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Methods

Proteins contained in the three tubes were separated by one-dimensional SDS-polyacrylamide gel electrophoresis on a Invitrogen X-cell SureLock using a precasted NuPage (Invitrogen) 10% polyacrylamide gel of 1-mm thickness.
Every lane (corresponding ti a different tube) was cut in 5 slices and trypsinized as previously described by Shevchencko et al. (*Anal.Chem*, 1996, *68*, 850–858.).
Peptides were desalted as described by Rappsilber et a. (Anal. Chem. 2003, 75, 663-670), dried in a Speed- Vac and resuspended in 7 µL of 0.1% TFA,
LC- ESI-MS/MS of 5µL of each sample was performed on a Fourier transformed-LTQ mass spectrometer (FT-LTQ) (Thermo Electron, San Jose, CA). Peptides separation was performed on a linear gradient from 100% solvent A (5 % ACN, 0.1% formic acid) to 20% solvent B (ACN, 0.1% formic acid) over 20 min and from 20% to 80% solvent B in 5 min at a constant flow rate of 0.3µL/min on Agilent chromatographic separation system 1100 (Agilent Technologies, Waldbronn, Germany) where the LC system was connected to a 10.5 cm fused-silica emitter of 100 µm inner diameter (New Objective, Inc. Woburn, MA USA), packed in-house with ReproSil-Pur C18-AQ 3 µm beads (Dr. Maisch Gmbh, Ammerbuch, Germany) using a high-pressure bomb loader (Proxeon, Odense, Denmark).
Data acquisition mode was set to obtain one MS scan followed by five MS/MS scans of the five most intense ions in each MS scan. MS/MS spectra were limited to one scans per precursor ion followed by 1 min of exclusion.
MGF file were extracted using DTASuperCharge (v.1.19, www.cebi.sdu.dk) and files obtained for each tube were manually merged.
Database search was performed using Mascot Daemon set up with the following parameters:
Database NCBInr, Taxonomy Homo Sapiens, enzyme Trypsin, Max missing cleavage 2, fixed modification carbamidomethyl (C), variable modification oxidation (M), peptide tolerance 10 ppm, MS/MS tolerance 0.5 Da, Instrument ESI-TRAP.
The N-15 search was performed according to the PRG2010 Supplementary information file.
The results file was automatically filtered by in house script (Daemex v2.1) followed by data interpretation according to literature.
Daemex v2.1 is usable to transform automatically the Mascot search result generated by Mascot Deamon into a complete list of excel file on which every protein is sorted by the numbers of peptides identified and their relative score, the total number of peptide identified and the total mascot score. A protein is considered "identified" if at least 2 peptides are identified, of which1 with mascot score higher than 33.

Results and Discussion

In Tube 1 we identified the following proteins: *1*) **catenin beta 1** (a cadherin-associated protein); *2*) Seven In Absentia Homolog 1 isoform b **(Siah1 E3)**; *3*) Chain B, Insights Into Scf Ubiquitin Ligases From The Structure Of The **Skp1-Skp2** Complex; 4) Calcyclin binding protein isoform 1 (**CacyBP);** 5) members of the family **S100 A** calcium-binding protein. The presence of these proteins organized in an active complex justified the ubiquitination activity found in the tube.

In Tube 2, Siah1 E3 Ligase was not identified, and indeed no ubiquitination activity was observed.

On the contrary, presence of Siah1 E3 Ligase in Tube 3 led to the reconstitution of the active complex, and ubiquitination can take place.