

## SEMIQUANTITATIVE ASSESSMENT OF DISHEVELLED-3 PHOSPHORYLATION STATUS BY MASS SPECTROMETRY

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The focus of this paper is the human Dishevelled 3 protein (hDvl3), an essential component of the Wnt signalling pathway that contributes to their regulation. Mass spectrometry-based analysis of hDvl3 phosphorylations induced by eight associated kinases was performed revealing several dozens of phosphorylation sites. The main outcome of this study was the description of Dvl phosphorylation "patterns" induced by individual kinases.

**Keywords:** phosphorylation, Dishevelled 3, mass spectrometry, CK1 $\epsilon$ , NEK2

### 1. Introduction

Reversible protein phosphorylation belongs to posttranslational modification which is of high biological significance mainly due to its role in regulating cellular processes such as gene expression, cell division, signal transduction, metabolism, differentiation, apoptosis, etc. [1]

Dishevelled-3 (Dvl3) is a protein involved in the Wnt signalling pathways and gets heavily phosphorylated in response to pathway activation by Wnt ligands [2].

In our study, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation of hDvl3 immunoprecipitates was combined with titanium dioxide phosphopeptide enrichment followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Data were processed using Skyline software to obtain a semiquantitative assessment with regard to the occupancy of phosphorylation sites induced by individual kinases.

### 2. Experimental

#### 2.1 Cell culture and transfection

The cell line HEK293 was used for this study. Cells were seeded in dishes 15 cm in diameter and transfected at a confluence of 60%. Two days after transfection, after reaching confluence, they were harvested for immunoprecipitation. A chilled NP-40 lysis buffer with protease inhibitors, dithiothreitol (DTT), phosphatase inhibitors and

N-ethylmaleimide (NEM) was used for cell lysis. The lysate was incubated with the antibody Anti FLAG M2.

#### 2.2 Gel electrophoresis, protein digestion and phosphopeptide enrichment

Immunoprecipitates were separated by SDS-PAGE, fixed with acetic acid in methanol, and stained with Coomassie Brilliant Blue for 1 hour then partially destained.

Corresponding 1-D bands were excised. After destaining, the proteins in gel pieces were incubated with 10 mM of DTT at 56 °C for 45 mins. After the removal of DTT excess samples were incubated with 55 mM of iodoacetamide (IAA) at room temperature in darkness for 30 mins, then an alkylation solution was removed and gel pieces were hydrated for 45 mins at 4 °C in a digestion solution (5 ng/ $\mu$ l trypsin, sequencing grade, Promega, Fitchburg, Wisconsin, USA, in 25mM ammonium bicarbonate (AmBic)). The trypsin digestion proceeded for 2 hours at 37 °C on a Thermomixer (at 750 rpm; Eppendorf AG, Hamburg, Germany). Subsequently, the tryptic digests were cleaved by chymotrypsin (5 ng/ $\mu$ l, sequencing grade, Roche, Basel, Switzerland, in 25mM AmBic) for 2 hours at 37 °C. Digested peptides were extracted from gels using 50% acetonitrile (ACN) solution with 2.5% formic acid (FA) and concentrated in a SpeedVac concentrator (Eppendorf AG, Hamburg, Germany). The aliquot (1/10) of the sample was transferred to an autosampler vial and concentrated under a vacuum. Water was used to produce 15  $\mu$ l of the peptide solution which was directly

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MGETKIIYHLDGQETPYLVKLPLPAERVTLADFKGVLQRP	SYKFFFKSMD	50
DDFGVVKEEISDDNAKLPCFNRRV	SWLVSAEGSHDPAPFCADNPSELP	100
PPMERIGGIGD	SRPPSFHPHAGGGSQENLDNDTE	150
GPEHATRLNGTAKGERRREP	GGYDSSSTILMSSSELETT	200
FSSTEQSSASRLMRRHKRRRRKQKVSRIER	SSSFSSITDSTM	250
TLNMEKYNFLGISIVGQ	SNERGGGIYIGSIMKGGAVT	300
QVNEINFENMSNDDAVRVLREIVHKPGPITLTVAKCWDPS	PRGCF	350
EPIRPIDPAAWV	SHTAAMTGF	400
LDDFHL	SIHSDMAAIVKAMASPE	450
LYHNVEGFTDRREARKYAS	NLLKAGFIRHTVNKIF	500
MANLSLHDHDGSSGASDQDTLAPLPHPGAAPWPM	AFYQYPPPPHPYNPH	550
PGFPELGYSYGGGSASQHSEGSRS	SGSNRSGSDRRKEKDPKAGDSKSGG	600
SGSES	DHTRSSLRGPRERAPSERSGPAASEHSHHSLASS	650
PSYGPPGVPPLYGPPMLMPPPPAAMGPPGAPPGRDLASV	PELTA	700
FRMAMGNPSEFFVDVM		

Figure 1: Comparison of phosphorylation sites of hDv13 induced by CK1 $\epsilon$  and NEK2. Experimentally determined phosphorylation sites by CK1 $\epsilon$  and NEK2 are indicated by green background color. Phosphorylation sites identified only by NEK2 are indicated by red background color.

analyzed by liquid chromatography coupled with mass spectrometry (LC-MS/MS) for protein identification (ID run).

The rest of the peptide mixture was used for phosphopeptide analysis. MS PhosphoMix 1, 2, 3 Light (Sigma Aldrich) was added to the samples before the phosphopeptide enrichment step in a concentration of 0.1 pmol. Phosphopeptides were enriched using a Pierce Magnetic Titanium Dioxide Phosphopeptide Enrichment Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the protocol of the manufacturer and eluted into an autosampler vial. The solution was concentrated under a vacuum to a volume of less than 5  $\mu$ l, dissolved in water and 0.6  $\mu$ l of 5% FA was used to obtain 15  $\mu$ l of the peptide solution before LC-MS/MS analysis.

### 2.3 Mass spectrometry

LC-MS/MS analyses of the peptide mixture were conducted using a RSLCnano system connected to a Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific) with a ABIRD (Active Background Ion Reduction Device; ESI Source Solutions) and a Digital PicoView DPV550 (New Objective) ion source (tip rinsing by 50% ACN with 0.1% FA) installed. Prior to LC separation, tryptic digests were concentrated online and desalted using a trapping column (100  $\mu$ m  $\times$  30 mm) filled with 3.5- $\mu$ m of XBridge BEH 130Å C18 sorbent (Waters). After the trapping column was washed with 0.1% FA, the peptides were eluted (flow rate of 300 nl/min) from the trapping column onto a Acclaim PepMap100 C18 column (3  $\mu$ m particles, 75  $\mu$ m  $\times$  500 mm; Thermo Fisher Scientific) along a 65 min-long gradient. Mobile phase A (0.1% FA in water) and mobile phase B (0.1% FA in 80% ACN) were used in both cases. The gradient elution

started at 1% of mobile phase B and increased from 1% to 56% during the first 50 mins (30% in the 35th and 56% in the 50th min), then increased linearly to 80% of mobile phase B over the following 5 mins and remained at this state for the next 10 mins. Equilibration of the trapping column and the analytical column was conducted prior to injection of the sample into the sample loop. The outlet of the analytical column was directly connected to the Digital PicoView DPV550 ion source.

MS data were acquired in a data-dependent strategy by selecting the top 6 precursors based on precursor abundance in the survey scan (350–2000  $m/z$ ). The resolution of the survey scan was 60,000 (at 400  $m/z$ ) with a target value of  $1 \times 10^6$  ions, one microscan and a maximum injection time of 200 ms. High resolution (resolution of 15,000 at 400  $m/z$ ) higher energy collisional dissociation (HCD) MS/MS spectra were acquired with a target value of 50,000. The normalized collision energy was 32 % for HCD spectra. The maximum injection time for MS/MS was 500 ms. Dynamic exclusion was enabled for 45 s after the acquisition of one MS/MS spectra and early expiration was disabled. The isolation window for MS/MS fragmentation was set to 2  $m/z$ .

The analysis of the mass spectrometric RAW data files was carried out using the Proteome Discoverer software (Thermo Fisher Scientific; version 1.4) with utilization of the in-house Mascot (Matrixscience; version 2.4.1) search engine. MS/MS ion searches were conducted against an in-house database containing the expected protein of interest with additional sequences from the cRAP (common Repository of Adventitious Proteins) database (downloaded from <http://www.thegpm.org/crap/>). Mass tolerance for peptides and MS/MS fragments were 7 ppm and 0.03 Da, respectively. Oxidation of methionine, deamidation (N, Q)

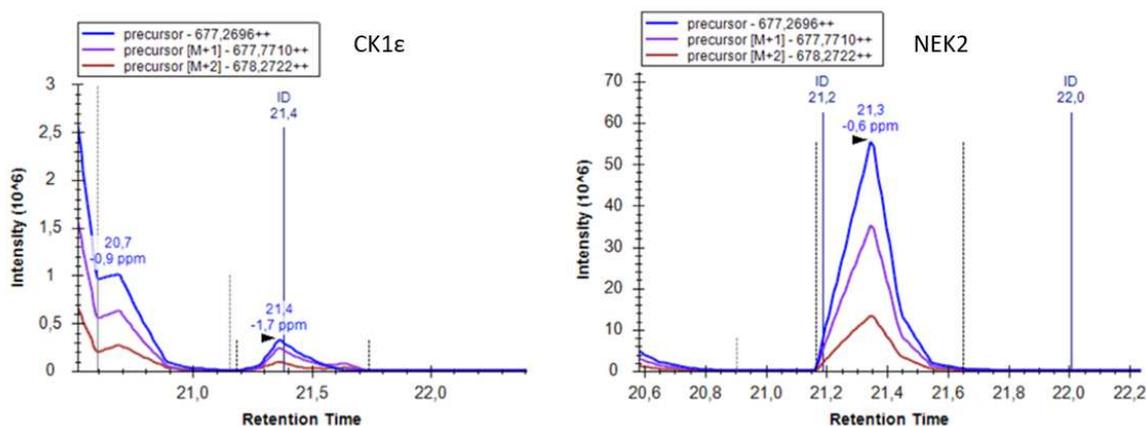


Figure 2: Semiquantitative analysis of phosphorylation site serine 204 (S204) on peptide FSSpSTEQSSASR induced by CK1 $\epsilon$  and NEK2. Precursor and selected fragment traces of corresponding hDvl3 phosphopeptides are shown for CK1 $\epsilon$  and NEK2 (in Skyline). The highest signal intensity was detected in the case of NEK2.

and phosphorylation (S, T, Y) as optional modifications, carbamidomethylation of C as a fixed modification and three miss cleavages of enzymes were set for all searches. The phosphoRS feature was used for phosphorylation site localization.

Quantitative information was assessed and manually validated in Skyline software (Skyline-daily 3.1.1.8884).

### 3. Results and Analysis

#### 3.1 Identification of phosphorylation sites

Phosphorylation is important for protein function and regulation. The phosphorylation status of human Dvl3 induced by eight individual Ser/Thr kinases that were previously reported or identified by an unbiased MS screen for Dvl-associated kinases was analysed. Dvl3 contains 131 serines/threonines, which can be potentially phosphorylated. In total, 88 Ser/Thr phosphorylation sites and one tyrosine phosphorylation site in Dvl3 were identified.

#### 3.2 Phosphorylations induced by CK1 $\epsilon$ and NEK2

Based on our experiment, a phosphorylation “map” of the Dvl protein was created that described the complex phosphorylation “fingerprint” for each kinase tested. Eight of the kinases used to induce phosphorylation include CK1 $\epsilon$  and NEK2. Fig. 1 shows a qualitative comparison of the identified phosphorylation sites using these two kinases. In the case of CK1 $\epsilon$  induction, 77 phosphorylation sites were identified, and in the case of NEK2, 87 phosphorylation sites were determined from a total of 131 possible Ser/Thr phosphorylation sites in the Dvl3 protein.

Next in terms of qualitative characterization, a semi-quantitative comparison with regard to the occupancy of phosphorylation sites induced by individual kinases was

conducted. The Skyline software was used for this evaluation. The individual phosphorylated peptides were compared based on their peak areas. A comparison of a selected peptide phosphorylated in the position of S204 by CK1 $\epsilon$  and NEK2 is shown in Fig. 2. The peak area was determined for CK1 $\epsilon$  as 7.60e6 and for NEK2 as 1.07e9. Subsequently, double normalization of the data was performed using a set of phosphopeptide standards (added to the sample prior to the phospho-enrichment step) and by unphosphorylated peptides identified in the identification run. The resulting areas (CK1 $\epsilon$ : 1.23e7 and NEK2: 1.10e9) were compared with each other.

### 4. Discussion

Our study focused on the determination of the phosphorylation sites of Dvl3 by MS induced by eight kinases. 88 Ser/Thr phosphorylations from a total of 131 sites and 1 tyrosine phosphorylation were identified which can be potentially phosphorylated.

CK1 $\epsilon$ -induced phosphorylation was identified at 77 unique sites and 10 more phosphorylation sites were induced by NEK2. Previous studies in various experimental systems identified several phosphorylation sites spread throughout the structure of the protein Dvl3 [3, 4]. Our data clearly demonstrate that the phosphorylation of the protein Dvl3 is extensive and the number of phosphorylated sites exceeds 60.

### 5. Conclusion

An approach based on the SDS-PAGE separation of Dvl3 immunoprecipitates, TiO<sub>2</sub> phospho-enrichment followed by LC-MS/MS analysis and data processing using Skyline software was utilized for the evaluation of semiquantitative differences in the phosphorylation level of hDvl3 at particular sites within the set of eight selected kinases.

Differences were observed in terms of the phosphorylation profiles induced by individual kinases, as indicated in Fig. 1. Based on our results, a “comprehensive map” of the phosphorylations of human Dvl3 will be created.

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