Investigation of Orc6- and ORCA-interacting Proteins

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Abstract

The Origin Recognition Complex (ORC) is a 6-subunit complex which binds to the origin of replication in all eukaryotes. When ORC is bound at origins of replication, it recruits the pre-replication complex to initiate DNA replication. The smallest subunit of ORC, Orc6, is essential in DNA replication and mitosis. Evidence from previous experiments shows that Orc6 is also involved in the DNA damage response. Additionally, Origin Recognition Complex-Associated (ORCA) is a pre-replication complex protein which localizes to regions of heterochromatin and stabilizes ORC. The aim of this study was to further define the role of Orc6 and ORCA by identifying unknown proteins which interact with them. With the goal of identifying these interacting proteins, we utilized a newly developed approach called BioID. The BioID method involves the expression of fusion proteins containing the BirA biotin ligase. We therefore stably expressed the BirA-HA-Orc6, BirA-HA-ORCA, or BirA-HA-EYFP fusion protein in mammalian cells and potential Orc6- and ORCA-interacting proteins were labeled upon subsequent addition of biotin to the cells. Finally, we performed a pull-down of biotinylated proteins which will be analyzed by mass spectrometry and we will further validate novel interacting proteins that may be identified by this method.

Experimental Approach

- Cloning of BirA-HA-ORC6, BirA-HA-ORCA, and BirA-HA-EYFP plasmids
- Stable transfection of BirA-HA-Orc6, BirA-HA-ORCA, and BirA-HA-EYFP
- Validation of biotinylation of proteins in the BirA-HA-Orc6, BirA-HA-ORCA, and BirA-HA-EYFP stable cell lines

Results

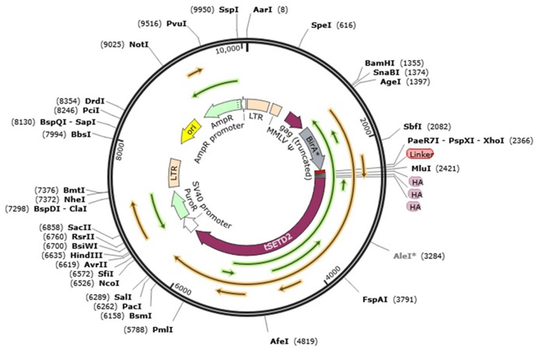

Figure 1. BirA-SETD2 plasmid map (10,061 bp): The BirA-HA-Orc6, BirA-HA-ORCA, and BirA-HA-EYFP plasmids were cloned using the backbone of the BirA-SETD2 plasmid. As shown here, the BirA-SETD2 plasmid contains the sequence for the BirA biotin ligase as well as the puro^R gene, which confers resistance to the drug puromycin. The SETD2 gene was replaced with the gene of interest (ORC6, ORCA, or EYFP) along with an HA tag sequence during the cloning procedures.

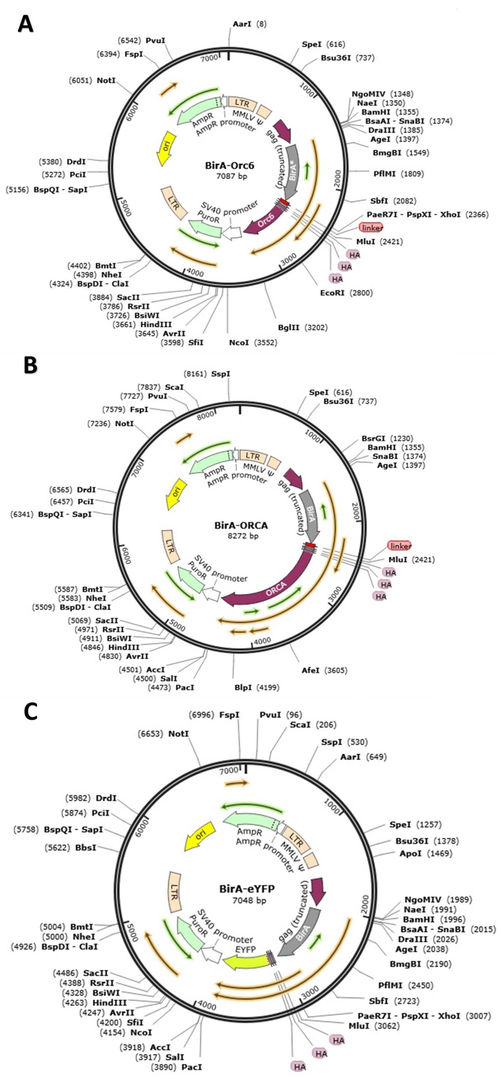

Figure 2. Maps of cloned plasmids: (A) BirA-HA-Orc6 plasmid cloning product, (B) BirA-HA-ORCA plasmid cloning product, (C) BirA-HA-EYFP plasmid cloning product. Note that the genes of interest were inserted adjacent to the BirA sequence along with the HA tag sequence.

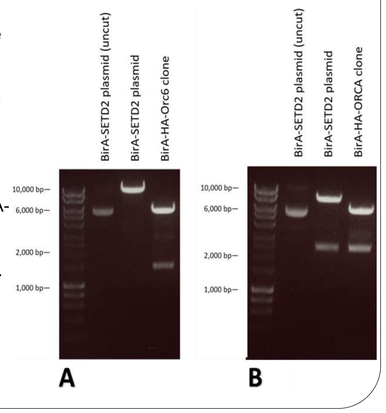

Figure 3. Validation of the BirA-HA-Orc6 and BirA-HA-ORCA plasmid constructs: (A) Restriction digestion of BirA-HA-Orc6 plasmid cloning product using EcoRI and BamHI restriction enzymes. (B) Restriction digestion of BirA-HA-ORCA plasmid cloning product using NotI and BamHI restriction enzymes. These figures are courtesy of Yo-Chuen Lin, graduate student in the Supriya Prasanth laboratory.

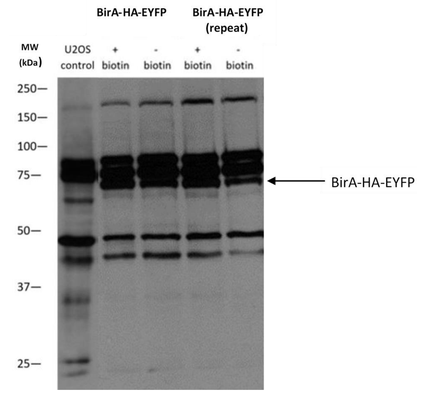

Figure 4. Validation of stable transfection of BirA-HA-EYFP: A Western blot using the HA tag primary antibody was performed on cell lysate obtained from the BirA-HA-EYFP stable cell line. This experiment yielded the data shown here, and the arrow indicates the location of bands corresponding to the molecular weight of BirA-HA-EYFP. Bands associated with this MW are found in the BirA-HA-EYFP stable cell line but not in the U2OS control sample.

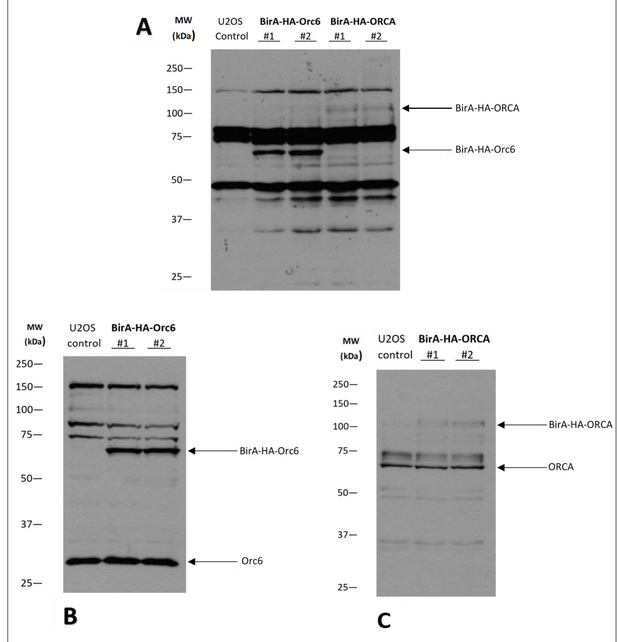

Figure 5. Validation of stable transfection of BirA-HA-Or6 and BirA-HA-ORCA: (A) Data from Western blot experiment performed on cell lysates from BirA-HA-Orc6 and BirA-HA-ORCA stable cell lines using the HA tag primary antibody. The arrows indicate locations of bands corresponding to BirA-HA-Orc6 or BirA-HA-ORCA. (B) Data from Western blot experiment performed on cell lysate from the BirA-HA-Orc6 stable cell line using the Orc6 primary antibody. The arrows indicate locations of bands corresponding to BirA-HA-Orc6 or endogenous Orc6. (C) Data from Western blot experiment performed on cell lysate from the BirA-HA-ORCA stable cell line using the ORCA primary antibody. The arrows indicate locations of bands corresponding to BirA-HA-ORCA or endogenous ORCA. Note that the "#1" and "#2" indicate two separate samples from the same stable cell line. These figures are courtesy of Yo-Chuen Lin, graduate student in the Supriya Prasanth laboratory.

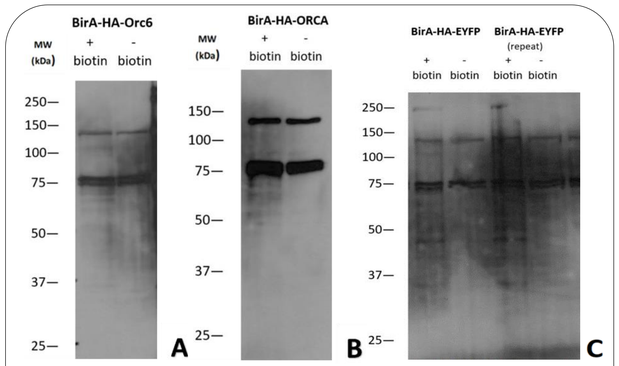

Figure 6. Validation of biotinylation in BirA-HA-Orc6, BirA-HA-ORCA, and BirA-HA-EYFP stable cell lines: (A) Immunoblot of cell lysate from the BirA-HA-Orc6 stable cell line using streptavidin-HRP. (B) Immunoblot of cell lysate from the BirA-HA-ORCA stable cell line using streptavidin-HRP. (C) Immunoblot of cell lysate from the BirA-HA-EYFP stable cell line using streptavidin-HRP.

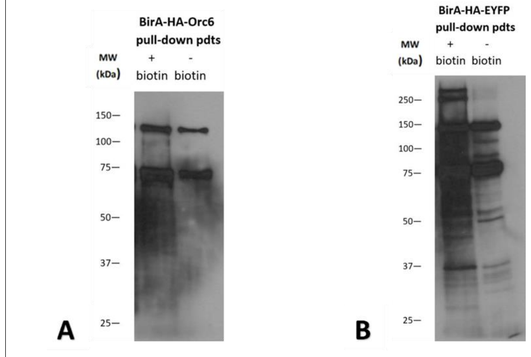

Figure 7. Validation of biotinylation in pull-down products: (A) Immunoblot of pull-down products which originated from the BirA-HA-Orc6 stable cell line using streptavidin-HRP. (B) Immunoblot of pull-down products which originated from the BirA-HA-EYFP stable cell line using streptavidin-HRP.

Conclusions

- We successfully cloned three separate plasmids containing the BirA-HA-Orc6, BirA-HA-ORCA, and BirA-HA-EYFP fusion genes, respectively.
- We successfully established the stable expression of each of our BioID fusion proteins.
- We validated that the BioID fusion proteins could label proximal proteins with biotin.
- *Future Directions:* We will further analyze candidate proteins that are identified by mass spectrometry analysis of pull-down products originating from the BirA-HA-Orc6 or BirA-HA-ORCA stable lines. This will be accomplished by first confirming the presence of candidate proteins in BirA-HA-Orc6 or BirA-HA-ORCA pull-down products.

Acknowledgments

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