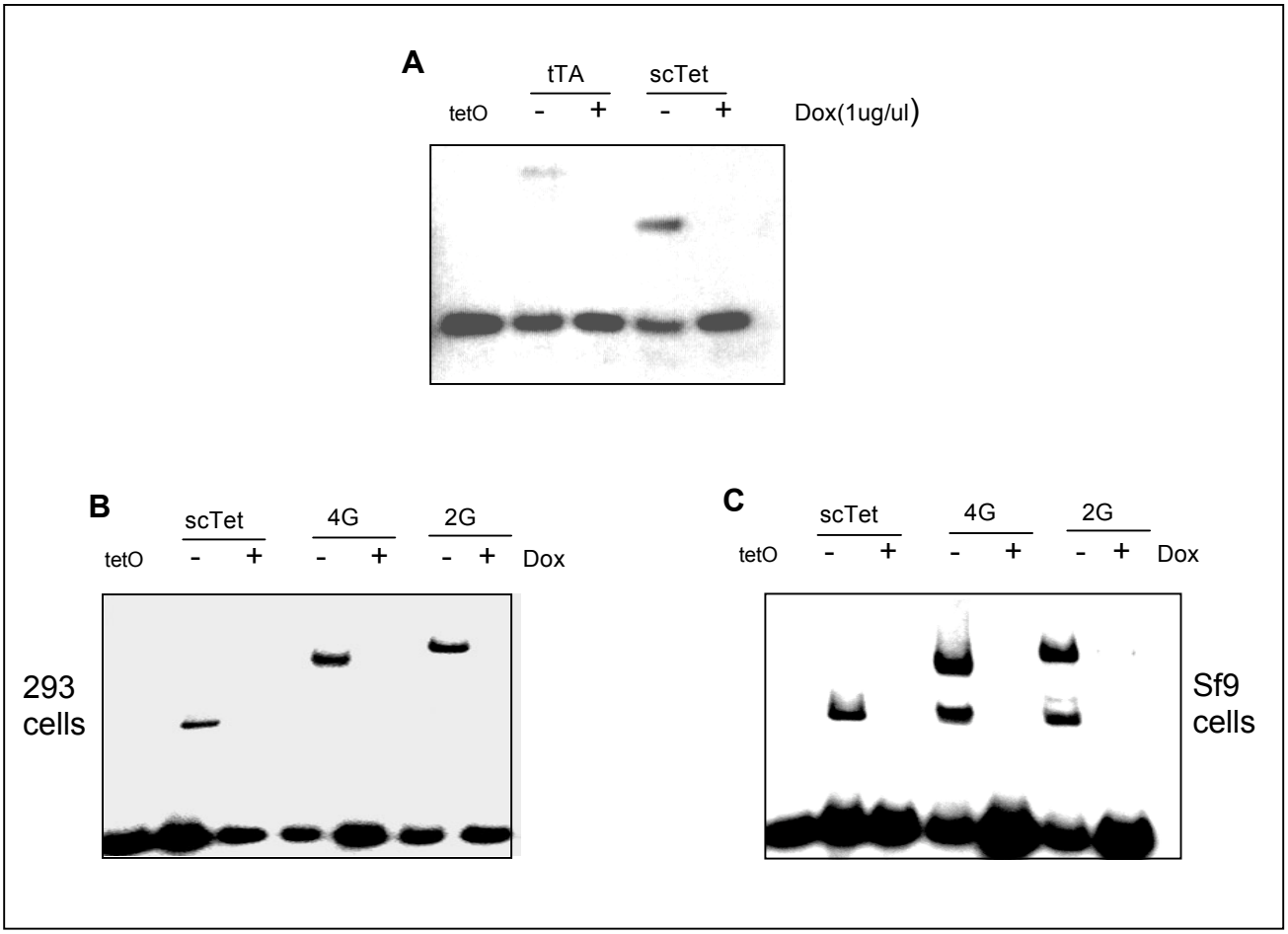


**Fig.11 Dox dependent tetO binding by Orc fusions.** A) tetO binding conditions were optimized for scTet. The disappearance of bands in presence of Dox shows that tetO binding by scTet can be controlled using doxycycline. B) Orc fusions expressed in HEK293 cells binds to tetO in Dox controlled manner. C) Protein extracts from Sf9 cells infected with Orc4G or Orc2G virus.

The same conditions were then employed to check the DNA binding activity of Orc fusions. Orc4G and Orc2G protein extracts were prepared from HEK293 cells were incubated with P-32 labeled tetO sequences in presence and absence of doxycyclin for 20 minutes at room temperature. The reaction mixture was loaded on a native polyacrylamide gel and run at room temperature at 150V. The gel was dried between two whatmann papers and exposed to phosphor plate. Both Orc2G and Orc4G show a band shift as expected and in a Dox controlled manner (Fig. 11B). Qualitatively, it was clear the each band shift corresponds to single protein subunit. However, it was expected that Orc fusions could partly interact with endogenous Orc subunits. To further confirm the shifts from only Orc subunit fused with TetR, we utilized our baculovirus system (see next chapter) system. Baculovirus expressing Orc4G and Orc2G were infected in Sf9 insect cells. The whole cell extracts prepared from these cells were used as protein source for the EMSAs. The band for Orc4G and Orc2G show the same shifts confirming the interaction of only the fused Orc subunit with tetO sequence. Baculovirus system overexpresses protein, due to which protease inhibitor cocktail in the extract preparation was apparently not enough to prevent some proteolysis, which was evident from the lower molecular weight bands at the level of scTet. However, the disappearance of these bands in the presence of Dox indicated them to be TetR proteolytic fragment. Taken together, these results showed that fusion with Orc subunits did not affect the DNA binding activity of scTetR moiety *in vitro*.

Next, we tested the DNA binding by Orc fusions *in vivo*. HeLa cells were co-transfected with TO4/LacZ (see appendix, Fig. IV) along with indicated plasmids (Fig. 12) in presence and absence of Dox. TO4/LacZ plasmid has 2XtetO upstream of TATA box. The binding of tet trans regulators to these sites will inhibit the transcription of the downstream lacZ gene. The pTR6 expressing wildtype TetR used as a positive control. Like wild type TetR, both 4G and 2G binds to tetO in the absence of Dox. This was observed by ß- galactosidase intensity. However, in the presence of Dox the ß- galactosidase intensity

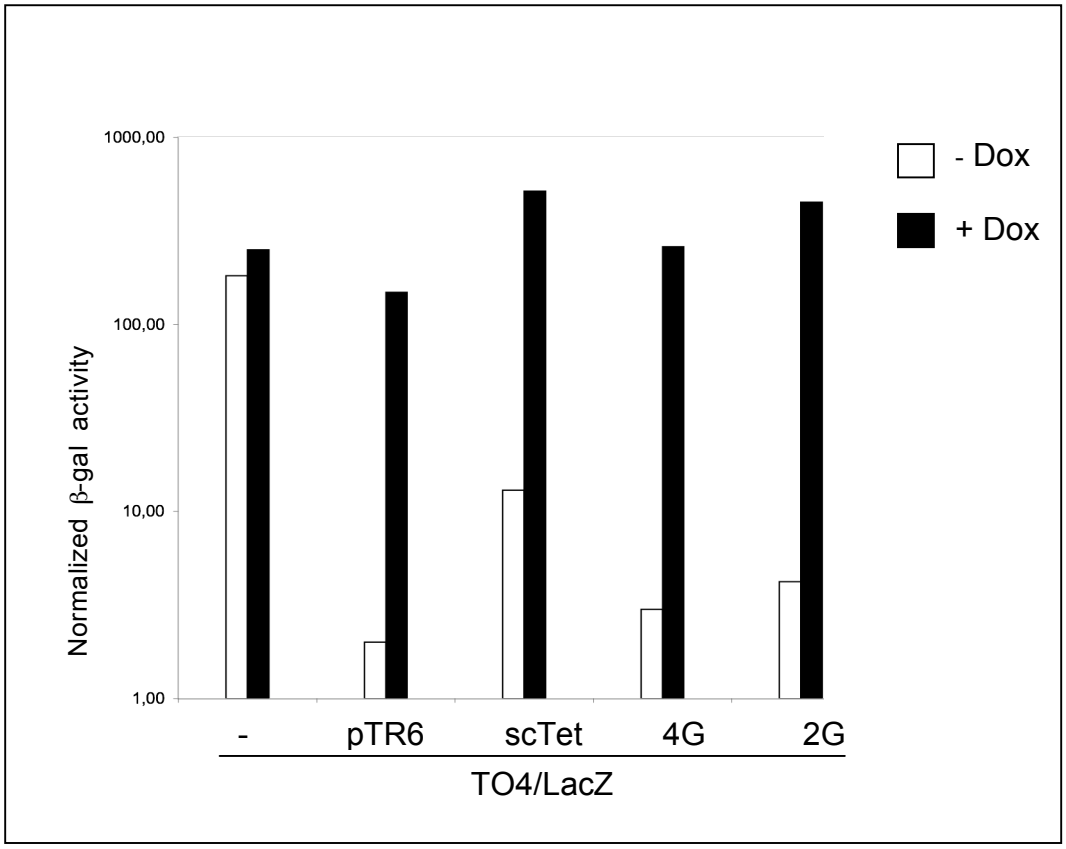


**Fig. 12 In vivo DNA binding by Orc fusions.** The repressor assay was used to investigate the DNA binding activity of Orc fusions. No trans regulator was transfected in negetive control, wild type tetR is co-transfected with TO4/LacZ as a positive control. scTet, 4G and 2G all responded to the Dox treatment as wild type TetR. For response plasmid scheme, see figure. IV in appendix.

increases approximately 100 folds, indicating that in the presence of Dox, TetR domain cannot bind to the tetO. As an internal control, firefly luciferase expression plasmid was transfected into each reaction and luciferase activity was used to normalize the ß- galactosidase activity. Hence, the Orc fusions did not affect the DNA binding activity of tetR moiety, *in vivo.*

In budding yeast origins of replication are found in intergenic regions, suggesting a mutual exclusivity between replication and transcription (Raghuraman et al., 2001; Wyrick et al., 2001). So we wanted to confirm that the Orc fusions were transcriptionally inactive. I used a pTRE/LacZ (see appendix, Fig. IV) plasmid where the expression of the downstream LacZ gene is driven by a minimal CMV promoter activated by upstream heptamerized tetO by action of tet trans

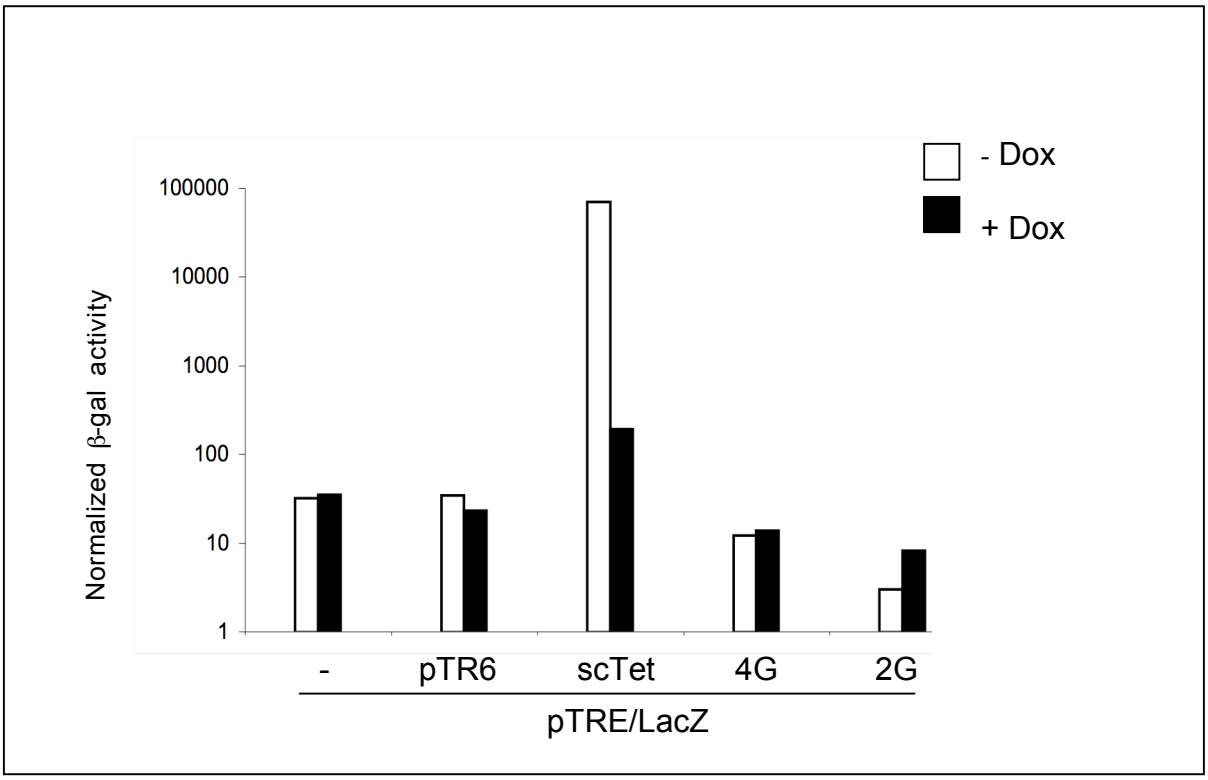


**FIG. 13 Orc fusions are transcriptionally inert.** HeLa cells were co-transfected with TRE/LacZ plasmid and other indicated plasmids. As a negetive control, where no trans activator is transfected. Except for scTet, which has the VP16 transcriptional activating domain, none of reaction activates the expression of the LacZ gene. 4G and 2G behaves as wild type TetR that is known to be transcriptionally inert.

activators. As an internal control firefly luciferase expressing plasmid was transfected in each reaction. The LacZ expression was measured by the ß-galactosidase intensity (Fig. 13). Wild type TetR show no transcriptional activity as evident from the low ß- galactosidase intensity in both presence and absence

of Dox. Only scTet with a transcriptionally active VP16 domain showed a high level of LacZ expression in absence of Dox. However, this activity decreases 100 folds in presence of Dox. Thus, 4G and 2G behaved more or less like transcriptionally inert bloc like wild type TetR.

**Stable cell lines expressing Orc fusion**

In the course of my study, I have faced serious problems to generate stable cell lines with Orc fusions using traditional antibiotic selection method. It was not possible to get the positive clone at first place. Even the positive signal in the transfected population died out after few days. At that time, part of our group was also elucidating the epigenetic modulations and its effect on transgene expression (Liu, W. et al., 2006). In this study, a novel method term as ‘ sorting subcloning’ had been shown to be a more effective method for generating stable and homogeneous transgene expression for long term (Liu W., unpublished data). The details of this method and its application are described in the appendix section.

To investigate the interaction of Orc fusions with endogenous Orc subunit, I generated cell lines that stably expressed 4G in HeLa cells. A modified version of ‘sorting subcloning method’ is utilized to obtain stable 4G clones. As a first step, 4G plasmid is co-transfected with EGFP-loxP plasmid (Fig. 14A). The cloned EGFP-loxP plasmid has EGFP flanked by two loxP sites in same direction. The functionality of loxP sites in the EGFP-loxP plasmid was tested both *in vitro* and *in vivo* (Fig. 14B). Purified CRE recombinase was used *in vitro* to excise the EGFP fragment flanked by loxP sites in same direction. For confirmation *in vivo* the *E.Coli* strain, EL350 where an arabinose promoter can be induced by addition of 10% arabinose to express CRE recombinase was transformed with EGFP-loxP plasmid. As shown in figure14B, both *in vitro* and *in vivo* results show that CRE expression reduced the size of plasmid by approximately 750bp, equivalent to size of EGFP fragment flanked by loxP sites.

Transfected HeLa cells were sorted for GFP positive cells. After two rounds of sorting cells were diluted to one cell per 200ul with complete media. After the colonies grew up cells were expanded to 35mm plates and screened for the positive 4G clones with immunoblots (Fig 15).

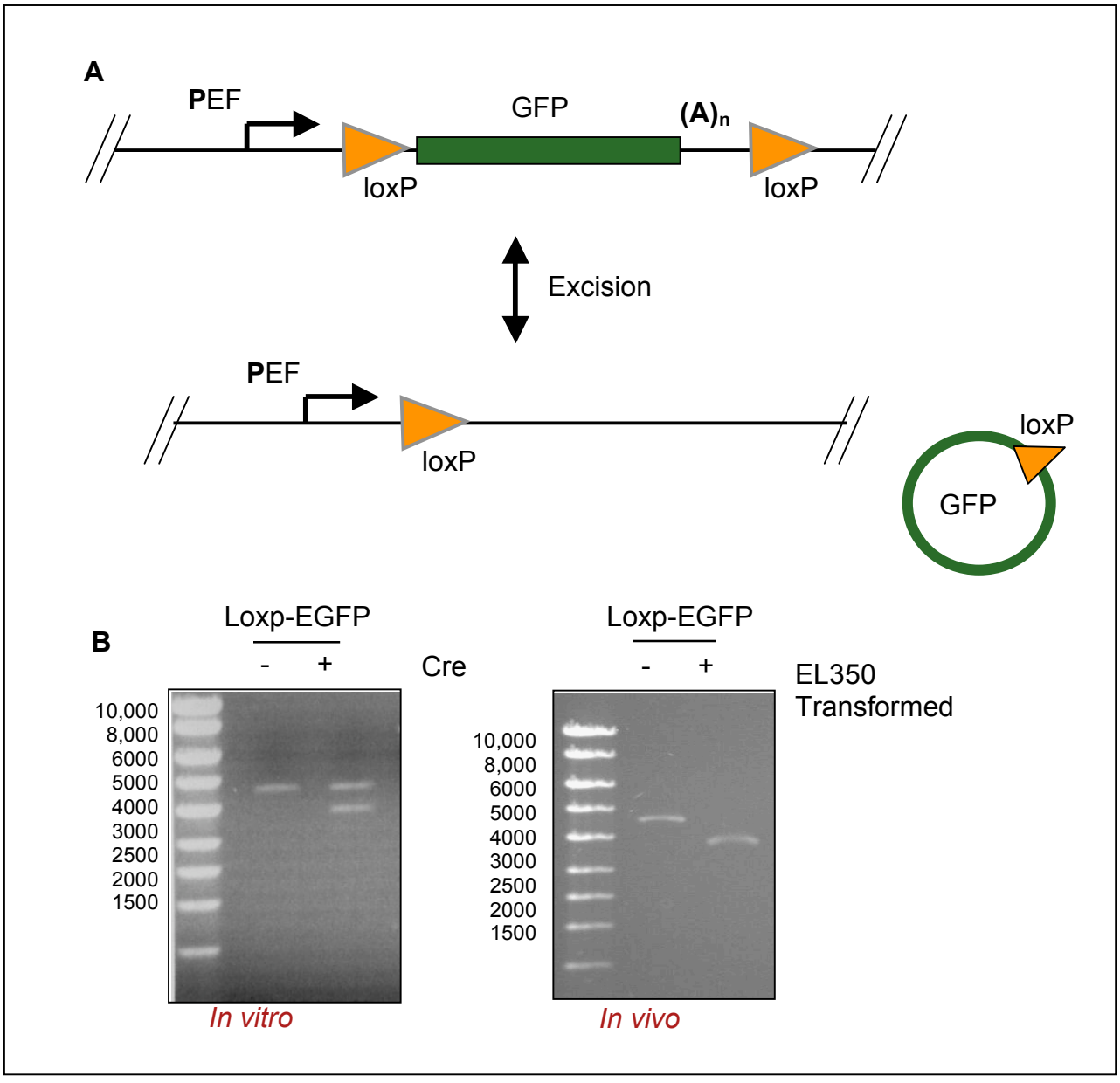

**Fig. 14 EGFP excision by CRE recombinase action on flanked loxP sites** A) The important features of plasmid EGFP-loxP. Two unidirectional loxP sites are flanked EGFP. EF promoter drives the expression of EGFP. B) Functionality of loxP sites *in vitro* and *in vivo*. Purified CRE recombinase was incubated with 100ng of EGFP-loxP plasmid for 1 hour. The lower molecular weight fragment showed the successful recombination event. EL350 cells were transformed by EGFP-loxP plasmid. Addition of 10%arabinose induced the CRE expression from arabinose promoter. The reduction of 750bp in linearized plasmid size when CRE was expressed confirmed the recombination

The endogenous Orc4 show the equal loading of all the lysates. A cross-reacting band was found only in the 4G clones and not in the untransfected control. It would be interesting to probe it further with an antibody other than αHsOrc4 to know if this band represents 4G-degradation product. Sorting subcloning' provided 2 positive clones from 6 clones that were screened. More examples about the utility of this method have been investigated, which are shown in the appendix (Fig. V).

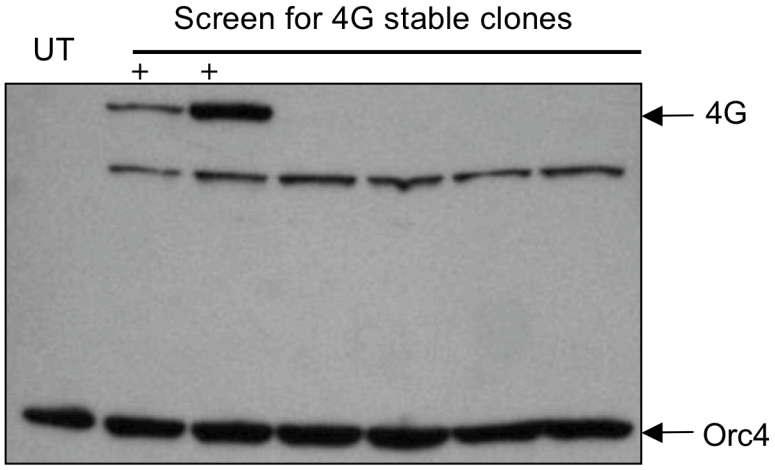


**Fig 15 4G stable clones screened with immunoblots.** HeLa cells were co-transfected with 4G and EGFP-loxP plasmid. Cells were sorted twice for EGFP and then seeded to a dilution of single cell per well of a 96 well plate. Clones growing from the single cell were probed for the positive 4G clones. Out of 6 clones probed with anti Orc4 antibody 2 showed a positive 4G band. Cross-reacting band in all the clones but not in untransfected cells could be degradation product of 4G.

Cell line thus formed expressed 4G along with EGFP (from co-transfected EGFP-loxP plasmid). EGFP that is flanked by loxP sites was excised by CRE recombinase expression. One of the stable clones was taken and transfected with a plasmid constitutively expressing CRE recombinase. 3 days after the transfection, a modest difference in the GFP signal was observed between CRE expressing and untransfected cells. Non-green cells were picked by limited dilution and positive clones were screened by western blots with Orc4 antibody (Fig. 16A). The GFP fluorescent intensity as measured by FACS scanning clearly show the loss of GFP signal in CRE treated (+CRE) cells as compared to stable 4G cells not transfected with CRE (-CRE; Fig. 16B) expressing plasmid.

CRE mediated recombination can induce genomic instability as CRE recombination involves excision of DNA fragment and re-ligating the genome again. Also, ORC has an important role in cell cycle regulation and introduction of an additional copy of Orc4 in the form of 4G could affect cell cycle profile of these cells. Therefore a negative effect of 4G stable integration and CRE recombination on the cell cycle profile was possible. To analyze this, we compared cell cycle profile of +CRE and - CRE stable 4G cells with untransfected cells. As clear from the fig. 16C, the cell cycle profiles of stable 4G (both - CRE and + CRE untreated) cells look the same.

To this end, I have characterized the DNA binding of Orc subunit fused with TetR moiety. Moreover, stable clones for 4G stable cells showed no negative effect on cell cycle profile, which was identical to HeLa cells. So, I tested the interaction of 4G with endogenous Orc subunits. To this end, nuclear extracts prepared from 4G stable cell lines were immunoprecipitated with Orc1 antibody and interacting proteins were immunoblotted with either Orc4 antibody (Fig. 17A) or Orc3 antibody (Fig. 17B). The immunoblot showed that when Orc1 is precipitated with αOrc1 antibody, it pulls down 4G together with endogenous Orc4. Which indicates that inside the cell there exit two sets of complex. One, where Orc1 forms complex with endogenous Orc subunits and the other where 4G integrates

into the complex instead of Orc4. As we know that ORC has single copy of each subunit, it's not possible that both 4G and Orc4 can interact together with Orc1 in single complex.

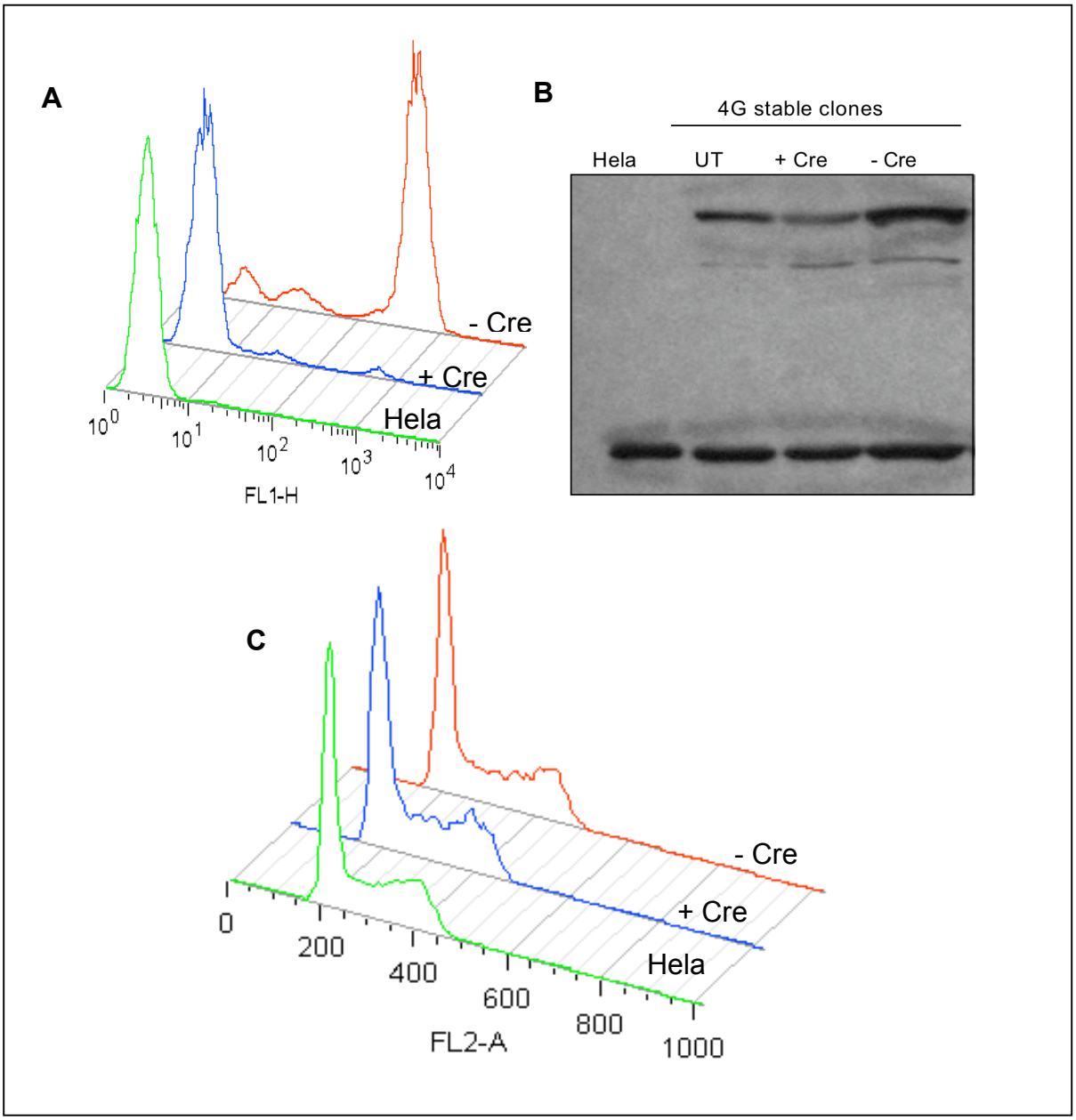


**Fig.16 CRE mediated recombination and analysis of 4G stable clones.** A) GFP fluorescent intensity of either HeLa or +CRE or –CRE cells was measured. –CRE cells represent the 4G expressing clones originating from single cell where CRE mediated recombination did not occur, + CRE cells represent 4G

expressing clones originating from single cell where CRE recombinase has excised the EFGP flanked by loxP sites.B) Immunoblot to confirm expression of 4G protein after the CRE transfection. UT cells represent clone without CRE transfection as a positive reference to 4G expression levels.C) Propidium iodide staining of various indicated clones for cell-cycle profile.

**ORC targeted plasmid replication**

In this study so far, I have shown efficient DNA binding of Orc fused to TetR, which integrates with endogenous Orc subunits. At this point, I hypothesized that this artificial ORC in its functional state would be capable of recruiting pre-RC proteins and could possibly initiate replication of plasmid carrying target DNA (tetO) sequences in mammalian cells.

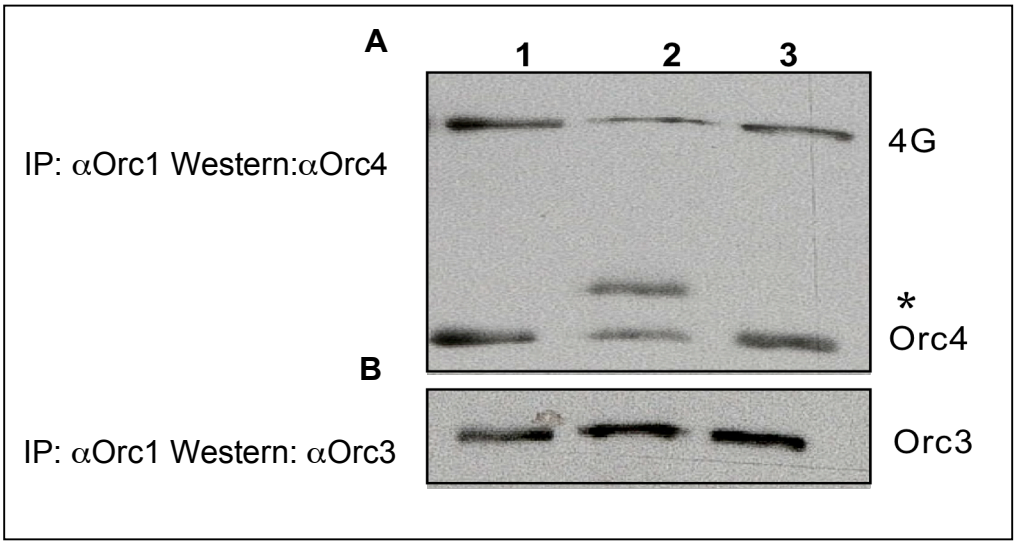


**Fig. 17 *In vivo* interaction of Orc fusions with endogenous Orc subunits**
Immunoblots of proteins resolved on SDS PAGE after immunoprecipitating nuclear extracts of 4G stable cells with αOrc1 antibody. A) Western blots were probed with Orc4 antibodies. 1% of total input for IP was loaded on lane 1. Lane 2 was loaded with immunoprecipitated proteins. Lane 3 was loaded with 2.5% of proteins in the supernatant after IP proteins were pulled down. B) Western blots were probed with Orc3 antibody. Asterix (∗) indicate the heavy chain of IgG used for immunoprecipitation.

To test the plasmid replication inside mammalian cells, I used a short-term replication assay. Here, mammalian cells were transfected with plasmid carrying tetO sequences. Three days later, plasmid was enriched by Hirt's protocol (Material and methods). The enriched plasmid was digested with DpnI an enzyme that digested all dam methylated input bacterial plasmid. However, mammalian cells have no dam methylase, hence, a DpnI resistant band in southern blot indicates the plasmid replication Initially, I chose HeLa cells stably expressing 4G to test the replication of a plasmid carrying heptamerized tetO sequences. However, I was never able to see any DpnI resistant bands above the background level.

So far, most of the plasmid replication assays have been reported in HEK 293 cells. One of the possible reasons could be the higher level of protein expression in these cells. So I established the replication assay for a plasmid carrying SV40 origin, in 293T cells, which are stably transformed with large tumor (T) antigen, a viral replication initiator. As a negative control, the same plasmid is transfected in 293 cells (cells without Large T antigen). Cells were harvested after 3 days, Hirt supernatant (see material and method) was prepared and finally extracted plasmid was subjected to DpnI digestion for 3 hours to overnight. A unique cutter was used to linearize the plasmid. After a phenol:choloroform extraction, digestion reactions were loaded on a 1% agarose gel. The resolved DNA was transferred on a charged nylon membrane by capillary method and the membrane was hybridized with P-32 labeled probe. The results were analyzed by phosphoimager. Initially, the quality of Hirt's plasmid extracts was tested by on southern blots (Fig. 18A). Next, 5 folds more of each sample was linearized along with DpnI digestion. The DpnI will digest all the dam-methylated plasmid of bacterial origin but not newly replicated plasmid in the HEK cells. The southern blot analysis (Fig. 18B) show a large amount of DpnI digested plasmid (lower panel, Fig. 18B), indicating that most of the Hirt supernatant consisted of input bacterial plasmid. However, 293T lane, show a clear DpnI resistant band with much higher intensity than background band observed in 293 lane. After, I have

established the replication assay with SV40 Ori containing plasmid, I tested the effect of ORC targeting on a plasmid replication.

Heptamerized tetO sequences were cloned in the 4G or 2G expression vector resulting in tetO7-4G or tetO7-2G (see appendix, Fig. III), respectively. Cloning was confirmed by DNA sequencing. Hetpamerized tetO sequences containing tetO7-4G or tetO7-2G were transfected in parallel with 4G or 2G plasmids in HEK293 cells.

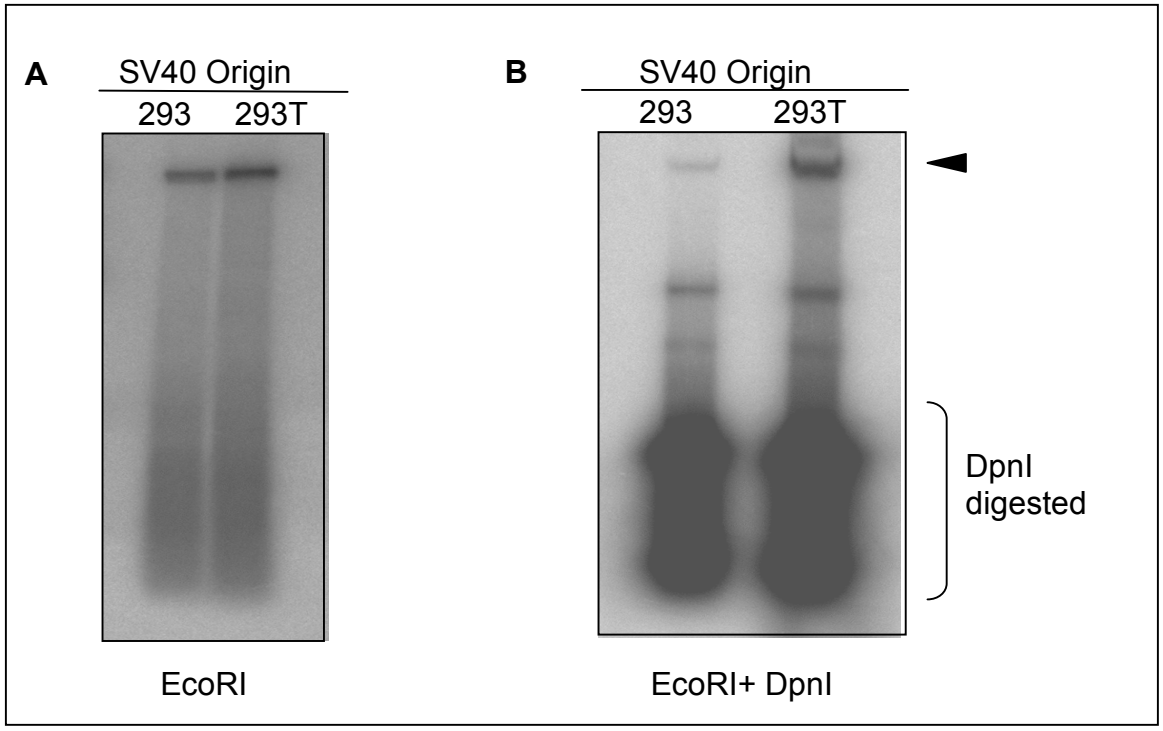


**Fig. 18 SV40 Ori mediated plasmid replication in 293T cells.** Hirt's supernatant extraction efficiency from 293 and 293T cells. EGFP-C1 plasmid carrying SV40 origin of replication was transfected both in 293 and 293T cells. 3 days later cells were harvested and plasmid DNA was extracted by Hirt's protocol. Each sample was linearized with EcoRI and probed by Southern blot. A) Results showed comparable plasmid yield in both 293 and 293T samples.
B) In addition to EcoRI, DpnI was added to plasmid DNA samples from 293 and 293T cells. DpnI digested all the input bacterial plasmid. As mammalian cells do not express dam methylase, the DpnI resistant band (shown by arrow) indicated plasmid replication in mammalian cell.

The replication efficiency of each plasmid is tested using the established short-term plasmid replication assay. A unique NheI site was used to linearize each plasmid extract. Enzymatic digestion of Hirt's supernatants with NheI and DpnI were incubated for 3 hours to overnight before loading on agarose gel. Southern blot was probed with Xcm*I*/Bgl*II* fragment cut out from scTet plasmid.

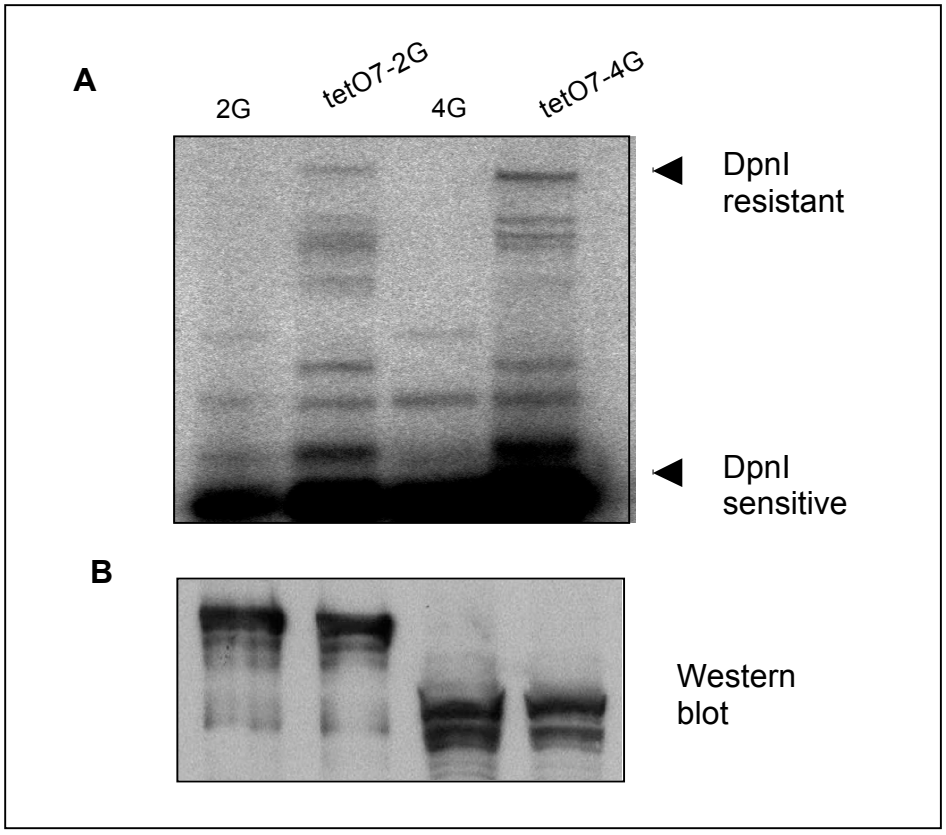


**Fig. 19 TetO dependent replication of plasmid.** A) Plasmid replication assay for 2G or tetO7-2G or 4G or tetO7-4G plasmids. Each plasmid was transfected in HEK 293 cells. 3 days later plasmid extracts were made and each sample is digested with NheI and DpnI. DpnI resistant bands in tetO7-2G and tetO7-4G lanes indicate plasmid replication. B) An aliquot each of the transfected cells in A) were lysed and proteins were immunoblotted with αOrc4 and αOrc2 antibody.

The southern blot analysis showed a DpnI resistant band (Fig. 19A) in tetO7-2G and tetO7-4G lanes. However, no such band is visible in 2G and 4G lanes. This indicated that replication of plasmid required tetO sites. These sequences formed

a preferential binding site for the binding of TetR-fused ORC. Moreover, as shown by western blot analysis of corresponding samples (Fig. 19B), the absence of DpnI resistant bands in 2G or 4G is not due to the inability of these plasmids to express respective fusion proteins. Taken together, these results indicated that targeting ORC to specific DNA sequences is sufficient for replication initiation.

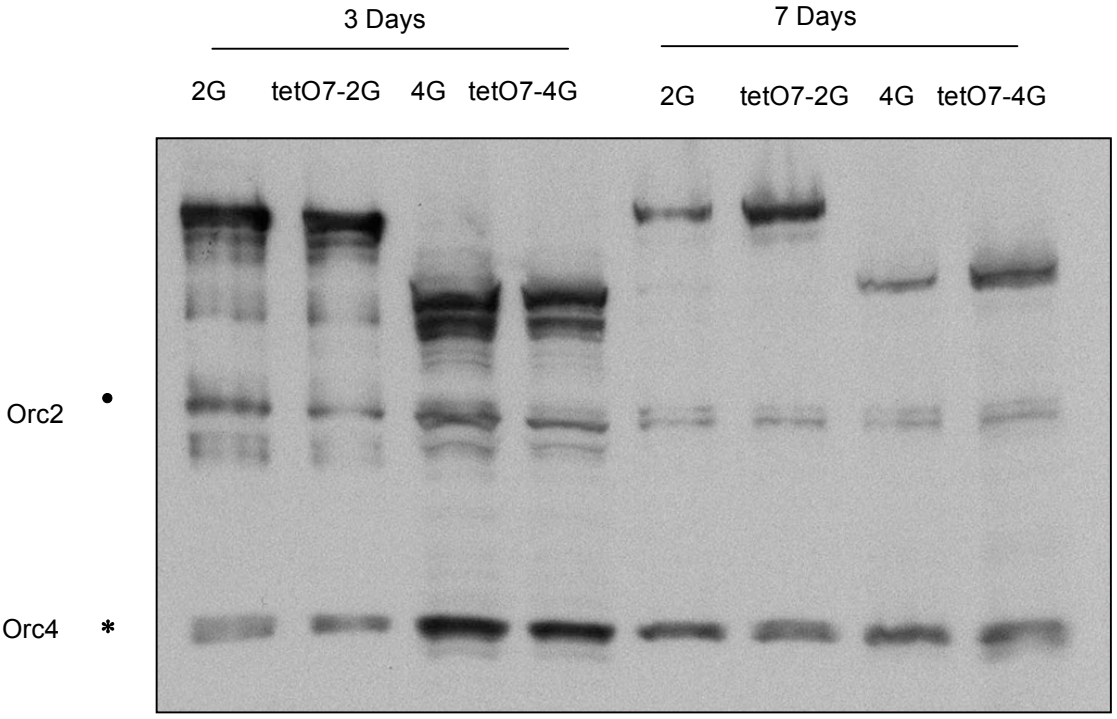


**Fig. 20 Comparison of protein expression levels between short term (3days) and long term (7 days).** Protein lysates for western blot analysis were made on day 3 and day 7. No selection pressure was applied during the time-course. The transfections were made in a small 35mm well, which was expanded to a 100mm dish after 3 days. The proteins were loaded in the order as indicated and probed with Orc4 antibody. The same membrane was then probed with Orc2 antibody. The endogenous proteins (Orc2 and Orc4) show the equal loading. Although no significant difference was seen in 3 days old samples a more intense band can be seen for tetO7-2G or tetO7-4G as compared to respective 2G or 4G lanes in 7 days old protein lysates.

Next, I did a time course of protein expression levels. Cells were again transfected with indicated plasmids (Fig. 20) in a 35mm plate. After 3 days, cells

were transferred to a 100mm dish and aliquot of 3 days protein lysate was saved. No selection pressure was applied during the time course. After 7 days, an aliquot of each sample was taken. Protein lysates from 3 days and 7 days old transfected cells were immunoblotted with Orc4 and Orc2 antibody (Fig. 20). The results showed that the protein levels during the 3 days sample were comparable. However over the period of 7 days tetO7-4G and tetO7-2G showed higher amounts of protein as compared with their counterparts 4G and 2G, lacking tetO sequences. In line with southern blot analysis where replication was detected with tetO7-4G and tetO7-2G plasmids, high protein level indicates higher protein expression through higher copy numbers as a result of replication in these plasmids. Due to the antibiotic regulation of TetR binding, we had expected the system to be Dox controlled. Surprisingly no Dox dependence was observed. There could be various reasons for it. One of the most important is the mechanism of ORC formation and its recruitment to DNA. Moreover, ORC itself is known to have intrinsic DNA binding, this would have resulted in a bipartite DNA binding. Consequently, Dox could efficiently control only TetR moiety. Where as ORC moeity would have Dox independent DNA binding. Never the less, we have created a system where recruitment of ORC to the target DNA sequences supports plasmid replication. Additionally, we have shown the utility of this system in long-term protein expression, which could be a very useful tool for gene delivery.

## Part II: DNA binding analysis of purified engineered ORC

### Baculovirus mediated expression of Orc fusion

Previous studies with yeast and fruitfly have shown the utility of insect cell system to express the Orc subunits. In our lab, we have previously purified human Orc using this expression system. I chose the baculoviral system to over express the Orc-TetR fusions, individually and with other Orc subunits. The open reading frames of all ORC fusions described before were inserted in Fastbac1 vector using compatible restriction sites. The expression in this vector is driven through the insect cell specific polyhedrin promoter. All the baculoviruses used in this study were amplified in Sf9 cells and expression and purification was done in Hi-5 cells. The expression of various Orc fusions is shown in Figure 21.

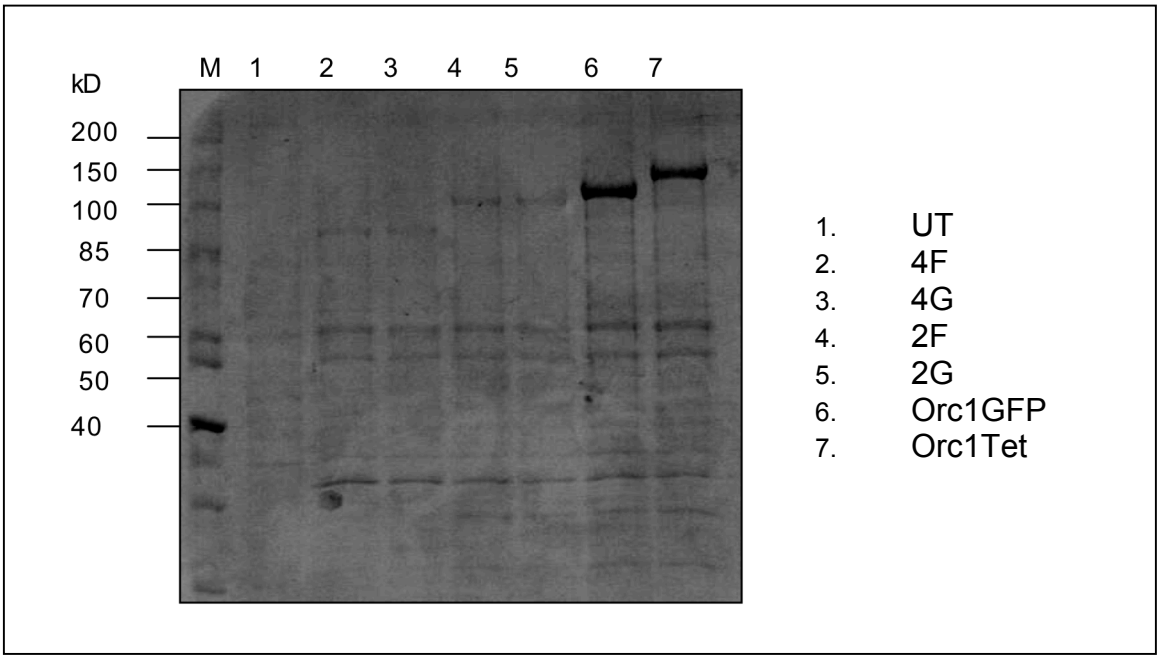


**Fig. 21 Expression of Orc fusions in insect cells**. Coomassie stained 10% SDS PAGE, showing the expression of Orc fusions. Protien lysates prepared from Hi-5 cells 60 hours post transfected were loaded on the gel. Protein bands that were absent in untransfected (Lane1) cells correspond to the expected protein.

Coomassie stained gel with whole cell lysates of individual Orc fusions showed clear bands for each subunit. 4G, 4F and 2G, 2F expression intensity was weaker as compared to Orc1GFP and Orc1Tet. Although, the expression levels vary from clone to clone, in our experience these amounts were sufficient for our biochemical studies.

**In vitro complex formation by Orc fusions**

It has been shown before that co-infection of insect cells with baculoviruses expressing Orc subunits from 1 to 5 resulted in a soluble complex that can be affinity purified (Dhar et al., 2001; Ranjan and Gossen, 2006; Vashee et al., 2003). Except for Orc4 or Orc6 subunits, individual Orc subunits were found insoluble. Moreover, Orc6 subunit does not integrate in complex *in vitro*.

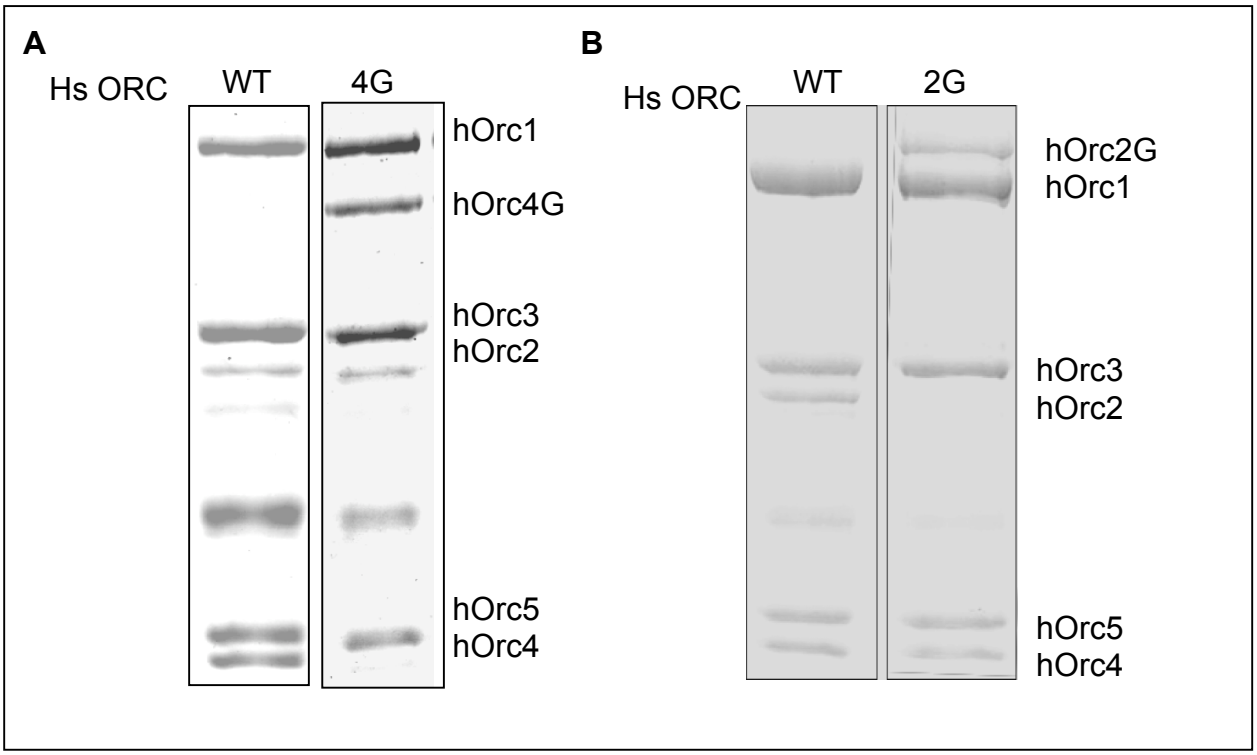


**Fig. 22. Complex formation by Orc fusions.** Affinity pull-down of ORC using HA antibody coupled agarose beads. Nuclear extracts prepared from insect cells

were incubated overnight at 4 degrees with HA beads. Bound proteins were centrifuged, washed thrice and boiled with SDS buffer before loading on SDS gel. Insect cells co-infected with indicated viruses along with hOrc4G A) or hOrc2G B). Both the fusion proteins integrate into the complex.

Therefore, I co-infected Hi-5 cells with baculoviurses expressing Orc subunits from 1 to 5. For wild type ORC formation only wild type Orc subunits were co-infected. In a parallel experiment, to test the complex forming ability of Orc-Tet fusions, either 4G or 2G was infected along with other wild type Orc subunits instead of Orc4 or Orc2, respectively. An HA affinity tag (Ranjan and Gossen, 2006) on the Orc1 subunit was used to pull down the interacting Orc subunits. Bound proteins were resolved on a 10% protein gel and stained in coomassie blue. The bands obtained show that 4G and 2G integrated into the ORC by interacting with other Orc subunits (Fig. 22). Where as Orc4 migrates at 45kD, 4G showed a band of approximately 100Kd (Fig. 22A) due to its fusion with the TetR moiety and 2G migrates at around 120kD size (Fig. 22B). There was no proteolytic band observed in the gel in either case.

**In vitro DNA binding analysis of Orc fusions**

After *in vitro* complex formation was confirmed, I studied the *in vitro* DNA binding by purified engineered ORC. In contrast to the previous protocol, the purification was done using his-tagged affinity purification. Talon beads were used to affinity-purify ORC from the nuclear extracts of insect cells. For a typical purification, 150mm dish of Hi-5 cells infected with Orc subunits gives about 2-3 ug of purified ORC using around 100ul of talon beads. All buffers contain 1mM of ATP and elution was done in lysis buffer containing 400mM imidazole, pH 5.0.

Target DNA sequences (tetO) were biotinylated at 5’ end of one oligo. The single stranded oligos were annealed and coupled with streptavidin coated paramagnetic beads, as described in method section. To confirm the integrity of biotinylated and streptavidin coupled tetO oligonucleotide, purified TetR was

incubated with it, in the presence or absence of Dox. The bound and unbound fractions were loaded on the gel and visualized by silver staining.

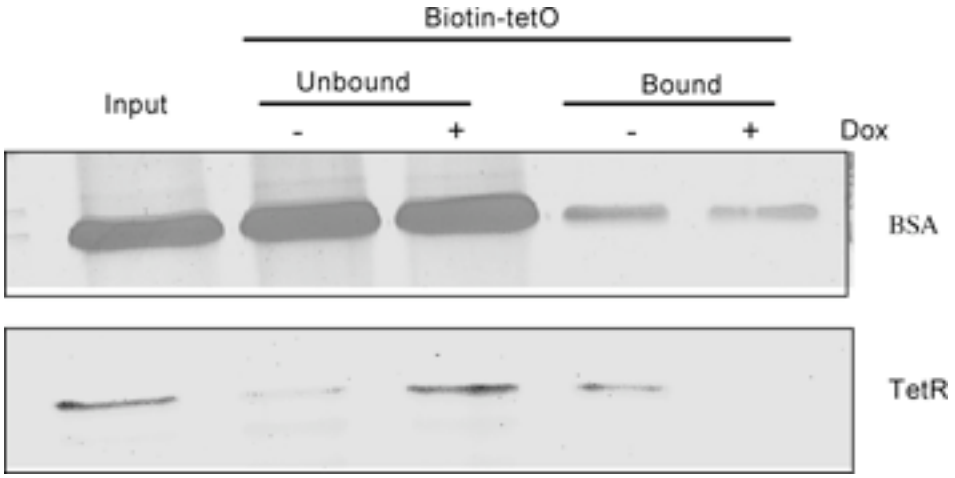


**Fig. 23. Biotinylated tetO interaction with purified TetR in a Dox. controlled manner.** 50ng of purified TetR (kind gift from Bujard) was used with 50pmol of tetO in a binding reaction, both in presence and absence of Dox. The reaction was incubated for 10 minutes at room temperature and bound DNA bound protein was pulled down by applying magnet. The supernatant shows the unbound segment. Bound fractions were wash thrice and boiled with SDS buffer to analyse on silver stained protein gel. BSA used in the reaction showed equal loadings.

Input lane show the amount of protein used for the reaction. Under unbound segment, which show the fraction of protein that didn't bind to DNA, TetR gave a weaker band in absence of Dox as compared to band observed in presence of Dox. This indicated that majority of protein couldn't bind DNA in presence of Dox. However, under the bound segment the majority of input protein was observed in absence of Dox. Indicating a high affinity of TetR to the tetO sequences in absence of Dox. However, in the presence of Dox, DNA binding is disrupted so no protein band was observed in bound fraction.

Next, I analysed the DNA binding of the affinity purified engineered ORC. As Orc subunit fused to TetR domain integrates to form a complex, it was imperative that

our engineered human ORC should bind to tetO sequences. I incubated purified ORC2G with tetO in presence of excess of salmon sperm DNA. The magnet was applied and bound protein was washed thrice and analysed on a sliver stained protein gel. A fraction of unbound protein was loaded to analyse the binding efficiency. Input lane (Fig. 24) shows the affinity-purified protein used for the DNA binding reaction. An enrichment of purified ORC2G on biotinylated tetO was observed.

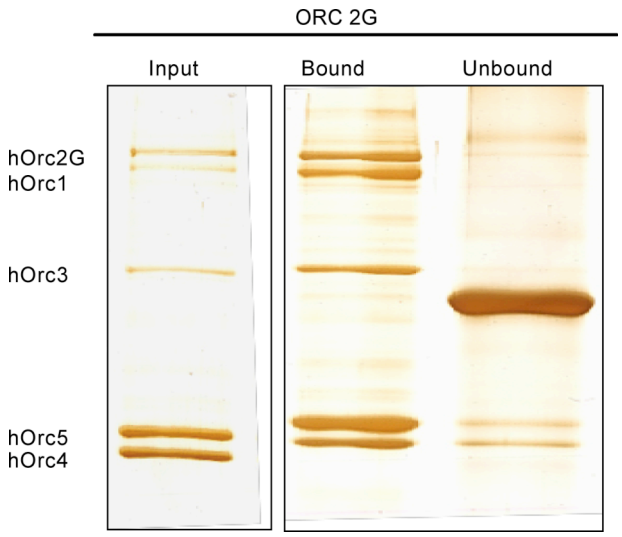


**Fig. 24 Biotinylated tetO mediated pull down of purified ORC2G**. 20pmol of biotinylated tetO was used in the reaction containing purified ORC2G. Reaction was incubated at 4 degrees for 30 minutes. Magnet was applied to pull down bound protein. After three washes, beads were boiled with SDS buffer and bound protein was analysed by silver staining. The prominent band in unbound fraction is BSA.

Next, I analysed the effect of Dox on the ORC-tetO interaction. Two parallel reactions as described before were made. To one reaction 1ug/ml Dox was added and protein bound in tetO sequences was analyzed. To our surprise, no Dox effect was observed; ORC can be pulled down by biotinylated tetO both in presence or absence of Dox (data not shown). The DNA used for these experiments were 77bp in length. Next, the experiment was repeated with 34bp (size of a single tetO repeat) long biotinylated tetO. Even then, only a modest effect was observed in the presence of Dox (Fig. 25A). To this end, we wanted to

confirm that engineered ORC has at least a preference for tetO. For this, we did a DNA binding assay in presence of competitors

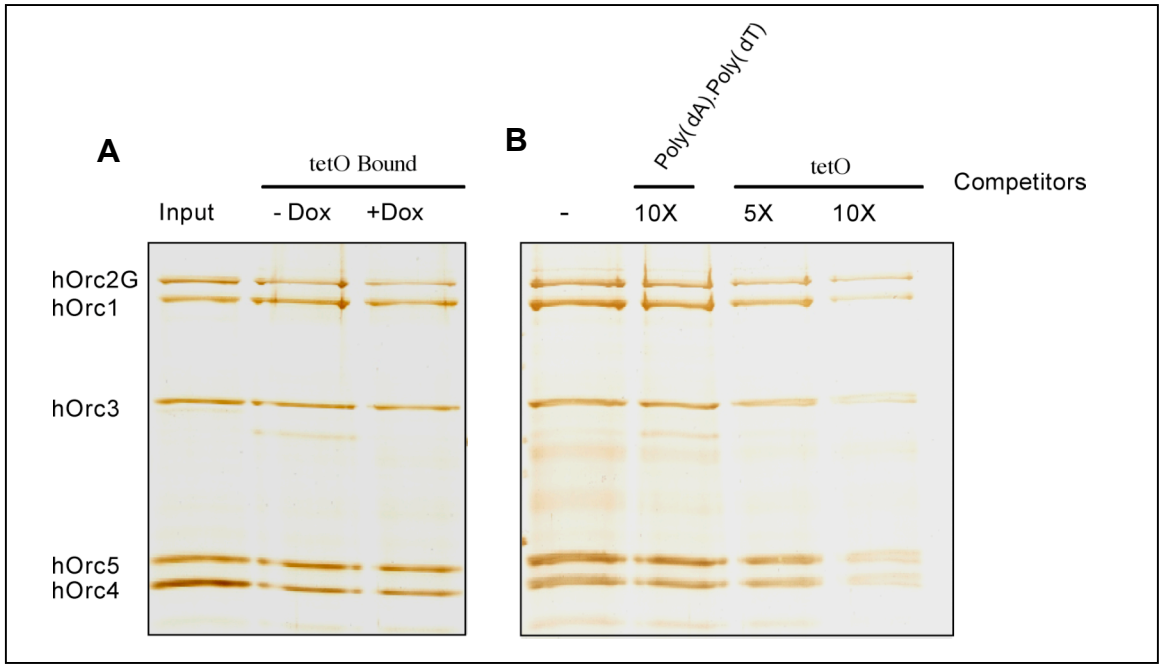


**Fig. 25 Engineered human ORC binds preferentially to tetO in a Dox independent manner.** A) tetO binding to ORC2G in presence and absence of 1ug/ml of Dox. No significant effect of Dox was observed. B) Effect of adding AT rich sequences or tetO on ORC2G binding with biointinylated tetO. Either no competitor was added or 10X Poly(dA).Poly(dT) or 5X tetO or 10X tetO was added 30 minutes before 10pmol of biotinylated tetO was added in the reaction.

It has been shown before that human ORC binds preferentially bind to AT rich sequences (Vashee et al., 2003). So we compared the ability of Poly(dA).Poly(dT) sequences and tetO to compete with the biotinylated tetO sequences (Fig. 25B). There is no significant change in the bound protein when 10 fold more Poly(dA).Poly(dT) was added to reaction before adding biotinylated tetO. However, significant reduction in the bound protein was observed when 10-fold excess of tetO was used. Taken together, our results show that although engineered human ORC binds to tetO sequences in a Dox independent manner, it still has a much higher affinity to tetO as compared to unrelated sequences.