Mechanism of Cas9 Ribonucleoprotein Uptake in Neural Progenitor Cells

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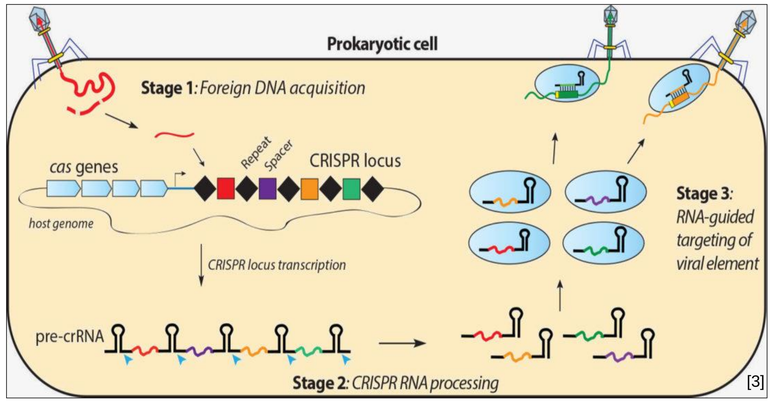
Abstract – The Cas9 protein, associated with the CRISPR system from bacteria and archaea, is a powerful tool that can be programmed to make precise cuts and edits in DNA[1]. Combining the Cas9 with a guide RNA forms the Cas9 ribonucleoprotein (RNP) complex. The native Cas9 RNP has no cell-penetrating activity when delivered onto cells; however, engineered RNPs have been reported to increase editing activity, indicating an increase in cell penetration[2]. The next question is how the engineered Cas9 RNP is entering the cells. Preliminary data points toward an endocytic mechanism of entry. By using small molecule inhibitors and endosome antibody markers that target various stages of endocytosis, we're able to determine the exact pathway by which the RNP enters and moves through the neural progenitor cells (NPCs). These studies will lead to better engineering of the RNP for increased uptake and editing efficiency. Ultimately, this will get the scientific and medical communities closer to developing a successful CRISPR-Cas9 human therapeutic to correct or inactivate genes that cause genetic disorders.

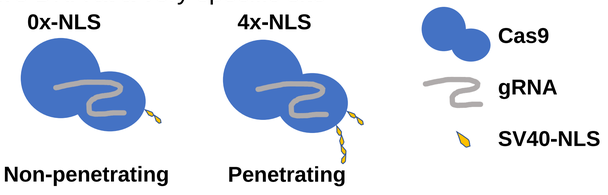
CRISPR



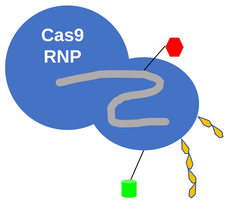
- Adaptive immune system in bacteria and archaea
- CRISPR locus contains previously encountered viral DNA and is then transcribed into RNA
- RNA complexes with CRISPR associated (Cas) proteins that recognize foreign DNA and proceed to cleave and degrade that DNA preventing infection

Engineering the Cas9 RNP

- Monogenic diseases have no cures nor treatments that target the underlying genetic cause
- The CRISPR-Cas9 system poses as a hopeful tool to treat such diseases and can be programmed with a single guide RNA to cleave DNA at a very specific site[1]

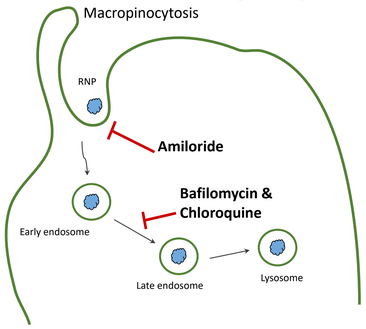


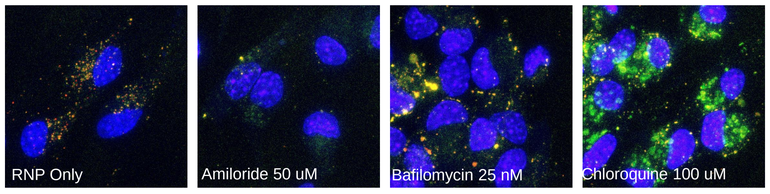
- Cas9 RNP has been engineered with Simian vacuolating virus 40 nuclear localization sequence (SV40-NLS) to improve its cell and nuclear penetrating abilities[2]
- It was specifically found that the 4x-NLS-Cas9-2x-NLS (4x RNP) had the highest editing efficiency[2]
- We tagged the Cas9 protein with green fluorescent protein (GFP) and the guide RNA with Atta 550, a red fluorescent dye
- These fluorescent tags allowed us to visualize our experiments using confocal fluorescence microscopy



Small Molecule Inhibitors

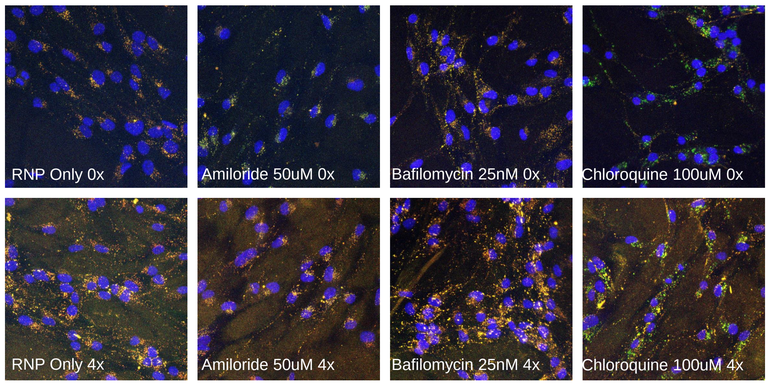
- Preliminary data using small molecule inhibitors pointed toward the RNP having an endocytic mechanism of entry
- **Amiloride** is known to be an inhibitor of macropinocytosis
- High enough doses of Amiloride show almost no RNP getting into the cells, compared to the control
- **Chloroquine** and **Bafilomycin** both inhibit endosome acidification, leading us to expect to see more RNP build up as it does not get degraded by acidification
- More distinct, brighter vesicles form in the presence of Bafilomycin and Chloroquine, compared to the control





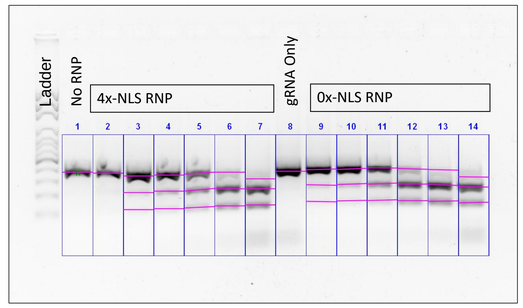
Blue = Nuclei stained with DAPI
Green = Green fluorescent protein tagged to Cas9
Red = Atto 550 (Red dye) tagged to guide RNA
Yellow = Cas9 RNP (co-localization of red and green)

4x-NLS vs. 0x-NLS RNP



- Qualitative analysis of confocal fluorescence microscopy images show 4x-NLS RNP is much brighter confirming more RNP gets into cells with the 4x-NLS
- Small molecule inhibitors affect 4x- and 0x-NLS RNP in the same way indicating they take the same pathway into the cells

In Vitro Cleavage Assay



Molar Ratios of RNP:DNA
Columns 2,9 – 0.125:1
Columns 3,10 – 0.25:1
Columns 4,11 – 0.5:1
Columns 5,12 – 1:1
Columns 6,13 – 2:1
Columns 7,14 – 4:1

- Combining DNA with 0x and 4x RNP in vitro at different molar ratios allows us to compare the RNPs outside of the cell
- By gel-electrophoresis, cleaved DNA is separated
- Helps us determine the amount of active RNP
- Assay shows 0x and 4x RNP have similar amounts of active RNP
- Allows us to be sure that reduced amount of 0x RNP seen in cells is solely dependent on cell penetrating ability

Future Work

- We began work with antibody markers against endosomes: EEA-1, against early endosomes and LAMP-2, against late endosomes/lysosomes
- Thus far, we've been unable to determine optimal conditions with these antibodies to provide us with any conclusive evidence
- Continued work with these would allow us to confirm an endocytic pathway and to track the RNP through that pathway by imaging and determining percentage of co-localization
- The work done to elucidate RNP mechanism of entry will lead to better engineered RNP with increased editing efficiency
- Ultimately, we will be able to shape the RNP into a effective therapeutic to treat genetic diseases

References

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[3] "CRISPR Systems." Doudna Lab, http://doudnalab.org/research_areas/crispr-systems/

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