However, despite MLKL’s importance in mediating necrosis, the downstream targets of MLKL remain unknown.

**STUDY OVERVIEW & OBJECTIVES**

- Transfect HEK-293 cells with GFP (control) and MLKL-Myc-FLAG plasmids
- Immunoprecipitate the MLKL-protein complexes using an anti-FLAG antibody
- Identify the proteins in the MLKL complexes using LC-MS/MS
- Verify the MLKL-protein interactions by co-immunoprecipitation assays
- Knockdown the candidate proteins in MEFs using siRNA and test the effects on TNFα-induced necrosis

**RESULTS**

**Overexpression of MLKL in 293 Cells**

- GFP
- MLKL-FLAG-Myc
- FLAG
- Myc
- GAPDH

**Immunoprecipitation of MLKL**

- FLAG IP:FLAG IB
- GFP
- MLKL-Myc-FLAG
- FLAG

**Proteomic Identification of MLKL-Binding Proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>GFP Count</th>
<th>MLKL Count</th>
<th>Subcellular Localization</th>
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<tbody>
<tr>
<td>EFhd2</td>
<td>0.7</td>
<td>2.7</td>
<td>Plasma Membrane (Lipid raft)</td>
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<tr>
<td>AnxA2</td>
<td>2.2</td>
<td>7.8</td>
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<td>CD59</td>
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<tr>
<td>RIP1</td>
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<td>PARP1</td>
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<tr>
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<tr>
<td>Tropomysin-3β</td>
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<tr>
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<tr>
<td>Calmodulin</td>
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</tr>
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</table>

**Candidate MLKL Binding Proteins**

**Effects of EFhd2 and Annexin-A2 siRNA on TNFα-Induced Necrosis**

- COsi
- EFhd2si
- AnxA2si

**CONCLUSIONS**

- We successfully identified several novel MLKL binding proteins.
- The lipid raft proteins EFhd2 and Annexin-2 interacted with MLKL.
- EFhd2, but not Annexin-A2, appears to be a negative regulator of TNFα-induced necrosis.
- Future studies will evaluate the other MLKL-binding proteins and their potential role in TNFα-induced necrosis.

**ACKNOWLEDGEMENTS**

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