Genome Editing and Engineering
Prof. Utpal Bora
Department of Bioscience and Bioengineering
Indian Institute of Technology, Guwahati

Module - 06
Transcription activator-like effector nuclease (TALEN) Technology
Lecture - 03
Application of TALEN - Part A

Welcome to lecture 3 of module 6 on TALEN technology. Today, we are going to discuss about the applications of TALEN in diverse fields particularly with respect to diverse organisms.

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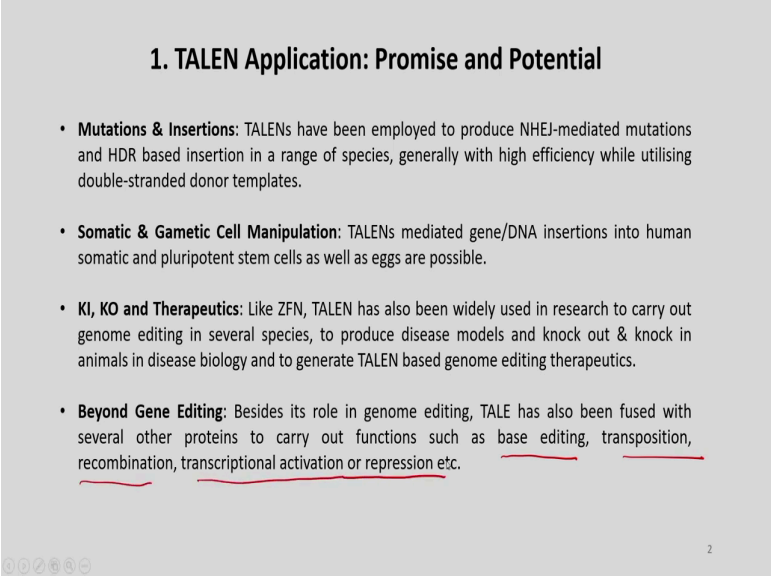

So, before we move into the various specific examples of TALEN applications, let us look into the promise and potential these technology holds overall.

We know about TALEN as a TALE based engineered nuclease which can cause double strand breaks and we can use the cell's internal repair methods triggered by these double strand breaks to insert DNA fragments as well as create point mutations.

So, one of the potential or promise of TALEN obviously, is creating mutations and genetic insertions and TALEN have been employed to produce non-homologous and joining

mediated mutations as well as homology directed repair based insertions in a range of species generally with high efficiency while utilizing the double stranded donor templates.

Somatic and gametic cell manipulation can also be carried out with TALENs. So, TALEN-mediated gene DNA insertions into human somatic pluripotent stem cells as well as embryos and eggs are possible. The other promise or potential is in the area of creating knock-ins, knockouts, as well as gene therapy.

So, like zinc finger nucleases, TALEN has also been used widely in research to carry out genome editing say in several species to produce disease models. And, knockout and knock-in animals in disease biology and to generate TALEN based genome editing therapeutics.

And even beyond gene editing we know TALEN can carry out a TALE based constructs can carry out many other functions when fused with functional domains such as for in base editing, then transposition, recombination, transcriptional activation or repression etcetera.

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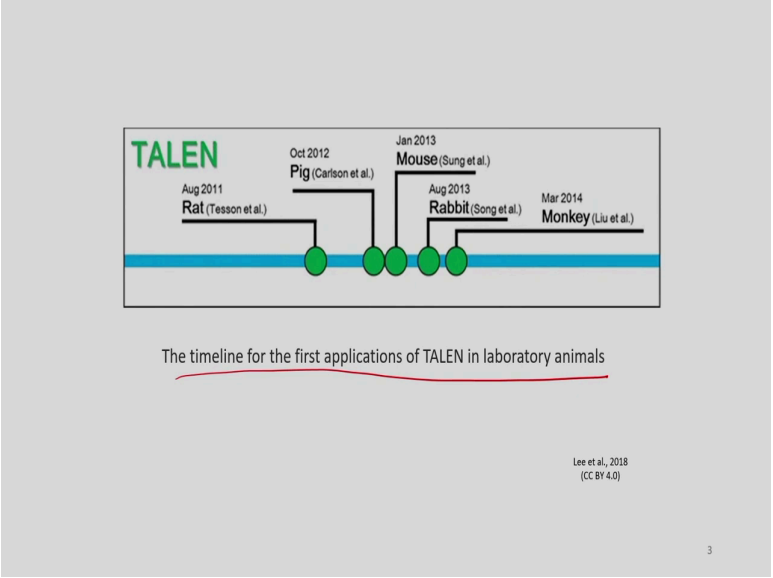

So, let us see the timeline for the first application of TALEN in laboratory animals. So, rat is the first animal in which a TALEN was applied in August 2011 by Tesson et al. Then the next year it was used by Carlson et al in 2012 in pigs directly and mouse by Sung et al. in 2013, then also rabbit by Song et al. the same year and in 2014 Liu et al. used these technology in monkeys.

So, this is in brief the timeline for the first applications of TALEN in some of the standard laboratory animals.

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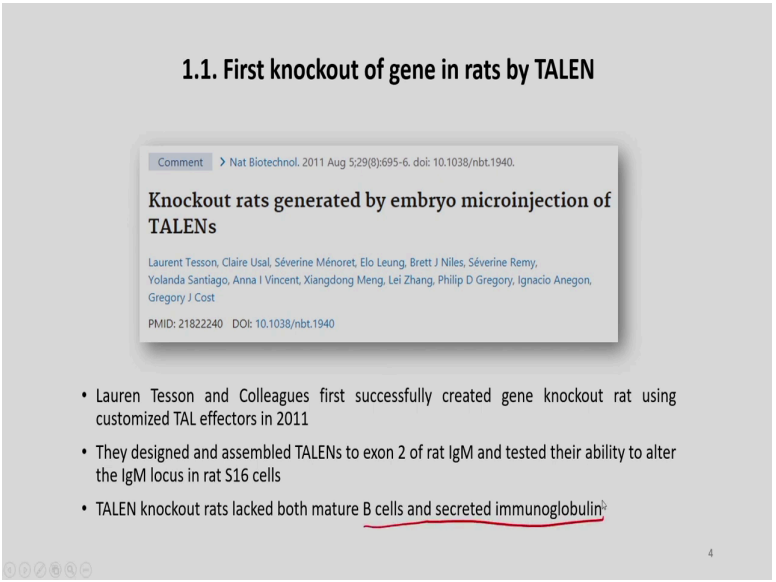

The first knockout of gene in rats was done by TALEN by Tesson et al. in 2011, as I already told you in the timeline discussion. So, Tesson and his colleagues first successfully created a gene knockout rat using these customized TAL effectors. They designed and assembled TALENs to exon 2 of rat IgM and tested their ability to alter the IgM locus in rat S16 cell lines.

TALEN knockout rats lacked both mature B cells and secreted immunoglobulin.

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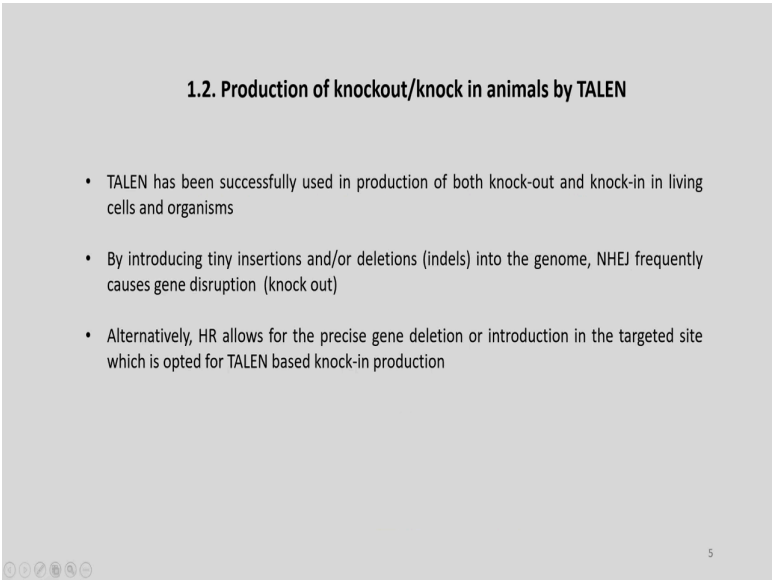

TALEN has been successfully used in production of both knock-out and knock-ins in living cells and organisms. By introducing tiny insertions and/or deletions indels into the genome. Non-homogeneous end joining frequently causes gene disruption which we call as knock-out.

Alternatively, homologous recombination allows for the precise gene deletion or introduction in the targeted site which is opted for TALEN based knock-in production.

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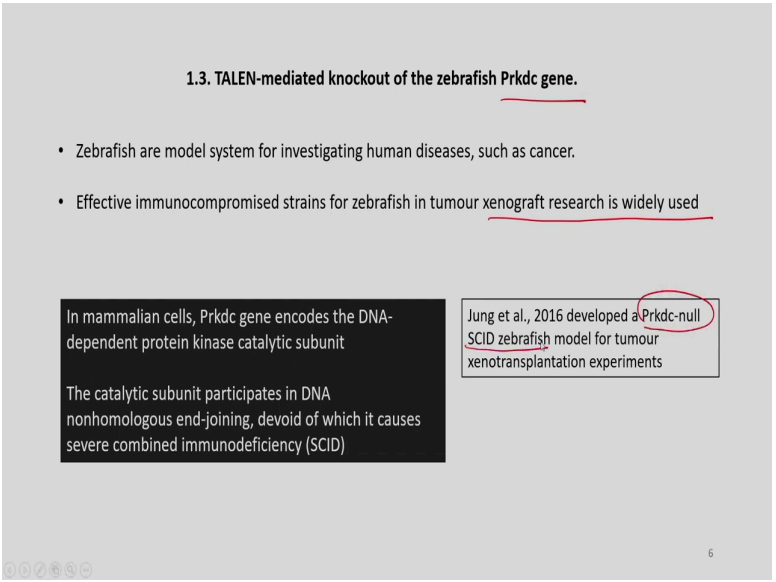

TALEN mediated knockout of Zebrafish Prkdc gene. Zebrafish are model system for investigating human diseases such as cancer. Effective immunocompromised strains of zebrafish in tumour xenograft research is widely used.

In mammalian cells Prkdc gene encodes the DNA dependent protein kinase catalytic subunit. The catalytic subunit participates in DNA non homologous end-joining, without which it causes severe combined immunodeficiency or SCID.

Jung et al., Developed a Prkdc-null or minus SCID zebrafish model for tumour xenotransplantation experiments.

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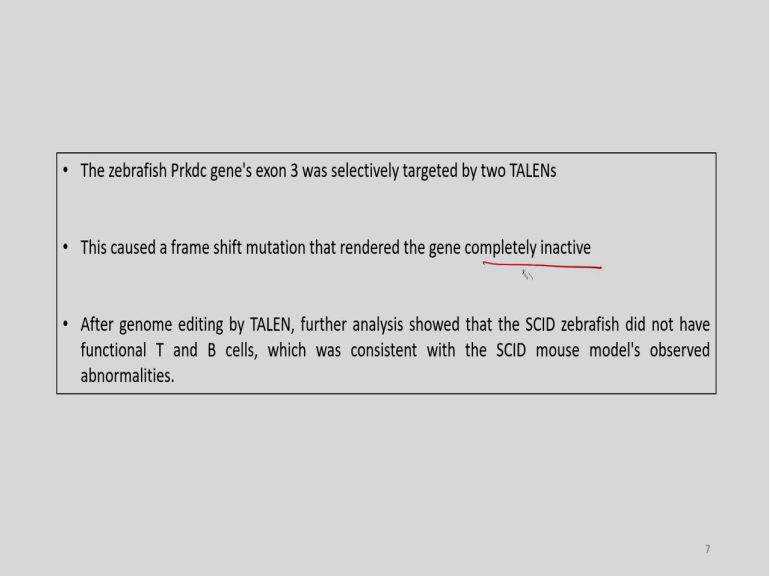

The zebrafish Prkdc genes exon 3 was selected to be targeted by 2 TALENs for this purpose and this causes a frame-shift mutation that rendered the gene a completely inactive. After genome editing by TALEN, further analysis showed that the SCID zebra fish did not have functional T and B cells which was consistent with the SCID mouse model's observed abnormalities.

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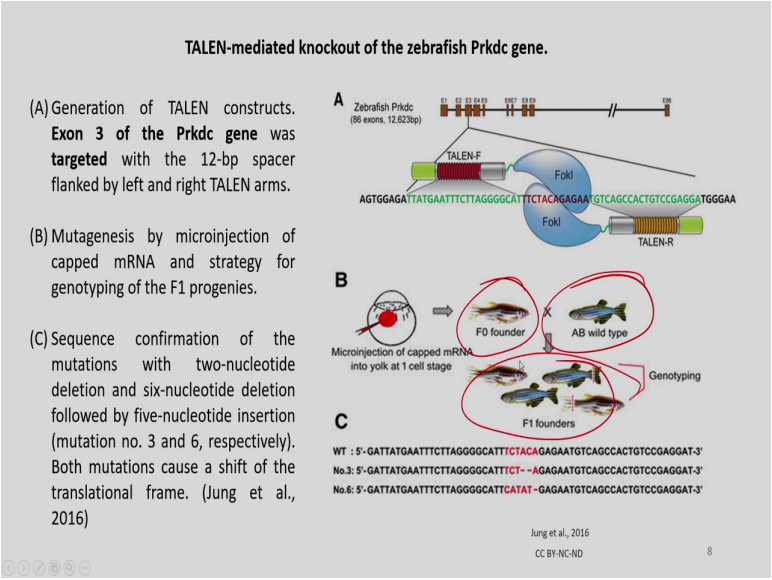

So, this is in details the experiment for TALEN mediated knockout of zebrafish Prkdc gene which has 86 exons and around 12,623 base pairs and you can see here the different exons and this is exon 3 to which these TALEN construct is targeted and as you can see we have TALEN-F and TALEN-R which are designed to bind across the DNA strands, so that the FokI domains come opposite to one another and dimerize and facilitate the cleaving of the DNA.

And, here in (B) this TALEN construct is delivered by micro injection of capped mRNA into yolk at 1-cell stage and then you have these F0 founders which are crossed with the wild types and the F1 founders are established as a result of this genotyping is done to confirm the successful crosses. So, step (A) you can see here the generation of TALEN constructs. Exon 3 of the Prkdc gene was targeted with the 12 base pair spacer flanked by left and right TALEN arms.

Mutagenesis by micro injection in step (B) of capped mRNA and strategy for the genotyping of the F1 progenies, as already told to you. And, in (C) you can see the sequence conformation of the mutations with two-nucleotide deletions here and six-nucleotide deletion followed by five-nucleotide insertion. Both mutations cause a shift of the translational frame.

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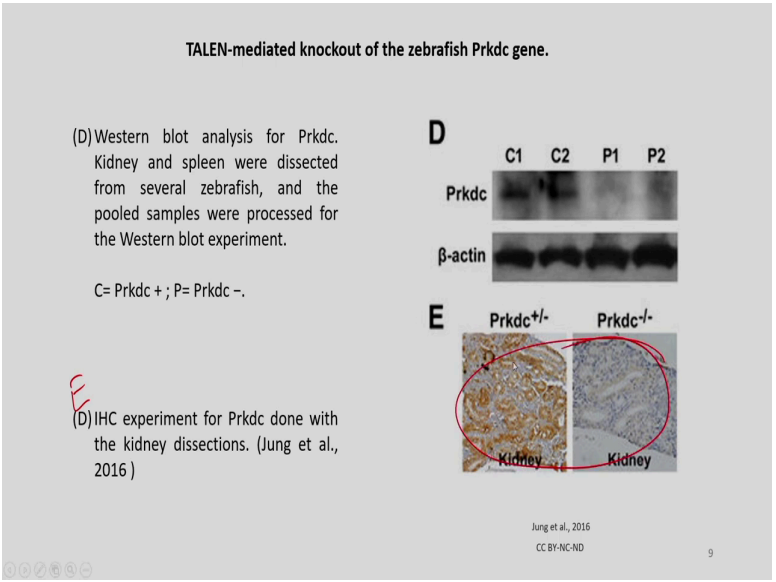

So, here you can see the Western blot analysis for Prkdc. Kidney and spleen were dissected from several zebrafish and the pooled samples were processed for the Western blotting experiment. In C you have the Prkdc positive. And, in P you have these Prkdc negative, these are not present over here. You can see here this is positive, but no any visible sign of Prkdc here in this Western blot analysis and beta-actin is used as a control positive control which is so that we can know that the experiment is going on correctly.

And, these experiments successfully showed the effect of the TALENs and here you have this is (E) Immuno-Histo Chemistry experiment for Prkdc which was done with the kidney dissections and the visual evidence supported the Western blot analysis as well.

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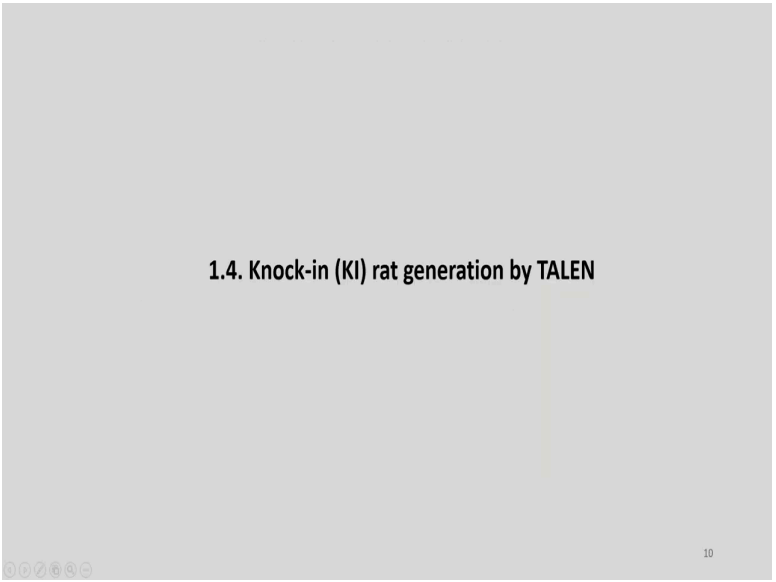

Let us also now look into other capabilities of TALENs and their applications particularly in knock-in rat generation where TALEN has been used.

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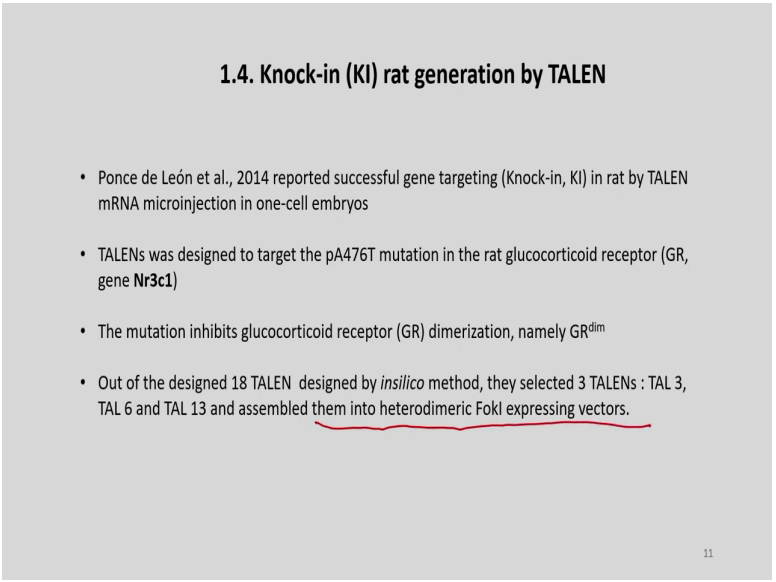

Leon et al., in 2014 reported successful gene targeting knock-in in rat by TALEN mRNA injection in one-cell embryos. TALEN was designed to target the pA476T mutation in the rat glucocorticoid receptor or GR gene namely as Nr3c1. The mutation inhibits glucocorticoid receptor dimerization namely GRdim. So, a mutation in this gene will inhibit the dimerization of glucocorticoid receptor.

Out of the designed 18 TALEN constructs by insilico method they selected 3 TALENs: TAL 3, 6 and 13 and assembled them physically into heterodimeric FokI expressing vectors.

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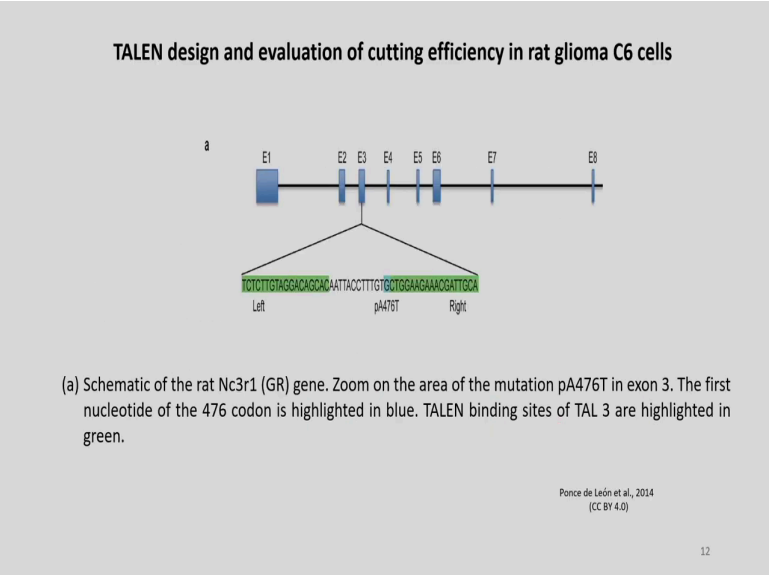

So, design TALEN design and evaluation of cutting efficiency in rat glioma C6 cells, this is the schematic of these rat Nc3r1 gene.

So, you can zoom into this area of mutation of pA476T in exon number 3. The first nucleotide of the 476 codon is highlighted in blue. The TALEN binding sites of the TAL 3 are highlighted in green in both the left side and the right side.

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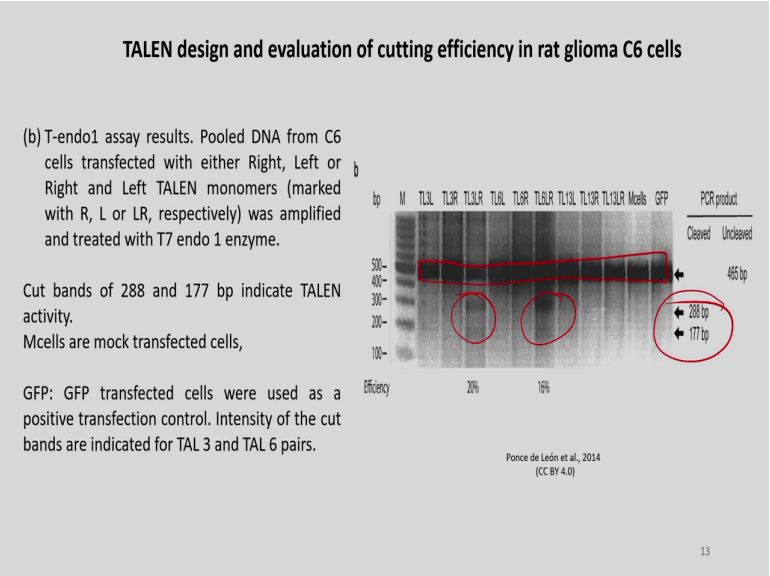

T-endo1 assay results showed that pool DNA from C6 cells transfected with either right, left or right and left TALEN monomers which are marked as R, L was amplified and treated with ah T7 endo 1 enzyme and those with left right are marked as error, ok.

So, you can see here 2 cut bands 288 and 177 base pairs, you can see some of them visible over here and this is the band for 465 base pairs which is being cleaved into 2 as shown in this figure.

The M cells are mock transfected cells and GFP are the GFP transfected cells which are used as positive. Transfection control intensity of the cut bands are indicated for TAL 3 and TAL 6 pair. So, here you this is around 20 percent and this is around 16 percent.

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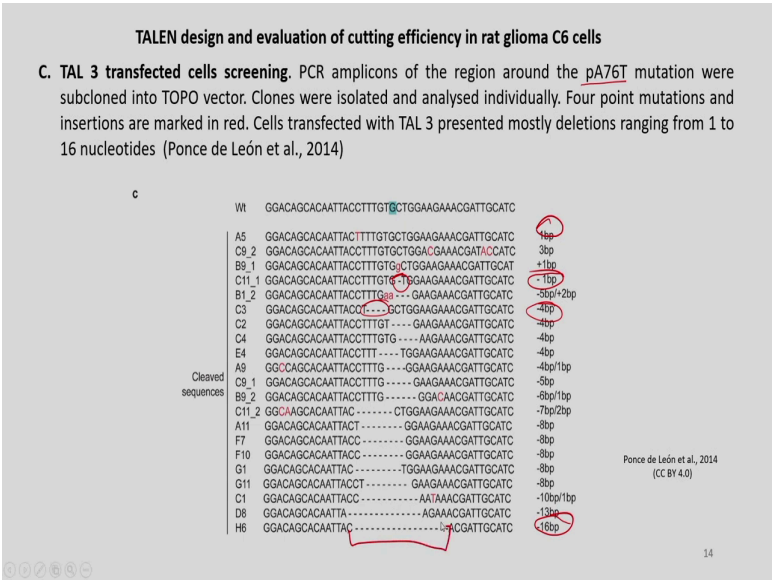

TAL 3 transfected cells were screened. So, here PCR amplicon of the region around the pA76T mutations were sub cloned into TOPO vector. Clones were isolated and analyzed individually. Four point mutations and insertions are marked in red. So, you can see here 1, 2, 3, 4 and so on.

Cells transfected with TAL 3 are presented mostly deletions ranging from 1 to 16 nucleotides. So, here you have 1 base pair, minus 1 base pair here, ok, this is the deletion and you have say here minus 4 base pairs and here you have larger ones minus 10, minus 13, and as big as 16 base pairs here.

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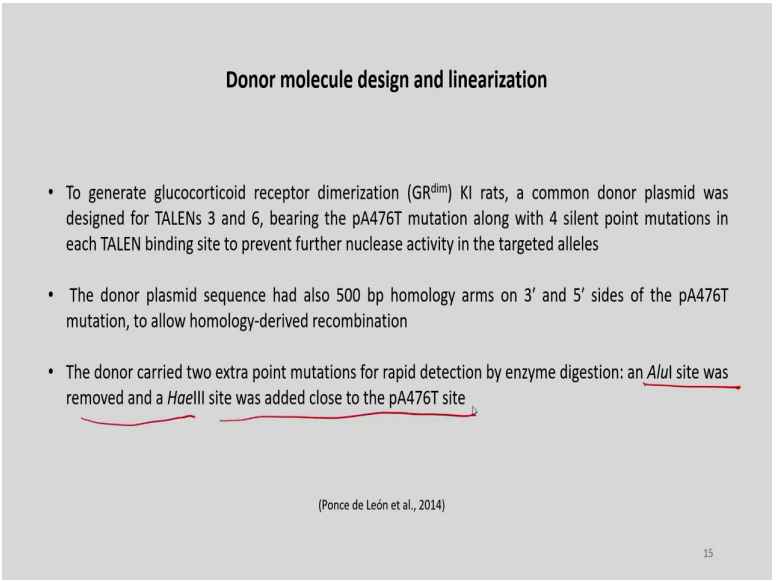

A donor molecule design and linearization. To generate glucocorticoid receptor dimerization GRdim knock-in rats a common donor plasmid was designed for TALENs 3 and 6 bearing the pA476T mutation along with 4 silent point mutations in each TALEN binding site to prevent further nuclease activity in the targeted alleles.

The donor plasmid sequence had also 500 base pair homology arms on 3 prime and 5 prime sites of the pA476T mutation to allow homology derived recombination. The donor carried 2 extra point mutations for rapid detection by enzyme digestion and an Alu site AluI site was removed and a HaeIII site was added close to the pA476T site.

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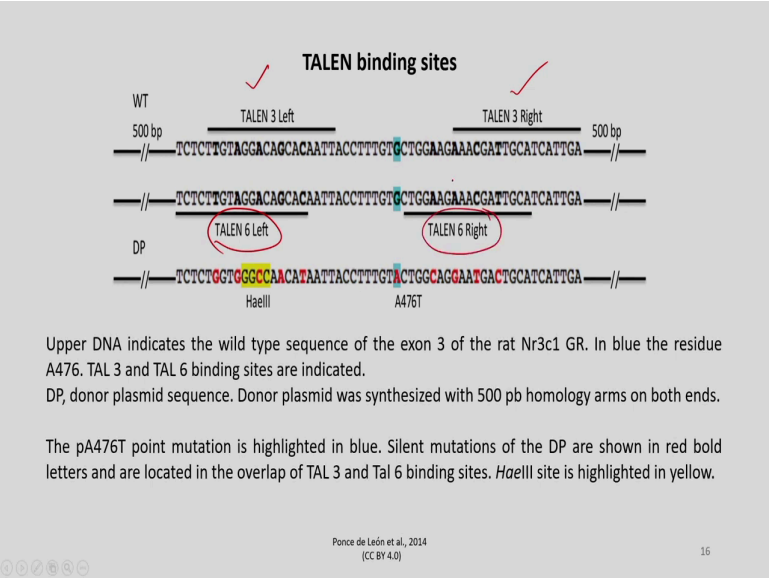

So, you can see here the wild type and this is the TALEN 3 left and this is the TALEN 3 right. And, this is these are the sites we were speaking about HaeIII, and this is TALEN 6 left and this is TALEN 6 right. So, the upper DNA indicates the wild type sequence of the exon 3 of the rat Nr3c1 GR.

In blue the residue A476 TAL 3 and TAL 6 binding sites are also indicated. DP is the donor plasmid sequence. Donor plasmid was synthesized with 500 base pair homology arms on both sides. The pA476T point mutation is highlighted in blue. Silent mutations of the DP are shown in red bold letters and are located in the overlap of TAL 3 and TAL 6 binding sites. HaeIII site is highlighted in yellow color.

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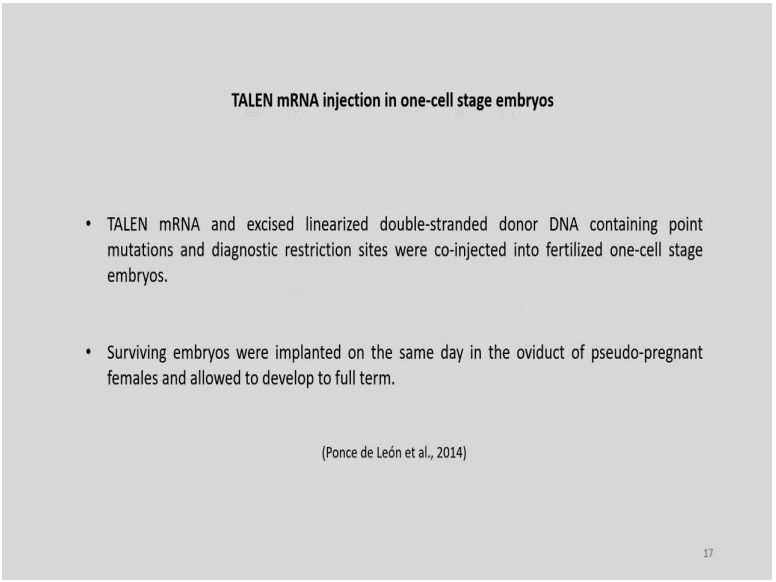

TALEN mRNA injection in one-cell stage embryo. TALEN mRNA and excised linearized double-stranded donor DNA containing point mutations and diagnostic restriction sites were co-injected into fertilized one-cell stage embryos.

Surviving embryos were implanted on the same day in the oviduct of pseudo-pregnant females and allowed to develop to full term.

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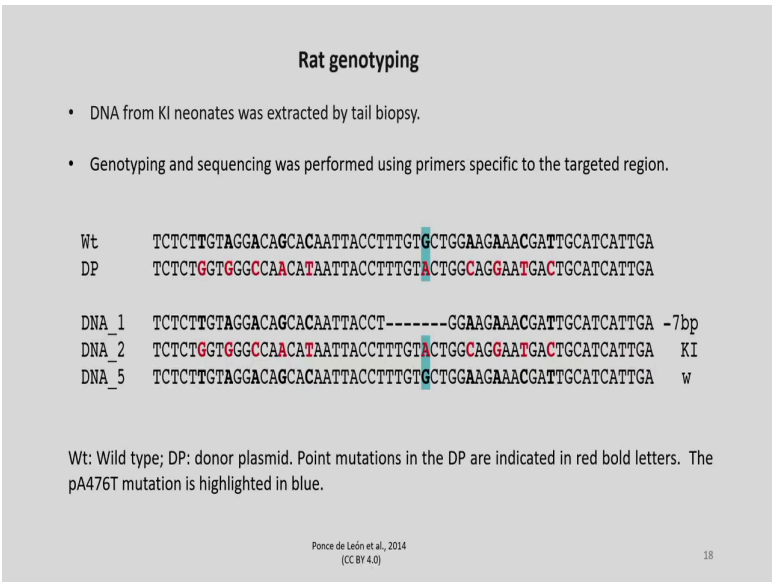

So, this is the rat genotyping. DNA from knock-in units was extracted by tail biopsy. Genotyping and sequencing was performed using primers specific to the targeted region. So, this is the wild type and donor plasmid DP.

Then you have the DNA 1, 2 and 5 point mutations in the DP are indicated in a red bold letters. The pA476T mutation is highlighted in blue.

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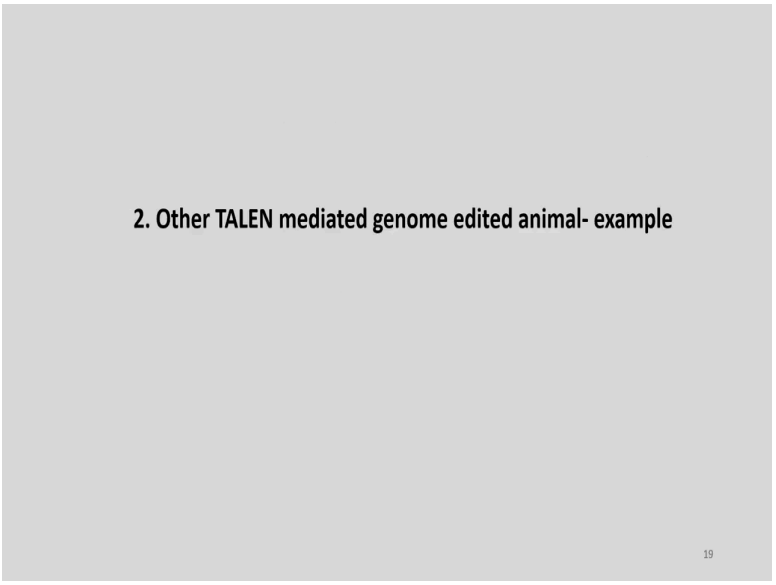

There are several other applications of TALEN for animal in animal editing.

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For example, there is a production of alpha 1, 3-galactosyltransferase targeted pigs using transcription activator-like effector nuclease-mediated genome editing technology. This paper was published by Kwang Wook.

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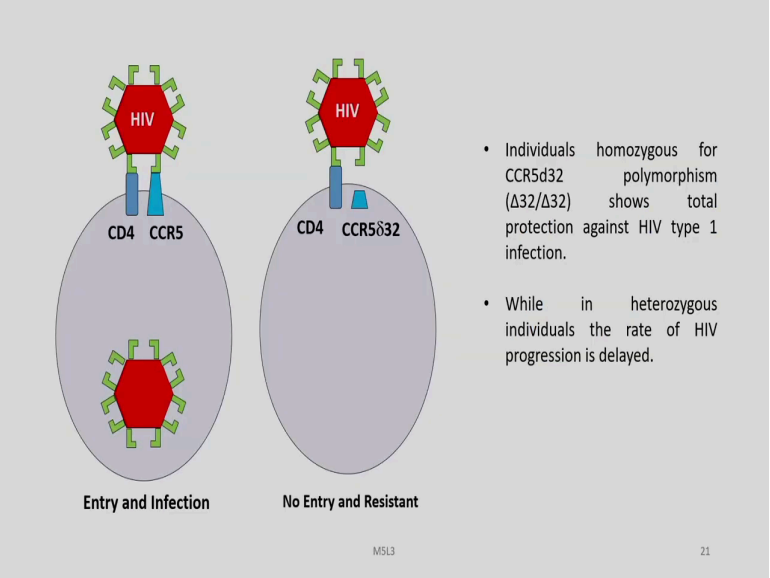

And, this is the discussion we had in the last module regarding the applications of zinc finger nucleases in genome editing HIV resistance cell lines. And, you are all aware about of the importance of CD4 as well as CCR5 co-receptor, and the HIV virus can proliferate or enter a cell only when both of them are functional.

If CD4 is functional, but there is a mutation in CCR5, most famously known as the CCR5 delta 32 mutation, the structure of the CCR5 receptor is changed. The exposed transmembrane domains are absent in such kinds of mutations. And, due to this the virus cannot enter cells and thereby this kind of cell population will become resistant to HIV.

And, we know that individuals homozygous for CCR5 delta 32 polymorphisms show total protections against HIV type 1 infection and those who are heterozygous will show delayed progression of HIV.

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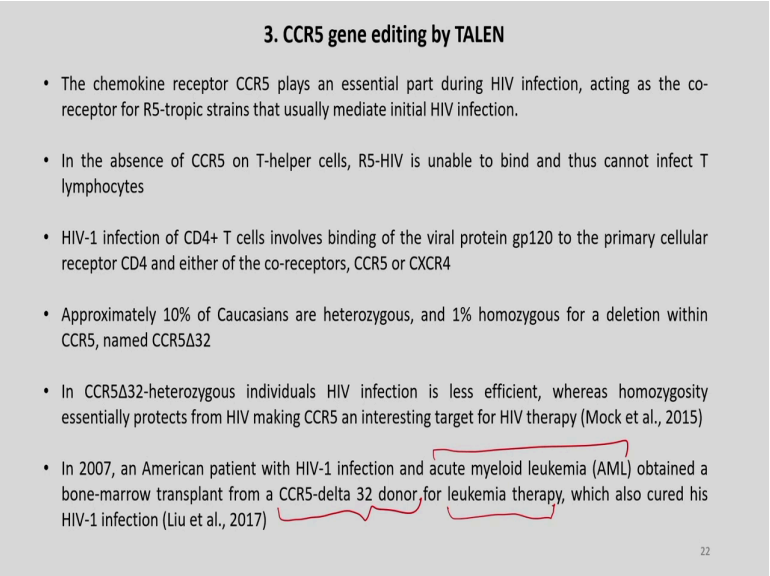

So, once again let us have a brief recap of the CCR5 gene in HIV pathogenesis. This is a chemokine receptor which plays an essential part during HIV infection and it act as the co-receptor of the R5-trophic strains that is usually mediate initial HIV infection. And, in the absence of CCR5 on T-helper cells R5-HIV is unable to bind and thus cannot infect T lymphocytes.

Approximately, 10 percent of Caucasians are heterozygous and 1 percent homozygous for a deletion within this particular gene named CCR5 delta 32. In 2007, an American patient with HIV infection and acute myeloid leukemia AML was given bone marrow transplantation from a CCR5-delta 32 donor for AML therapy leukemia therapy and this was a kind of an accidental finding.

Due to this bone marrow transplantation from a CCR5-delta 32 donor the patient who was HIV positive got cured of his HIV infection. So, this is an accidental finding and this has given us a clue about genome editing to modify the CCR5 receptor and thereby use this methodology as a HIV therapeutics approach.

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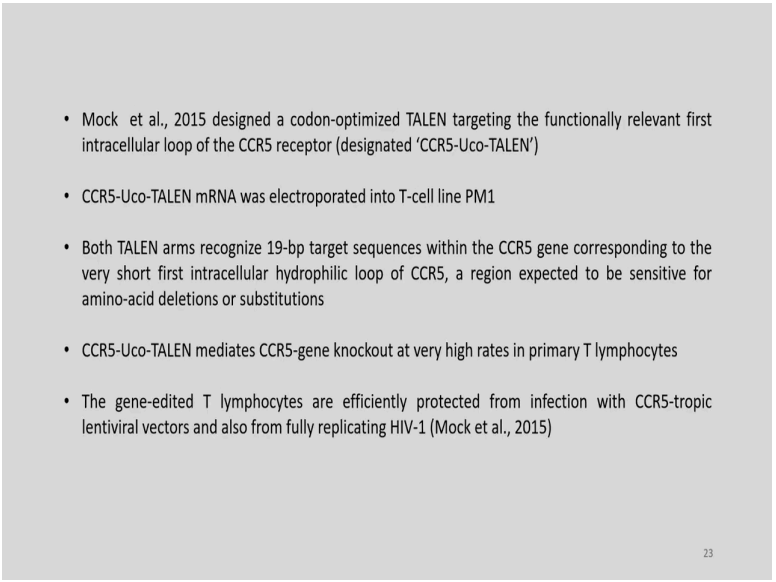

Mock et al., in 2015 designed a codon-optimized TALEN targeting the functionally relevant first intracellular loop of the CCR5 receptor. This is designated as CCR5-Uco-TALEN. CCR5-Uco-TALEN mRNA was sent into the T cells lines PM1 through electroporation.

Both TALEN arms recognized the 19-base pair target sequences within the CCR5 gene corresponding to the very short first intracellular hydrophilic loop of CCR5, a region expected to be sensitive for amino acid deletions or substitutions. CCR5-Uco-TALEN mediates CCR5-gene knockout at very high rates in primary T lymphocytes.

The gene-edited T lymphocytes are efficiently protected from infection with CCR5-tropic lentiviral vectors and also form fully replicating HIV-1 as reported by Mock et al., in 2015.

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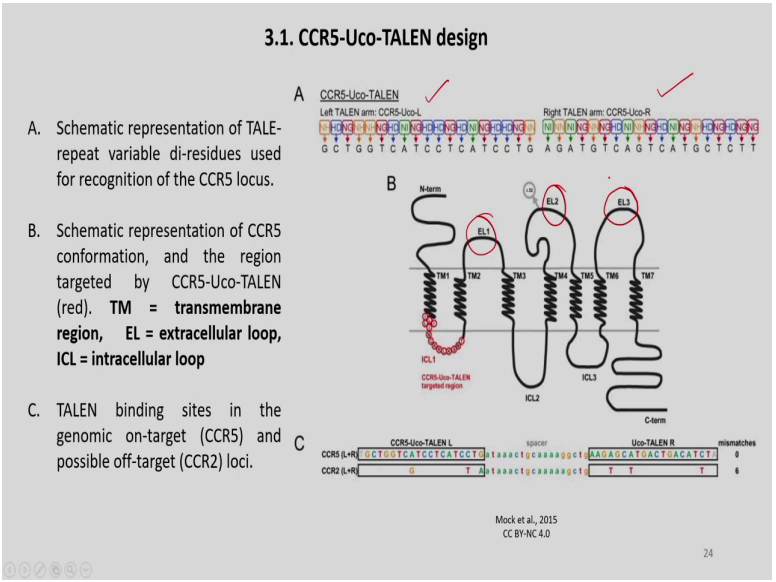

So, let us look into the construct of CCR5-Uco-TALEN design. So, if there is a left TALEN arm CCR5-Uco-L and a right TALEN arm CCR5-Uco-R. Here you can see the various transmembrane domains, total 7 in number, of the CCR5. You have these EL1, EL2 and EL3 and you can see here in EL2 the delta 32 mutation which occurs over there.

And, this is a schematic representation of the tale repeat variables di residues used for recognition of the CCR5 locus in (A) and (B) is the schematic representation of CCR5 conformation. And, the region targeted by CCR5-Uco-TALEN which is shown as red here; TM is trans membrane region, EL is extracellular loop and ICL is intracellular loop.

TALEN binding sites in the genomic on-target CCR5 and possible off target CCL loci is shown in figure number C.

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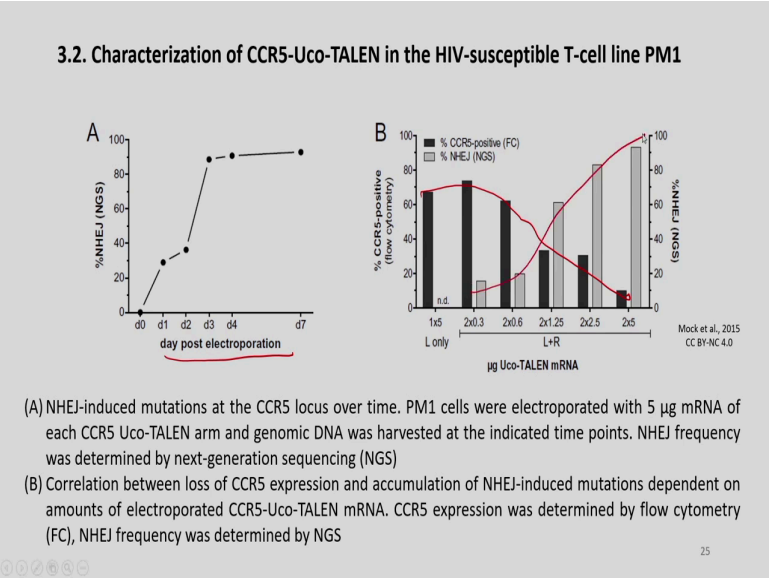

So, following these, they characterize CCR5-Uco-TALEN in the HIV susceptible T-cell line PM1. So, you can see the percent non-homologous end joining going up post electroporation day 0 nothing, day 1 you have around 20-30 percent, then slowly day 2 you have around roughly 38 percent, but then it jumps to over 80 percent in day 3 and day 4 and little bit higher in towards day 7.

So, non-homologous end joining induced mutations and the CCR5 locus over time can be seen in this figure A. PM1 cells were electroporated with 5 microgram mRNA of each CCR5-Uco-TALEN arm, and genomic DNA was harvested at the indicated time points. The NHEJ frequency was determined by next-generation sequencing.

And, in B you can see the correlation between loss of CCR5 expression and accumulation of NHEJ induced mutations which is dependent on the amounts of electroporated CCR5-Uco-TALEN mRNA. CCR5 expression was determined by flow cytometry, NHEJ frequency was determined by NGS.

So, as the positivity of CCR5 goes down you can see the percentage of non-homologous end joining is going up.

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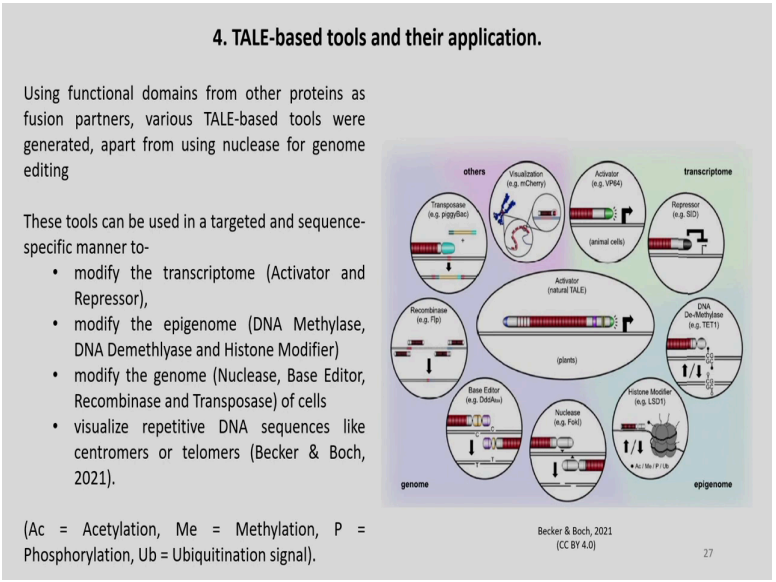

Let us now discussed about tale based tools and their applications in diverse fields. So, you can see here they are being used to make recombinases, transposes, and activation and repression, methylation as histone modifiers, base editors and well-known function of nucleases. So, we will discuss some of these.

So, using functional domains from other proteins as fusion partners various TALE-based tools can be generated apart from using nuclease for genome editing. These tools can be used in a targeted and sequence specific manner to modify the transcriptome, modify the epigenome, modify the genome. And visualize repetitive DNA sequences like centromeres or telomeres.

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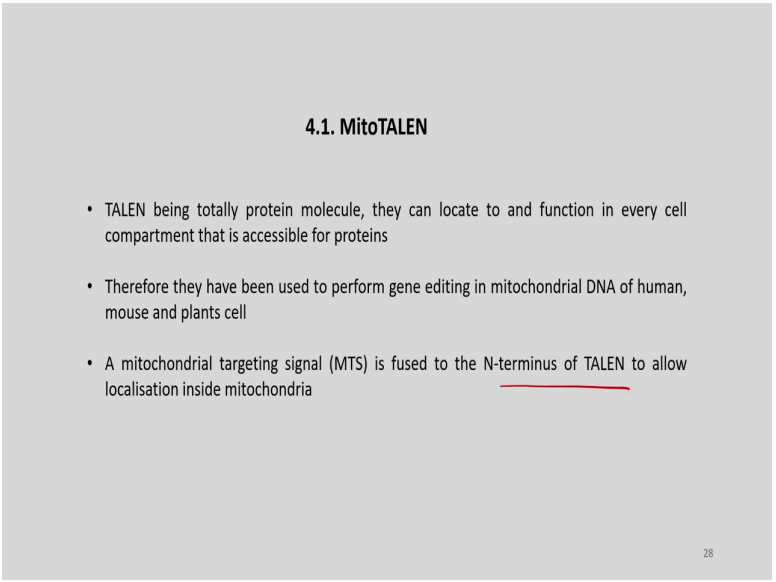

MitoTALEN – Talen being totally a protein molecule, they can locate to and function in every cell compartment which is accessible for proteins. Therefore, TALENs have been used to perform gene editing in mitochondrial DNA of human, mouse and plant cells.

A mitochondrial targeting signal (MTS) is fused to the N-terminus of TALEN to allow localization inside mitochondria.

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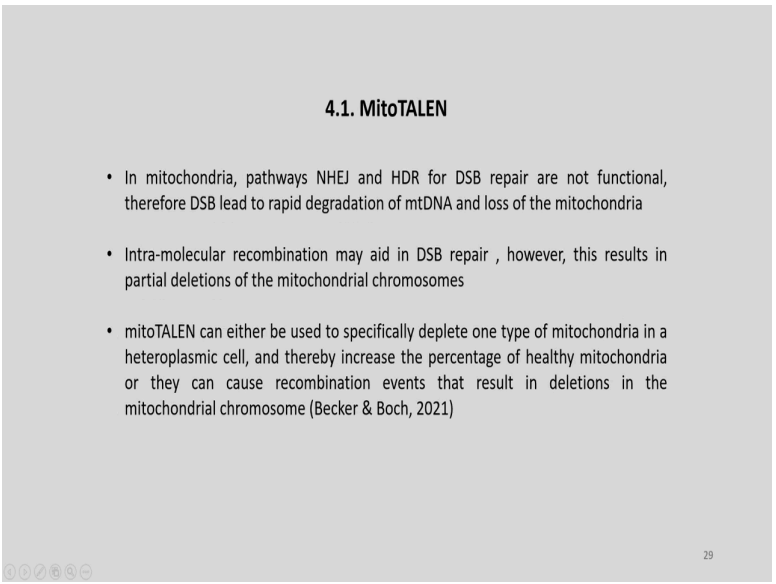

In mitochondria, pathways NHEJ and HDR for DSB repair are not functional, therefore, DSB lead to rapid degradation of mitochondrial DNA and loss of the mitochondria.

Intra-molecular recombination may aid in DSB repair, however, this results in partial deletions of the mitochondrial chromosomes. MitoTALEN can either be used to specifically deplete one type of mitochondria in a heteroplasmic cell, and thereby increase the percentage of healthy mitochondria or they can cause recombination events that results in deletions in the mitochondrial chromosomes.

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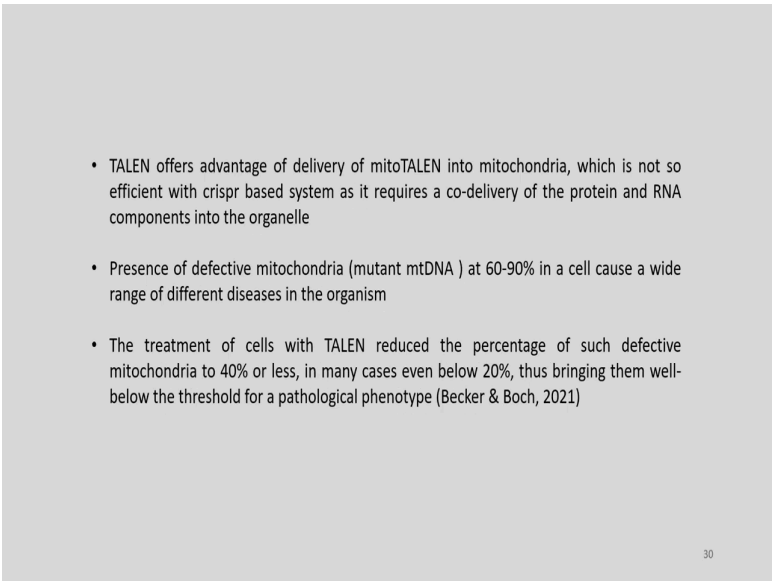

TALEN offers advantages of delivery of mitoTALEN into mitochondria which is not so efficient with CRISPR based system as it requires a co-delivery of the protein and the RNA components into the organelle.

Presence of defective mitochondria, mutant mitochondria at 60 to 90 percent in a cell causes a wide range of different disease in the organisms. The treatment of cells with TALEN reduce the percentage of such defective mitochondria to 40 percent or less, in many cases even below 20 percent, thus bringing them well below threshold for a pathological phenotype.

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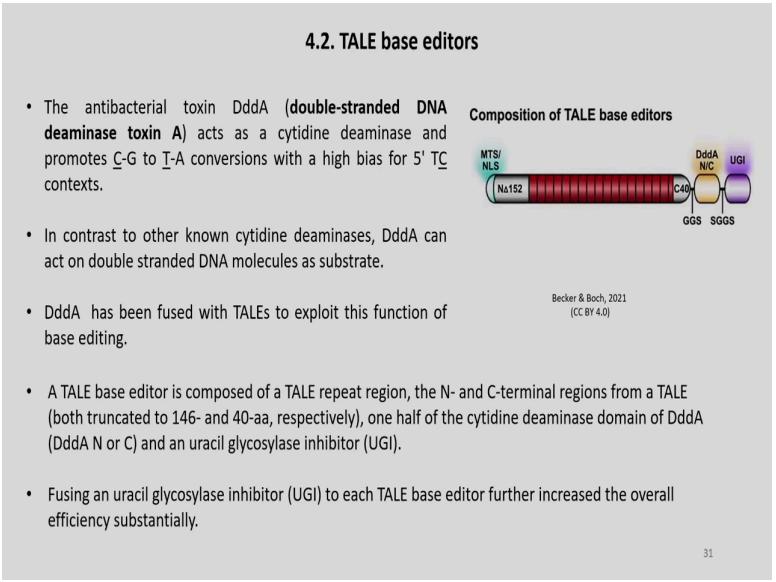

So, these are some of the TALE based editors. The antibacterial toxin DddA which stands for double-stranded DNA deaminase toxin A acts as a cytidine deaminase and promotes C-G to T-A conversions with a high bias for 5 prime TC context.

In contrast to other known cytidine deaminases, double-stranded DNA deaminase toxin can act on double-stranded DNA molecules as substrate. DddA has been fused with TALEs, ok. So, DddA is here and it has been fused with TALEs to exploit the function of base editing.

A TALE-based editor is composed of a TALE repeat region, N and C-terminal regions from a TALE both truncated to 146 and 40 amino acids respectively, one half of the cytidine deaminase domain of DddA N or C terminus and a uracil glycosylase inhibitor UGI.

So, this is the construct basically part of UGI part of DddA and these TALE constructs. Fusing an uracil glycosylase inhibitor to each TALE base editor further increase the overall efficiency substantially.

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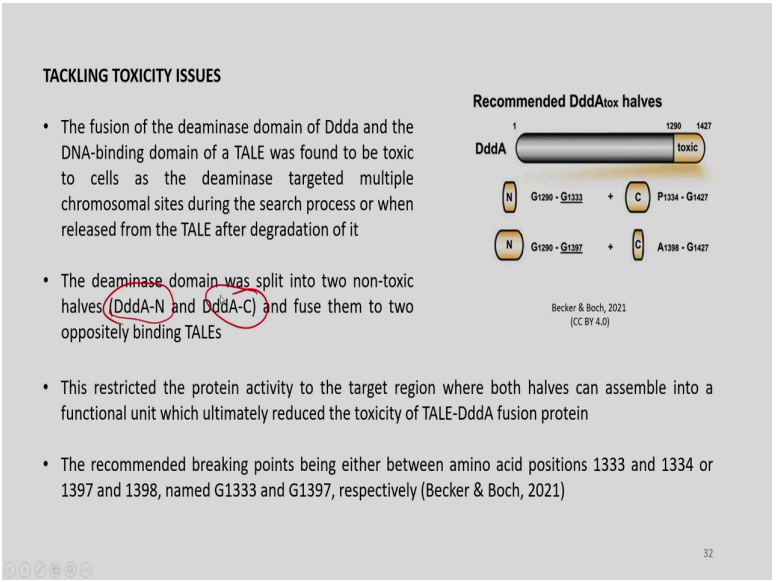

Now, there are issues of toxicity associated with this entire approach. So, those has to be tackled the fusion of the deaminase domain of triple DddA and the DNA binding domain of TALE was found to be toxic to cells as the deaminase targeted multiple chromosomal sites during the search process or when released from the tale after degradation.

The deaminase domain was split into 2 non-toxic halves DddA-N and DddA-C and fused them to form to oppositely binding TALEs. This restricted the protein activity to the targeted region where both halves can assemble into a functional unit which ultimately reduced the toxicity of the TALE-DddA fusion protein. The recommended breaking points being either between amino acids portions 1333 and 1334 or 1397 and 1398, named G1333 and G1397 respectively.

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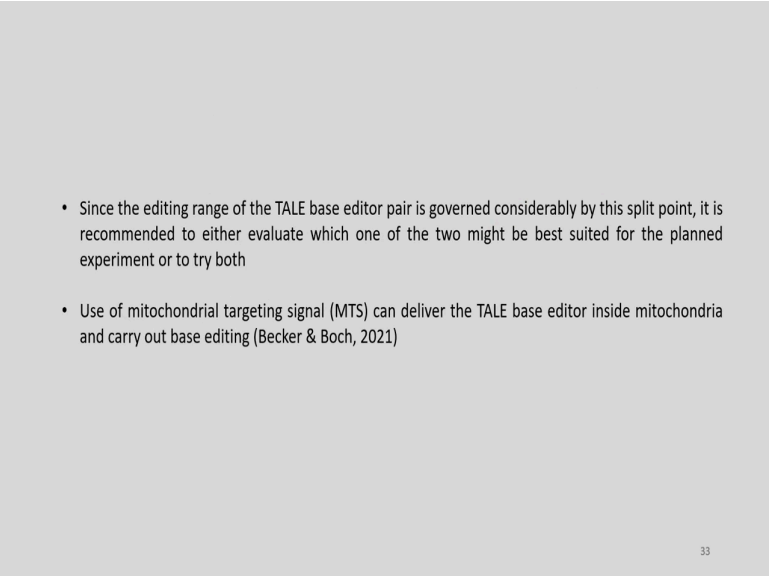

Since the editing range of the TALE based editor pair is governed considerably by this split point, it is recommended to either evaluate which one of the 2 might be best suited for the planned experiment or to use both. Use of a mitochondrial targeting signal can deliver the TALE-based editor inside mitochondria as well and carry out base editing there.

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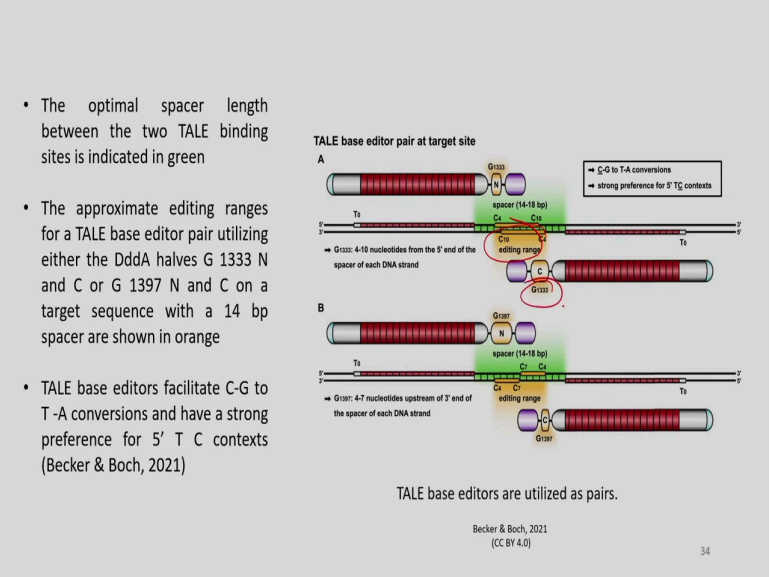

The optimal spacer length between the 2 TALE binding site is indicated in green. You can see here around 14 to 18 base pairs.

The approximate editing range for a TALE based editor pair utilizing either the DddA halves G1333 N or C or G1397 N and C on the target sequence with 14 base pair spacers are shown in orange. This is the editing range and this is G1333 and this is G1397. TALE base editors facilitate C-G to T-A conversions and have a strong preference for 5 prime T-C context.

So, with this we come to end of this part. We will continue this lecture in the next part.

Thank you.