Confirming Gene Mutation by CRISPR-Cas9 at the Protein Level and Identifying Proteome-Wide Changes

Using SWATH® Acquisition on TripleTOF® Systems

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Genome editing tools are invaluable to life science researchers, allowing changes to be made to an organism's DNA and then studying the effects to gain biological insights. The most recent gene editing tool is CRISPR-Cas9 which has rapidly gained adoption as it is faster, simpler and cheaper than previous methods. To ensure fidelity of biological conclusions, it is important to confirm the success of any gene editing at the protein level. Immunoblotting is typically used; however, this strategy can be limited by the availability and quality of antibodies.

Here, a label-free mass spectrometry approach has been applied both to confirm protein level changes induced by gene editing and to measure the proteome wide changes that occur in response to the system perturbation in a single experiment. Additionally, the method does not require antibodies, making it broadly applicable to many organisms.

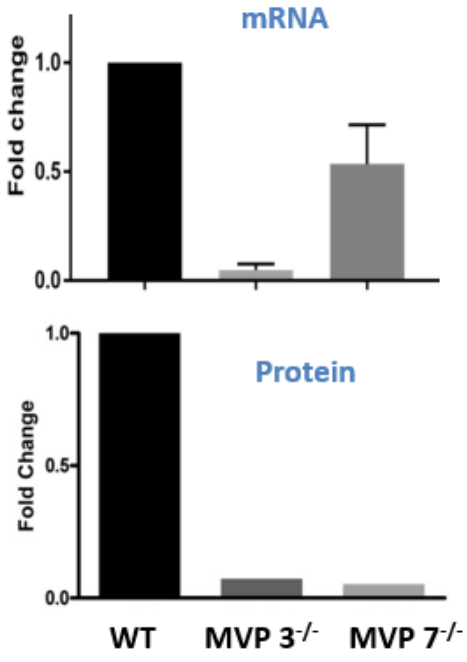

Figure 1. Ensuring Gene Editing and Loss at the Protein Level. qPCR analysis indicates total loss of mutant *mvp* mRNA in the first CRISPR allele (MVP3$^{-/-}$) but residual mutant mRNA in the second allele (MVP7$^{-/-}$). No antibodies are available for this protein in zebrafish therefore SWATH acquisition was used to confirm that gene editing was indeed effective and a significant reduction in the amount of MVP was observed at the protein level for both knockout alleles.

To demonstrate feasibility, CRISPR-Cas9 was used to knock out the Major Vault Protein (MVP) in zebrafish, and SWATH Acquisition was used to confirm the protein knockdown (Figure 1) and to study the proteome-wide changes that were induced (Figure 4).

Key Feature of SWATH Acquisition in Combination with CRISPR-CAS9 Studies

- SWATH® Acquisition[1], a data independent acquisition strategy, is the method of choice due to the comprehensive nature of the data that allows both high quality protein level quantitation as well as data re-interrogation for specific peptides to proteins of interest, without the need to rerun the sample.
- No antibodies are required, making the method more broadly and readily applicable to any protein and any organism.
- Provides a higher throughput workflow for screening gene editing experiments, allowing direct protein measurement.

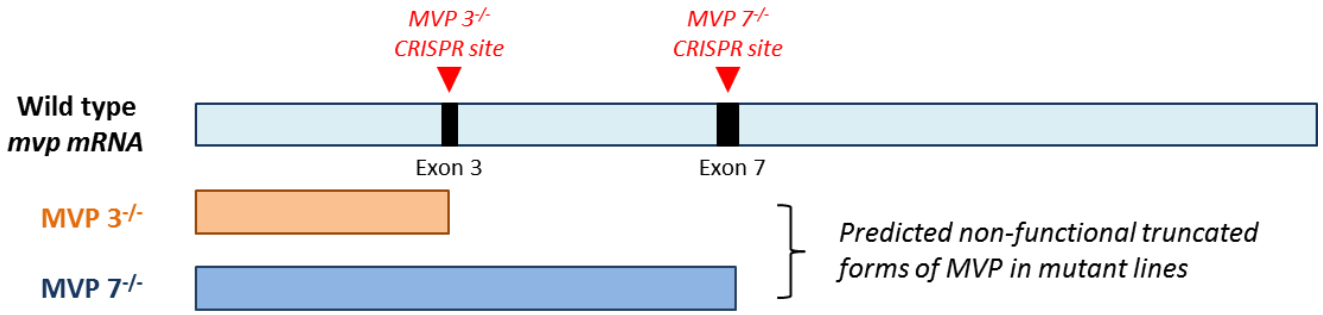

Figure 2. Mutant Alleles Generated by CRISPR-Induced Frameshift. Two mutations were made in the Major Vault Protein (MVP) in the Zebrafish model system, one in exon 3 (MVP $3^{-/-}$) and one in exon 7 (MVP $7^{-/-}$). Embryos were grown and studied however no significant change in phenotype was observed.

Methods

Sample Preparation: Exons 3 and 7 of the zebrafish (*Danio rerio*) *mvp* gene were targeted using CRISPR-Cas9[2] (Figure 2). Three biological replicates each of deyolked zebrafish 30 hours post fertilization embryos (300 per replicate) were prepared for wild type and *mvp* mutants. Samples were lysed using 50mM Tris-HCl, 100mM NaCl, 2% sodium deoxycholate. Proteins were reduced, alkylated, and digested first with Lys-C followed by trypsin. Resulting peptides were then cleaned-up using reverse phase chromatography.

Chromatography: Separation of the digested samples was performed on a NanoLC™ 425 System (SCIEX) operating in trap elute mode. Peptides were first trapped on a 350µm x 0.5mm trap, then eluted onto a 75µm x 15cm column (both packed with ChromXP™ column (SCIEX)). Elution was done with a long two-phase gradient (10-18% solvent B in 55 min, then 18-30% in next 60 mins). Total protein injected on column was 2 µg.

Mass Spectrometry: The MS analysis was performed on a TripleTOF® 6600 system (SCIEX) using a NanoSpray® Source III. Data dependent acquisition (DDA) was performed using 50 MS/MS per cycle with 50 msec accumulation time. Variable window SWATH® Acquisition methods were built using 120 variable windows with 25 msec accumulation time.

Data Processing: A spectral library was created by processing 3 DDA runs from a pool of wild type samples with ProteinPilot™ 5.0 Software using a thorough search. SWATH data was processed using the OneOmics™ platform in the SCIEX Cloud[3]. Manual data processing was performed using PeakView® Software.

Confirming Protein Level Mutation of the Edited Gene

Two different mutant indel alleles were generated for this study by a CRISPR-induced frameshift and were predicted to generate two non-functional truncated forms of MVP in the mutant lines; MVP $3^{-/-}$ and MVP $7^{-/-}$ (Figure 2). Observation of the mutant embryos revealed no significant phenotype change in either mutant. Additionally, mRNA analysis indicated significant mRNA decay of one allele, but not the other (MVP $7^{-/-}$) mutant (Figure 1, top). The lack of an observable change in phenotype in addition to the ambiguous mRNA data, made protein-level validation critical.

Typically, protein-level validation would be accomplished using an antibody-based technique such as western blot or ELISA. However, in this case, no antibody was available for zebrafish MVP. Instead, protein level analysis was performed by mass spectrometry using SWATH acquisition for quantitation of all proteins within the mutant lines. The results indicated that MVP protein levels were, in fact, negligible for *both* mutant lines (Figure 1, bottom).

Further scrutiny of the SWATH data indicated loss of peptides across the entire MVP protein sequence. Within the SWATH acquisition, 7 peptides across the entire MVP sequence were compared between the wild type and two mutant alleles. All 9 peptides were of significantly lower abundance within the mutants relative to the wild type (Figure 3). Even peptides within portions of the mutant MVP non-truncated sequences were also found to be of significantly lower abundance. This confirms that the CRISPR-Cas9 editing successfully targeted the gene and resulted in the loss of the entire MVP protein, and not just the portion located beyond the CRISPR site. This indicates instability of the truncated mutant MVP protein of both alleles, and thus abolition of the whole protein had been accomplished.

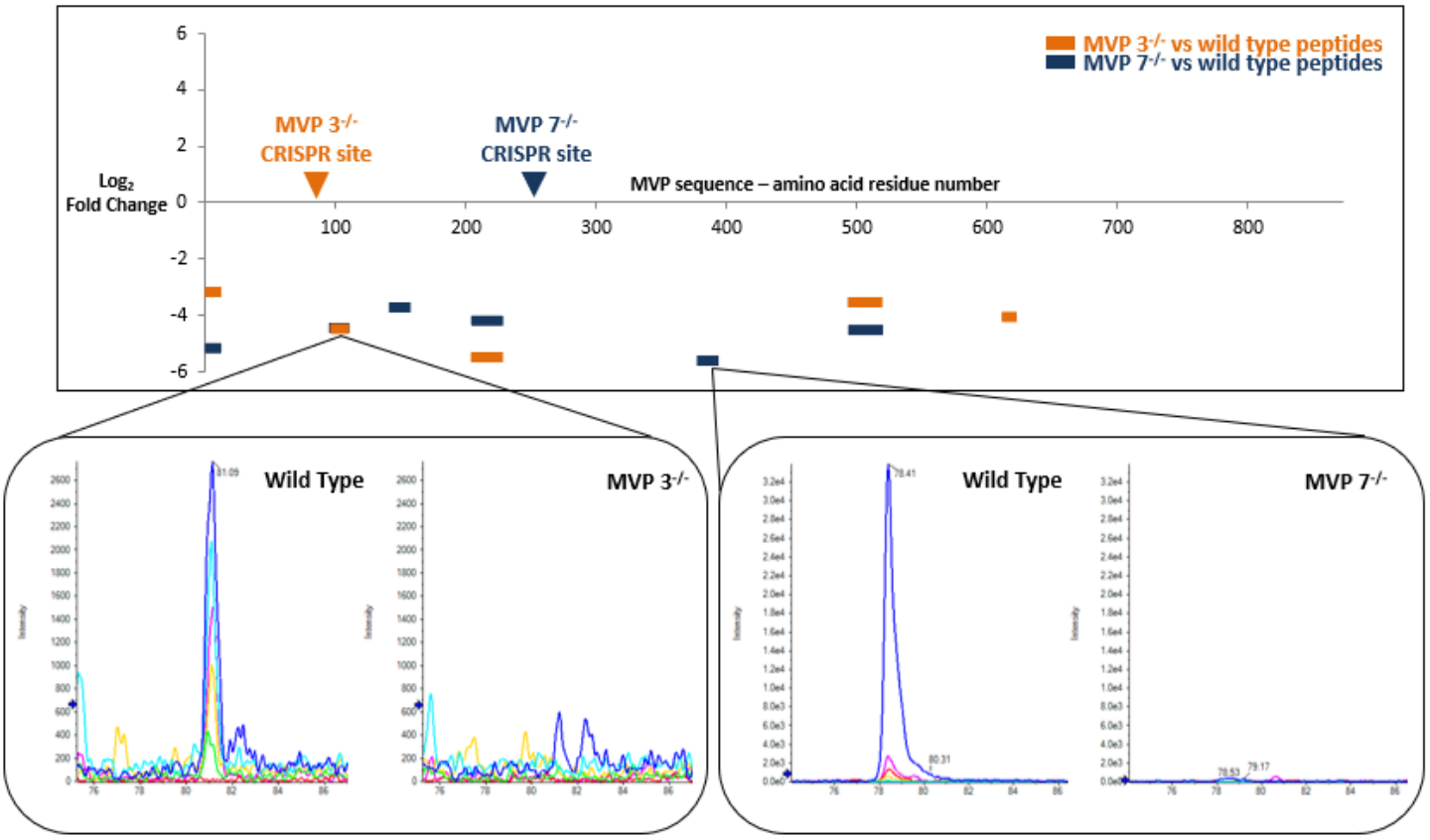

Figure 3. Measured MVP Peptide Signals Across the Full Protein Sequence. Extraction of the 7 peptides for MVP from the SWATH acquisition data allows for quantitation of the peptide level changes between the 9 samples. Comparing the peptide areas to those observed in the wild type samples using Browser shows that all peptides across the protein sequence were of significantly lower abundance relative to wild type. This confirms that the CRISPR-Cas9 editing successfully knocked down the protein, resulting in loss of the entire MVP protein (not just post CRISPR site). This provides more direct measurement of knock down success than mRNA experiment (Figure 1). Examples of the raw XIC data for 2 peptides is shown (bottom).

A Global View of All Proteins Impacted by the Edited Gene

A huge advantage of SWATH Acquisition for confirmation of genome editing at the protein level is that large numbers of proteins are analyzed in each experiment and not simply the protein product of the gene editing event. This allows a comprehensive picture of any biological changes the editing may induce. In this study, about 3800 proteins were quantified across the 6 samples (3 biological replicates each for wild type and mutant). As shown in Figure 4, within the MVP $7^{-/-}$ mutant, a subset of 65 proteins were significantly over expressed while 93 proteins were under expressed compared with the wild type.

A Venn diagram comparison of the number of proteins that were significantly changed within each mutant is shown in Figure 5. Only a small number of the same proteins were up-regulated (11) and down-regulated (18) for both mutants, and the number of unique up-regulated proteins and unique down-regulated proteins was higher for the MVP 7-/- mutant.

Investigating the Induced Biology from Gene Knockdown

To gain further knowledge of the impact of the gene editing event, the proteins that were significantly up- and down-regulated were more closely investigated. Figure 6 shows the biological process information associated with the proteins over- and under-expressed for the MVP $7^{-/-}$ mutant. While the MVP protein currently has no known function, proteins belonging to multiple groups of related processes are affected by the gene editing including cell adhesion, inflammation, and cytoskeleton processes. However, there is emerging evidence that MVP may play a role in infection or stress responses. This could explain why minimal changes were observed in the mutant phenotypes as there was no cellular stress invoked in the zebrafish prior to sample isolation.

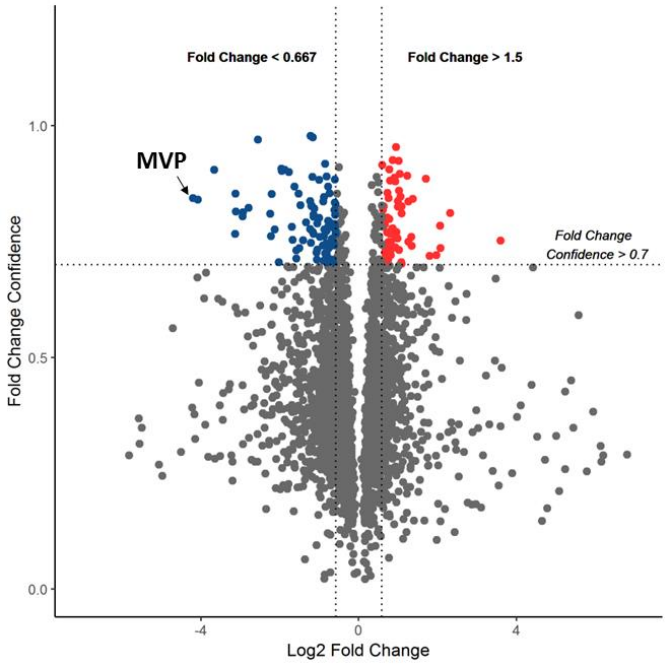

Figure 4. Proteome Wide Changes Measured by SWATH Acquisition. About 3800 proteins were quantified in the 6 samples. In the volcano plot shown here for log fold change vs protein confidence for MVP $7^{-/-}$ vs wild type. Using a filter of >1.5-fold change, >0.7 confidence and ≥2 peptides, there were 42 proteins increased and 65 proteins decreased in expression.

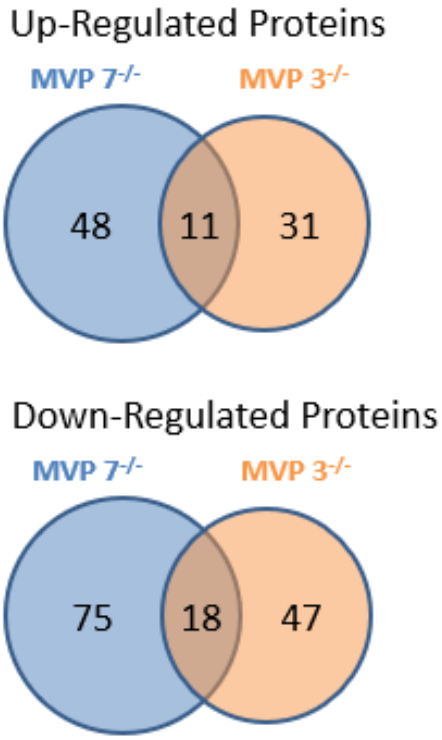

Figure 5. Comparison of Significantly Up and Down Regulated Proteins. Using a filter of >1.5-fold change, >0.7 confidence and ≥2 peptides, a total of 90 proteins were up-regulated for both mutants with only 11 of those in common between the two alleles. Conversely, a total of 140 proteins were down-regulated for both mutants with only 18 of those in common.

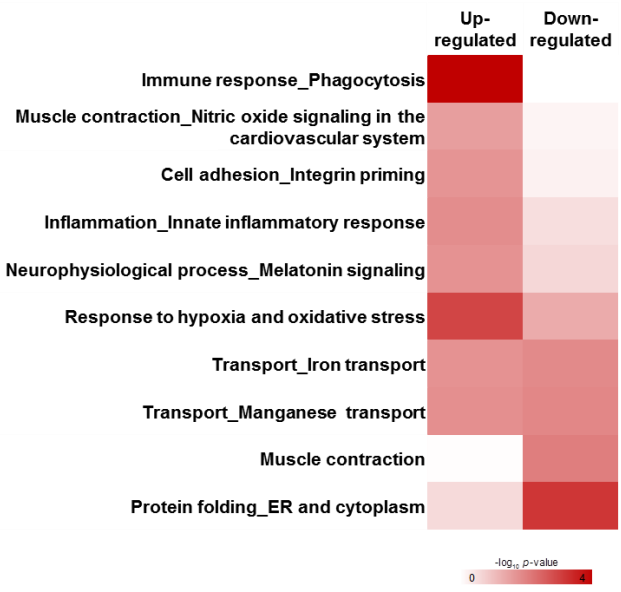

Figure 6. Biological Process Network Functional Enrichment. Top ten biological process networks significantly enriched among differentially regulated proteins in MVP $7^{-/-}$ mutant. Differentially regulated zebrafish proteins were mapped to their respective human orthologues before functional enrichment analysis using Metacore (Clarivate Analytics). MVP proteins currently have no known function however proteins belonging to several different groups of related processes are significantly affected. Overall, lack of a coherent pathway modulation supports the "Sentinel hypothesis" for Vaults, a new emerging theory that MVP is responsible for inflammatory or stress response.

Conclusions

Gene editing techniques have come a long way. With the advent of the CRISPR-Cas9 technique, genes can now be edited with incredible accuracy. mRNA experiments are typically used to verify gene editing but often mRNA and protein levels do not correlate. Thus, confirmation at the protein level is absolutely critical. With SWATH Acquisition, not only is protein level verification easily accomplished, but the additional advantages and information that SWATH provides makes this technique ideally suited for deeply exploring any organism after gene editing experiments.

- SWATH Acquisition verifies gene editing at the protein level (mutations, knockouts, truncations, etc.) without the need for antibodies
  - No costly delays waiting for antibody production
  - Easily applied across diverse organisms
  - Fast experimental protocol allows research to move more rapidly
  - Single LC-MS method for investigating all samples
- Besides verifying the protein product of the gene editing event, additional information is obtained around global changes in biology that are induced
  - Global quantitative profiling measures large numbers of proteins, including additional protein expression differences due to the effects of the gene editing
  - Linked ontology information to all affected proteins provides further understanding of the biological implications of the edited gene
- SWATH Acquisition is rapid and comprehensive
  - A digital archive is created. No need to re-run samples when new questions arise
  - Can be used for high throughput profiling for screening arrays of CRISPR experiments.

References

1. Evolution of SWATH® Acquisition Provides Large Gains in Quantified Proteins, SCIEX Technical notes RUO-MKT-02-5772-A.
2. Tracking genome-editing and associated molecular perturbations by SWATH mass spectrometry. Q. Lin, L.W.L. Low, A. Lau, H.L.F. Swa, E.W.L. Chua, Y. Matsuoka, A. Monteiro, S. Tate, J. Gunaratne, T.J. Carney. *Manuscript in preparation*.
3. OneOmics™ Project in the Cloud - Getting Started with the Proteomics Cloud ToolKit. SCIEX Technical note RUO-MKT-02-6969-B.

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