Q1.

1. Build targeting vector (the length of the construct is recommended between 6-12 kb for efficient homologous recombination), propagate, linearize and purify the construct.
2. Electroporate the targeting construct into ES cells (from BROWN mouse) and PN select (G418 for positive selection and GANC for negative selection, as shown in Fig.1). Grow up the correct ES cells, expose them to 2.0 mg/ml G148 to cause chromosomal lose followed by chromosomal duplication. Use restriction enzyme *Hind*III digestion and Southern blotting to screen ES cells and collect those with homogenous point mutation (*Cub*/Cub**) for further usage (*Cub*/Cub** ES cell will show one band in Southern blotting with correct size, *Cub*/Cub* ES cell will show three bands and *Cub*/*Cub* ES cell will show two bands).
3. Microinject the chosen ES cell into blastocyst-stage embryo (from GRAY mouse). Transfer the microinjected embryo into gray surrogate mother.
5. Recover chimeric male (brown+gray) and mate with gray female.
6. Screen for mice that are heterogynous for the point-mutant gene *Cub*/Cub* (screen strategy is all most the same as screen for *Cub*/Cub** ES cell).
7. Mate heterogynous mice (*Cub*/Cub*) and screen for homozygous progeny mice (*Cub*/Cub**).

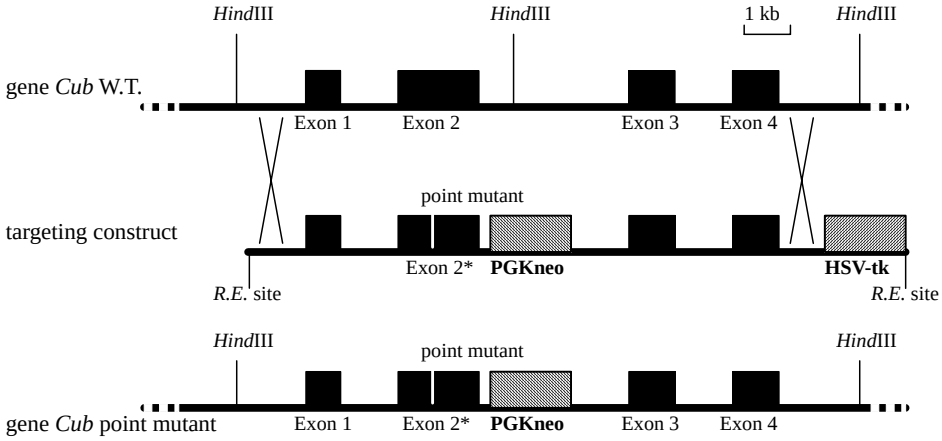


Fig.1 The PNS procedure to enrich for ES cells containing the targeted point mutation of gene Cub.

* I suppose that the interested point mutation is in the second exon of gene *Cub*.

Q2.

The sequence of AL699862 is:

```
>AL699862
TGGCCGGGGGCCCCGCCCCCGGCAGCGACACCCGATAAGGAGTCAGGCCAGGGCGGGATGACACTCATTGATTCTAAAGCATCTTT
AATCTGCCAGGCGGAGGGGGCTTTGCTGGTCTTTCTTGGACTATTCCAGAGAGGACAACTGTCATCTGGGAAGTAACAACGCAGGA
TGCCCCCTGGGGTGGACTGCCCCATGGAATTCTGGACCAAGGAGGAGAATCAGAGCGTTGTGGTTGACTTCCTGCTGCCCACAGGG
GTCTACCTGAACTTCCCTGTGTCCCGCAATGCCAACCTCAGCACCATCAAGCAGCTGCTGTGGCACCGCGCCCAGTATGAGCCGCT
CTTCCACATGCTCAGTGGCCCCGAGGCCTATGTGTTCACCTGCATCAACCAGACAGCGGAGCAGCAAGAGCTGGAGGACGAGCAAC
GGCGTCTGTGTGACGTGCAGCCCTTCCTGCCCGTCCTGCGCCTGGTGGCCCGTGAGGGCGACCGCGTGAAGAAGCTCATCAACTCA
CAGATCAGCCTCCTCATCGGCAAAG
```

The gene is corresponds to Homo sapiens phosphoinositide-3-kinase.

NM_005026.2 may represent full length cDNAs.

The expression of gene has temporal and spatial distribution. The gene you interested may only express in some specific tissue at specific point of life cycle, so the material you used to extract mRNA should be considered. Random primers may let you don't have to think more about the reverse transcription. But designed primers based on the gene's special terminals will make your experiments more efficient. As cDNAs have different sizes, you should consider which vector to use. Bacteriophage lambda vector is an effective choice, and you can also use bacterial plasmid (the mRNA here is about 5kB, which is difficult to be inserted into a plasmid vector) to construct your cDNA library. Efficient cDNA library screen strategy should also be considered too.

The **homologous** sequences share common evolutionary ancestor. Two sequences are **orthologous** if their last common ancestor existed at the speciation time. Two sequences are **paralagous** if last common ancestor existed at the gene duplication time.

I can find 5 human paralogs of this sequence from UniGene Cluster Hs.426967.

The orthologous in *Drosophila* is 1-phosphatidylinositol 3-kinase [pir: T13950], in *Arabidopsis* is phosphatidylinositol 3-kinase putative [ref:NP_176251.1], and in *S. cerevisiae* is 1-phosphatidylinositol 3-kinase [A36369].

Q3.

a.

Cross *nuc1-1*$^{ts-}$ *Sup* with *NUC1* then tetrad analysis.

From the tetrad analysis, if you can find some spores can grow at 25 °C but can not grow at 37 °C, the suppressor is extragenic. If all the spores can grow at both 25 °C and 37 °C, the suppressor is intergenic. [The table below is the possible tetrad score sheet.]

	Extragenic Suppressor			**Intergenic Suppressor**		
	Growth at:	**25°C**	**37 °C**	**Growth at:**	**25 °C**	**37 °C**
PD	*nuc1-1*$^{ts-}$ *Sup*	+	+	*nuc1-1*$^{ts-}$ *Sup*	+	+
	nuc1-1^{ts-} *Sup*	+	+	*nuc1-1*$^{ts-}$ *Sup*	+	+
	NUC1	+	+	*NUC1*	+	+
	NUC1	+	+	*NUC1*	+	+
NPD	*nuc1-1*$^{ts-}$	+	-	/	/	/
	nuc1-1^{ts-}	+	-	/	/	/
	NUC1 Sup	+(?)	+(?)	/	/	/
	NUC1 Sup	+(?)	+(?)	/	/	/

b.

Cross *nuc1-1*$^{ts-}$ *Sup#1*, *nuc1-1*$^{ts-}$ *Sup#2*, *nuc1-1*$^{ts-}$ *Sup#3*, *nuc1-1*$^{ts-}$ *Sup#4* with each other then tetrad analysis. From the tetrad analysis, if you can find some spores can grow at 25 °C but can not grow at 37 °C, these two suppressors are in different genes. If all the spores can grow at 25 °C and 37 °C, these suppressors are in the same gene. Then you can summary how much genes are represented. [The table below is the possible tetrad score sheet.]

	Sup1 & Sup2 in One Gene			**Sup1 & Sup2 in Different Genes**		
	Growth at:	**25°C**	**37 °C**	**Growth at:**	**25 °C**	**37 °C**
PD	*nuc1-1*$^{ts-}$ *Sup1*	+	+	*nuc1-1*$^{ts-}$ *Sup1*	+	+
	nuc1-1^{ts-} *Sup1*	+	+	*nuc1-1*$^{ts-}$ *Sup1*	+	+
	nuc1-1^{ts-} *Sup2*	+	+	*nuc1-1*$^{ts-}$ *Sup2*	+	+
	nuc1-1^{ts-} *Sup2*	+	+	*nuc1-1*$^{ts-}$ *Sup2*	+	+
NPD	/	/	/	*nuc1-1*$^{ts-}$ *Sup1 Sup2*	+(?)	+(?)
	/	/	/	*nuc1-1*$^{ts-}$ *Sup1 Sup2*	+(?)	+(?)

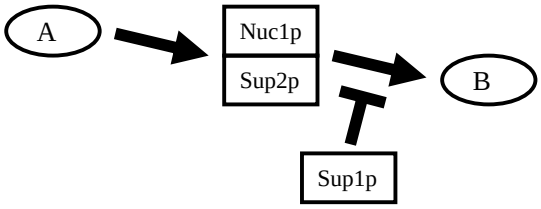
	/	/	/	*nuc1-1*$^{ts-}$	+	-
	/	/	/	*nuc1-1*$^{ts-}$	+	-

c.

SUP2-1 is dominant suppressor, and *sup1-1*, *sup 1-2* and *sup1-3* are recessive suppressors.

d.

SUP1-1, *sup1-2*, *sup1-3* and *sup1-4* are extragenic suppressors, and *nuc1-1*$^{ts-}$, *nuc1-2*$^{ts-}$ and *nuc1-3*$^{ts-}$ are mutants in *NUC1*. As *SUP2-1* can suppress *nuc1-1*$^{ts-}$, but not suppress *nuc1-2*$^{ts-}$ or *nuc1-3*$^{ts-}$, there should be a specific interaction between Sup2-1p and Nuc1-1p which can recover yeast's ability to grow at 37 ^{o}C. As recessive mutants of *sup1* can suppress all the recessive mutants of *NUC1*, Sup1p should be a downstream regulator of Nuc1p. Here is a possible functional relationship between Sup1p, Sup2p and Nuc1p.



From A to B is a pathway related to yeast's ability to grow at 37 ^{o}C. Nuc1p, interacted with Sup2p, charges this pathway. And Sup1p is a negative regulator of the downstream of Nuc1p and Sup2p. If Nuc1 is mutated, Nuc1p*'s function decreases and the yeast can not grow at 37^{o}C. But if Sup1 is also mutated, Sup1p* can not negative regulate Nuc1p*'s function as Sup1p, and the yeast will be recovered! If the yeast wants to recover Nuc1p* by Sup2p*, Sup2 should be mutated in a specific site which may recover these two proteins' interaction.

e.

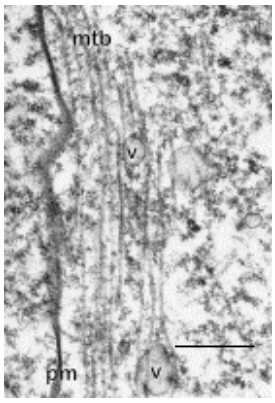
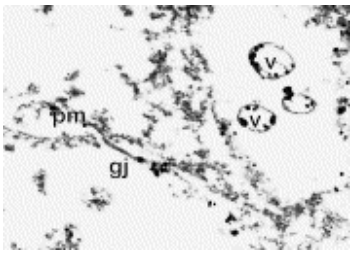
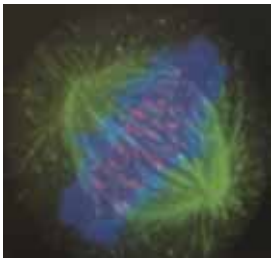
The table below is the possible tetrad score sheet. *SupX* is a non-authentic *SUP2-1* gene, and supposed to have dosage suppression.

	Authentic *SUP2-1* gene				**Non-authentic *SUP2-1* gene**			
	Growth at:	**25^{o}C**	**37 ^{o}C**	**-ura**	**Growth at:**	**25 ^{o}C**	**37 ^{o}C**	**-ura**
PD	*nuc1-1*$^{ts-}$ *URA3-SUP2-1*	+	+	+	*nuc1-1*$^{ts-}$ *URA3-SupX*	+	+	+
	nuc1-1^{ts-} *URA3-SUP2-1*	+	+	+	*nuc1-1*$^{ts-}$ *URA3-SupX*	+	+	+
	nuc1-1^{ts-} *SUP2-1 ura3-52*	+	+	-	*nuc1-1*$^{ts-}$ *SUP2-1 ura3-52*	+	+	-
	nuc1-1^{ts-} *SUP2-1 ura3-52*	+	+	-	*nuc1-1*$^{ts-}$ *SUP2-1 ura3-52*	+	+	-

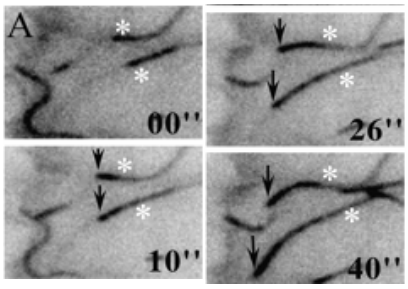
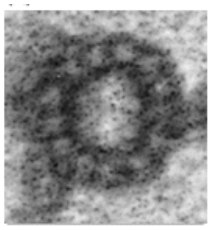
NPD	/	/	/	/	*nuc1-1^{ts-} URA3-SupX SUP2-1*	+	+	+
	/	/	/	/	*nuc1-1^{ts-} URA3-SupX SUP2-1*	+	+	+
	/	/	/	/	*nuc1-1^{ts-} ura3-52*	+	-	-
	/	/	/	/	*nuc1-1^{ts-} ura3-52*	+	-	-
TT	/	/	/	/	*nuc1-1^{ts-} URA3-SupX SUP2-1*	+	+	+
	/	/	/	/	*nuc1-1^{ts-} URA3-SupX*	+	+	+
	/	/	/	/	*nuc1-1^{ts-} ura3-52*	+	-	-
	/	/	/	/	*nuc1-1^{ts-} SUP2-1 ura3-52*	+	+	-

As the tetrad score sheet of Class A only has phenotype of PD, Class A clone contains the authentic *SUP2-1* gene. The tetrad score sheet of Class B has phenotype PD:NPD:TT=1:1:4, this clone does not contain *SUP2-1* gene.

Q4.

Technique	Description
1. Atomic force microscopy	The AFM detects and analyzes the force between the tip and the sample. By taking sequential tapping-mode AFM images and measuring the change in the microtubule end position as a function of time, the dynamics of microtubule is followed. [1]
2. Thin section TEM	High magnification showing the close structural relation between microtubule bundles (mtb) and membranous vesicles (v) along the plasma membrane (pm) of a fiber cell (Bar, 0.5 um) [2]. And the pictures from TEM revealed that microtubule is composed of three molecules that are associated to form a triple helix. 
3. Immunogold labeling TEM	The right figure shows that MIP26 membrane proteins immune labeled with gold particles are present in the vesicles and are transported by microtubules during fiber cell elongation and growth (Bar, 0.5 um). [2] 
4. Immunofluorescence microscopy	Immunofluorescence microscopy use antibody to label the target, and monitor the fluorescence. Here, The spindle microtubules are revealed with a green fluorescent antibody, centromeres in red, and DNA in blue. The distribution of microtubules during cell mitosis is revealed. [*MBOC4*: 9-14] 
5. Total internal reflection microscopy	TIRFM provides a means of direct imaging of processes within very

[1] *Ultramicroscopy*, 2003, **97**:239-247

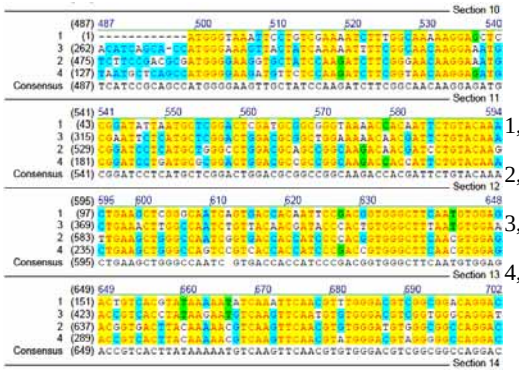
	close proximity to the coverslip. Here, observation of microtubule plus ends by TIRFM indicates that microtubules lift away from the substrate during shrinkage, and shows microtubule tracking along common paths. [3] 
6. Cryo-TEM	Here, electron micrograph of a microtubule seen in cross-section, with its ring of 13 distinct subunits, each of which corresponds to a separate tubulin molecule (an a/b heterodimer). Richard Linck based on Cryo-TEM data deciphered how tubulins polymerize into microtubule. [*MBOC3* 16-21] 
7. Dark field light microscopy	Shiny structures are seen in front of a dark background. Dark-field illumination clearly demonstrated the "hoop" shape of cytoskeletons in unfixed suspensions where the microtubule coils had a mean diameter of 2.87 microns. Microtubules were uncoiled by brief exposure to trypsin or by NaCl[4].
8. Electron diffraction crystallography	EDC resolved the structure of tubulin (monomer of microtubules). [www.pdb.org] 
9. Fluorescence recovery after photobleaching	FRAP could reveal the average dynamics of one or more subpopulations of molecules. You may use FRAP to determine the polarity of microtubule dynamics during fluorescence recovery.
10. Nipkow disk confocal microscopy	It is a time-multiplexed non-linear excitation microscope exhibits significantly less background and therefore a superior axial resolution as compared to a multifocal single-photon confocal system, which is very useful in studying microtubule' structure and dynamics.

[2] *Exp Eye Res*, 2003, **77**:615-626
[3] *J Cell Biol*, 2003, **161**:853-859
[4] *Biol Cell*, 1988, **64**:283-291

Actually there are still many other microscopy techniques are employed in investigating the microtubule. Negative stain TEM reveals that microtubules are in a bundle, *etc*. The microtubule is a polymer [Cryo-TEM, Thin section TEM]: tubulins [Electron diffraction crystallography resolves its 3D structure] form a series of rings, 25 nm in diameter; in cross section, each ring consists of 13 beads. And microtubule will elongate followed the axis [video enhanced differential interference contrast microscopy]. Microtubules are polar with a plus end (fast growing) and a minus end (slow growing). And microtubule keeps assemble and disassemble [immunofluorescence microscopy], called dynamics instability. Half-life of individual microtubule is about 10 minutes [FRAP]. Modern techniques [AFM, immunofluorescence microscopy, Nipkow disk confocal microscopy, *etc*] found that microtubules are involved in many cellular events, such as cell mitosis and vesicle transport, interact with many parts in the cytoplasm [Immunogold labeling TEM] and will “move” follow the cell’s shrinkage [TIRFM]. Microtubule is a very important part in cytoskeleton.

Q5.

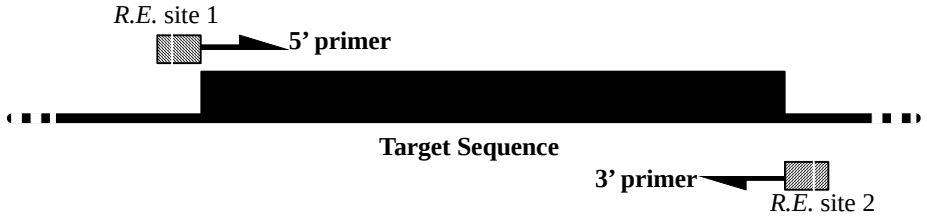
Construct your CHO cell cDNA library is the traditional way. Use standard method to extract total mRNA from CHO cell. Use reverse transcription to make cDNA from mRNA. Choose suitable bacterial plasmid (usually I will use lambda phage to construct cDNA library; but this time as the length of ARF-6 in mouse cell is only 1561bps, I would like to use plasmid to exclude relatively long cDNAs) as vector, and construct the library. Select for the clones that have insertions and use designed probe to screen the library. Further validate the positive clones and get the clone that contains ARF-6 finally.



1, *C. elegans*;
2, *Mus musculus*;
3, *Drosophila*;
4, *Xenopus laveis*.

As ARF-6 has been studied in mouse and many other model organisms, I tried multi-alignment of ARF-6 gene and found that the center part of ARF is highly conserved (the above diagram only show part of the alignment results). So I have a brave strategy to clone ARF-6 in CHO cell: based on the center part sequence of *Mus musculus* ARF-6 (about 800bp) design primers to **fish** in the mRNA from CHO cell; if we can get specific bands which is sequenced to show conserved with ARF-6, then extend this region by PCRs. If lucky enough, we can get full-length ARF-6 cDNA of CHO cell.

The MCS of pMSCVpuro contains *Bgl*II, *Xho*I, *Hpa*I and *EcoR*I. Use restriction enzymes have compatible overhand (c.o.), *BamH*I [c.o.*Bgl*II], *Sal*I [c.o.*Xho*I], *Xap*I [c.o.*EcoR*I] *etc*. If you find that *BamH*I and *Xap*I are not in the clone, use them to design the primers (diagram shown below) and got the ARF-6 sequences double-digested with *BamH*I and *Xap*I. At the same time, double-digest pMSCVpuro with *Bgl*II and *EcoR*I. Then mix, and ligate them!



Q6.

a.

		Ms. Davis	
		XX	***Y***
Mr. Goldblum	***X***	dead	Male
	Y	female	dead

b.

Usually, the male with XY will determine the progeny's sex: the progeny gotten Y from the father and X from the mother will be male, and the progeny gotten X from the father and X from the mother will be female. Here, as the female has XX/Y, she also takes part in the sex determination: if the progeny gets Y from the father and gets Y from the mother, he will die; but if gets XX from the mother, he will be female!

c.

If barfly mutation is dominant,

Genotype is:

		Ms. Davis	
		bar	***bar***
Mr. Goldblum	***BAR***	*BAR/bar*	*BAR/bar*
	bar	*bar/bar*	*bar/bar*

Phenotype is:

		Ms. Davis	
		bar	***bar***
Mr. Goldblum	***BAR***	barfly	barfly
	bar	normal	normal

If barfly mutation is recessive,

Genotype is:

		Ms. Davis	
		BAR	***BAR***
Mr. Goldblum	***bar***	*BAR/bar*	*BAR/bar*
	bar	*BAR/bar*	*BAR/bar*

Phenotype is:

		Ms. Davis	
		BAR	***BAR***
Mr. Goldblum	***bar***	normal	normal
	bar	normal	normal

d.

Barfly is recessive mutation. Puffy lips is dominant mutation.

e.

Genotypes are listed:

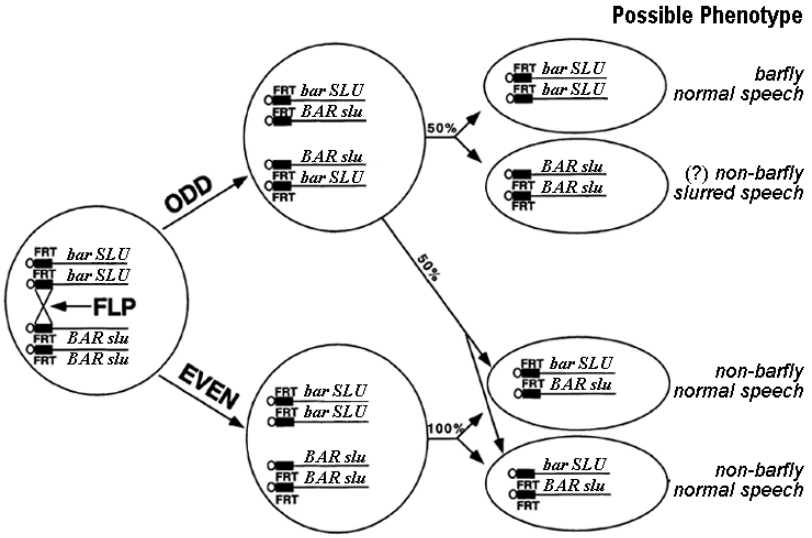
Jeff - *X/Y, bar/bar, puf/puf* or *X/Y, bar/bar, +/+*

Geena - *XX/Y, BAR/BAR, PUF/PUF* or *XX/Y, +/+, PUF/PUF*

The neighbor – *X/X, BAR/bar, PUF/puf* or *X/X, +/bar, PUF/+*

f.

I show the schematic diagram of mitotic recombination first.



As the wild type *slu* gene product is required simultaneously in both the tongue and brain to be wild type, the progeny with the initial cell genotype of (*BAR slu/BAR slu*) will not be wild type in the tongue or brain. If the wild type *bar* gene product is required in the brain: the progeny with the initial cell genotype of (*bar SLU/bar SLU*) from the cross between Mr. Goldblum and Neighbor#2 will die, and you can not find any *barfly* phenotype in the progenies. If the wild type *bar* gene product is required in the tongue: the progeny with the initial cell genotype of (*bar SLU/bar SLU*) from the cross between Mr. Goldblum and Neighbor#3 will die, and you can not find any *barfly* phenotype in the progenies. So the progeny with normal speech is the phenotypic class that will be informative to answer the question. Though the progeny with the initial cell genotype of (*bar SLU/BAR slu*) will continue mitotic recombination, 75% subsequent cells will have both the

wild type gene products of *slu* and *bar* to keep the progeny wild type.

g.

The wild type *barfly* gene is not required in either brain or tongue.

Q7.

Usually there is a tag in the retroviral cassette. You can use the blank cassette to test the infection efficiency. You can just microinject siRNA (targeting HP1) into NIH-3T3 cells and see what will happen. If the cells die, you should improve your system as the knockdown of HP1 is lethal.

If the knockdown of HP1 is lethal, I will use inducible expression system, such as "Tet-On" system to control the knockdown process. Grow up cells introduced with the RNAi cassette, and record the ultrastructure of chromatin. Add Dox into the cell media to activate the RNAi knockdown, and record the ultrastructure of chromatin again.

There are several ways to look at the chromatin structure.

i. Fluorescence in situ hybridization (FISH) [5]: use Alu fragment (widely spreads in the chromosome) or interested gene as probe (HP1 here, labeled with fluorescence); in situ hybridize with fixed cells after RNAi; detect using fluorescent microscopy.

ii. Immunogold staining and transmission electron microscopy[6]. Use gold particles labeled antibody to interact with chromatin in fixed cells, and TEM will show where gold particles are. From the gold particles' position, you will get information about the ultrastructure of chromatin.

iii. Constitutively express a GFP-tagged histone H2B in the cell. Make sure the expression of GFP-H2B fusion protein will not change the cell cycle parameters[7] and will not conflict with RNAi treatment. After RNAi, you can trace the fine nuclear chromatin structure by fluorescent microscopy in "live" cell.

iv. Microinjection of fluorescent nucleotides into mammalian cells followed by long chases of several cell cycles and random chromosome segregation produced cells containing a small number of labeled chromosome regions[8]. Through fluorescent microscopy, you can in vivo watch the dynamics of native chromosome domains.

[5] Lawrence JB, Singer RH, McNeil JA. *Science*, 1990, **249**:928-932
[6] Robinett CC, Straight A, Li G *etc*. *J Cell Bio*, 1996, **135**:1685-1700
[7] Kanda T, Sullivan KF, Wahl GM *etc*. *Currl Biol*, 1998, **8**:377-385
[8] Zink D, Cremer T, Saffrich R *etc*. *Hum Genet*, 1998, **102**:241-251