Deliverable 3.2-3:
Structural analysis of target protein interactions

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1. SUMMARY

This project is based on the early drug discovery phase for new effective therapies and aims at developing new tools for the rational design of novel compounds with anti-obesity or anti-diabetes activities.

Development of compounds with high specificity and reduced secondary effects requires the knowledge of the three-dimensional structure of the selected targets. The compounds (ligands) with potential biological effects can be precisely designed to have complementary shape and chemical properties to their target macromolecule.

The target protein DOR has been purified and its stability and interaction with intracellular partners has been structurally analyzed. From those results, the stoichiometry of complex formation has been determined and a peptide containing the interaction site has been produced for co-crystallization experiments. The structural characterization of the interaction between DOR and its interacting partner was crucial for the growth of crystals for 3D structure determination and led to the new results presented in this report.

2. INTRODUCTION

Obesity is increasing exponentially worldwide and represents a serious public health problem. Together with the lack of physical exercise and a inadequate diet, obesity contributes to the escalating levels of Type 2 diabetes, particularly in young adults. It is thus urgent to develop new approaches to the treatment and prevention of these modern-life pathologies. This project is based on the early drug discovery phase for new effective therapies and aims to develop new tools for the rational design of new compounds with anti-obesity or anti-diabetes activities.

The main goal of this specific aim (Task 2) within Group task 3 is the determination of the three-dimensional structure of target proteins by X-ray crystallography. Those experimental three-dimensional models will be crucial tools for the rational, structure-aided design of novel compounds to be screened for their anti-diabetic and anti-obesity potential. The determination of the 3D structures of proteins by X-ray crystallography comprises several steps, starting with the expression of the target molecule in sufficient amounts for crystallization trials, often resorting to heterologous (bacterial, yeast, insect or mammalian cells) expression systems. The selected target proteins are Diabetes and obesity related gene protein (DOR), Mitofusin-2 (Mfn2) and Vascular adhesion protein 1 (VAP-1). Research data from partners at IRB and INSERM indicate that those proteins may play a central role in obesity and/or diabetes, therefore constituting new potential drug targets (Zorzano *et al.*, 2009, Yraola *et al.*, 2006).

We were successful in obtaining milligram amounts of DOR for structural analysis. However, this protein is extremely unstable and rapidly degrades, even when stored in the presence of glycerol at -80ºC. Despite extensive

attempts to crystallize this protein unliganded and in complex with interacting proteins, we could not yet obtain diffraction-quality crystals. Our efforts where thus directed towards the detailed characterization of the factors affecting the stability of this protein and the structural characterization of its interaction with known macromolecular partners.

3. OBJECTIVES AND METHODOLOGY

3.1 OVERVIEW

The main goal of this group task is to determine the shape or three-dimensional structure of selected proteins correlated with obesity and type-2 diabetes. Those structures will provide a 3D framework for the structure-based design of specific molecules (ligands) with complementary shape and/or charge. The main method used for determining the three-dimensional structures of proteins is X-ray crystallography. The expression and purification of recombinant protein is the first of many steps towards the determination of the 3D structure of a macromolecule. In order to obtain high quality crystals, the sample must be chemically pure and devoid of heterogeneities. Although a protocol for obtaining high yields of pure protein was previously established, the instability and rapid degradation of the protein has so far prevented growth of diffraction-quality crystals. Therefore, the team invested in the characterization of the interaction between DOR and intracellular partners. The main goal was to characterize the nature of the interaction and potentially identify a stable complex for crystallization and three-dimensional structure determination.

3.2. SPECIFIC OBJECTIVES:

The main aim of this deliverable was the crystallization and three-dimensional structure determination of the target protein DOR unliganded and/or in complex with its interaction partner, microtubule-associated protein light chain 3 (LC3). In order to increase the success of the crystallization trials, the interaction between NusA-DOR and LC3 has been quantified by surface plasmon resonance and a DOR peptide, corresponding to the interacting region of LC3 has been prepared and used for co-crystallization.

3.3. METHODOLOGY

Analysis of the stability of NusA-DOR fusion alone and in complex with LC3.

The thermal stability of the NusA-DOR fusion was analyzed by differential scanning fluorimetry (Niesen et al., 2007) using a Bio-Rad iQ5 real-time PCR. For the analysis of the LC3 interaction with DOR peptide the melting curves were obtained using 50 ul of a solution containing 100μM of each protein in 20mM HEPES, pH7.5, 1mM EDTA, 100mM NaCl. For obtaining the melting temperatures of NusA, NusA-DOR and their complexes with LC3 in concentrations varying between 0.4 and 0.8 mg/ml in 20mM HEPES, pH7.5, 100mM NaCl, 1mM EDTA, 10% glycerol. Sypro Orange was used at 1:20 dilution and all experiments were performed in quadruplicate.

LC3:NusA-DOR complex: measuring the interaction affinity by Surface Plasmon Resonance (SPR)

LC3 was initially diluted in NaAc pH 4.5 to a final concentration of 1 µg/ml. This protein solution was immediately used for immobilization in CM5 Sensor chips (Biacore S series). For that, a sensor chip was activated with NHS/EDC followed by coupling of approximately 300 RUs (Chanel 1), 100 RUs (Chanel 3) and 50 RUS (Chanel 4) of LC3 and deactivation with ethanolamine. Channel 2 was left blank and was used as reference. For that, it was activated by NHS/EDC followed by deactivation with ethanolamine. Kinetic interaction experiments using NusA-DOR or NusA (negative control) as analytes were performed at 10ºC, at a flow rate of 30 µl/min during 60 seconds. Regeneration was achieved by injecting a solution of 25 mM NaOH at a flow rate of 60 µl/min for 60 seconds at the end of each cycle of interaction.

Identification of the DOR residues involved in the interaction with LC3 and co-crystallization experiments.

The plasmid expressing the NusA-DOR fusion protein was mutated in the conserved residues within the putative LC3-interacting motif (LIR motif). This region (SEEDEVDGWLIIDLPDSYAA) shares homology with the binding region on other LC3-interacting proteins such as p62 (Ichimura et al., 2008). The W35A/I38A MBP-DOR double mutant was expressed in *E. coli* and co-purified (metal-affinity chromatography followed by affinity chromatography on chitin agarose) with Trx-LC3 as previously described (deliverable 3.2-1). Negative controls where MBP/Trx-LC3 and Trx/MBP-DOR

were co-purified did not show any significant interactions either between LC3 and MBP or between DOR and Trx.

The more stable construct (NusA-DOR) was used for co-crystallization experiments at the EMBL High-throughput Crystallization Laboratory (Grenoble, France). The complexes, prepared by mixing LC3 and NusA-DOR or LC3 and DOR peptide at variable ratios, followed by incubation on ice for 1h, were used for setting up the crystallization assays by sitting drop vapour diffusion.

4. RESULTS ACCHIEVED

4.1. PURIFIED PROTEINS

The DOR (in fusion with the solubility tag NusA, NusA-DOR) and LC3 proteins were purified to over 95% purity and analyzed by SDS-PAGE (Figure 2).

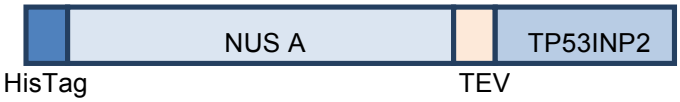


Figure 1- Schematic representation of the NusA-DOR(TP53inp2) fusion recombinantly expressed in *E. coli*.

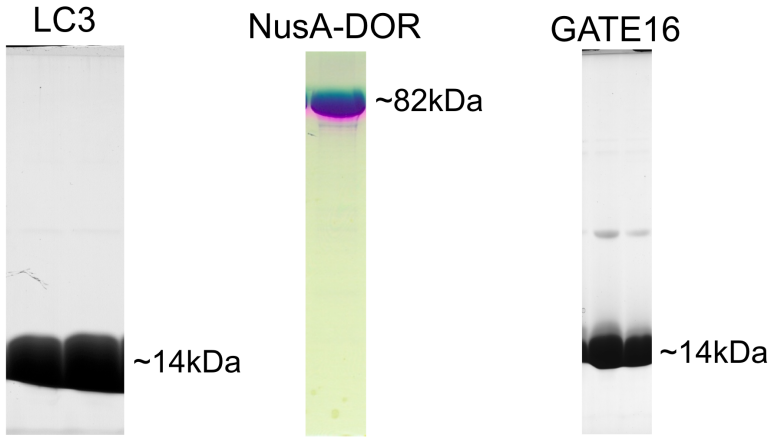


Figure 2- Representative SDS-PAGE analysis of the proteins purified and used for miniaturized crystallization assays using the HTX lab, Grenoble (https://embl.fr/htxlab/). All proteins are over 95% pure as judged by SDS-PAGE and gel filtration analysis.

4.2. ANALYSIS OF THE THERMAL STABILITY AND SECONDARY STRUCTURE OF NUSA-DOR ALONE AND IN COMPLEX WITH LC3

The secondary structure of DOR was analyzed by circular dichroism (Figure 3). The CD spectra show that the NusA-DOR fusion is preferentially alpha-helical as is the structure of the NusA tag alone. Subtraction of the NusA spectrum from that of NusA-DOR reveals that DOR is mostly unstructured. The lack of secondary structure of DOR partially explains the difficulty in obtaining diffraction-quality crystals of this protein.

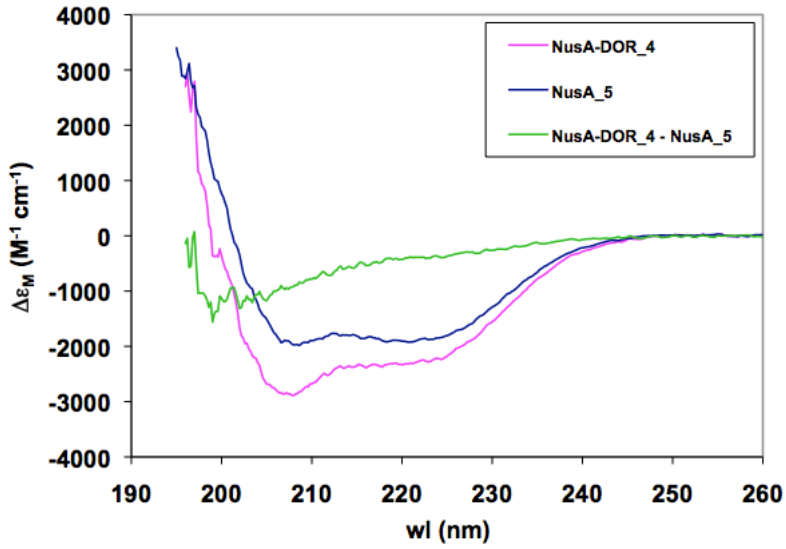


Figure 3- CD spectra of NusA-DOR and NusA. The difference between the two spectra (green line) reveals the secondary structure of DOR.

The thermal stability of the NusA-DOR protein (Tm 57ºC) was analysed by differential scanning fluorimetry (DSF) and compared with that of the NusA-DOR/LC3complex (Figure 4). DSF was also used to find the optimal buffer conditions, which by increasing protein stability might increase the success of the crystallization trials.

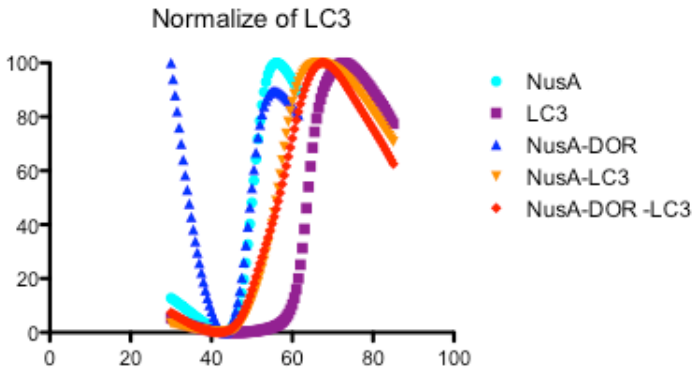


Figure 4- Analysis of the thermal unfolding of unliganded NusA-DOR and of the NusA-DOR/LC3 1:1 complex. (TmLC3=63.5ºC; TmNusA=50.2ºC; TmNusA-DOR=50.4ºC; TmNusA+LC3=55.3ºC; TmNusA-DOR+LC3=55.4ºC). All assays were done in 20mM HEPES, pH7.5, 100mM NaCl, 1mM EDTA, 10% glycerol.

The data show that the NusA-DOR fusion displays a thermal unfolding profile commonly observed in proteins that do not crystallize (Ericsson et al., 2006). The thermal unfolding profile of the complexes NusA-DOR/LC3 and NusA/LC3 display similar transition temperatures and in the absence of additional information, these results are difficult to interpret. The observed transition for the complexes between LC3 and NusA or between LC3 and NusA-DOR could represent the temperature required for disassembly of the complex (Kopec and Schneider, 2011), although the Tm of LC3 alone is significantly higher. Since these results did not allow us to conclude if the interaction between LC3 and NusA-DOR was strong enough for NusA-DOR stabilization, and given the apparent dominant effect of the NusA tag in the unfolding profiles of NusA-DOR, the interaction was characterized by surface plasmon resonance (SPR).

4.3. ANALYSIS OF THE INTERACTION BETWEEN DOR AND LC3 BY SURFACE PLASMON RESONANCE

Solutions with different concentrations of either NusA-DOR or NusA were randomly injected and interaction measured (Figure 5). As shown in Figure 5, NusA-DOR is able to interact with LC3, with interaction curves fitting to a 1:1 type of interaction.

Kinetic analysis (Table I) using the BiaEval software based on a bimolecular model limited by mass transport revealed an association constant (Ka) of 2.02E+5 $M^{-1}s^{-1}$ and a dissociation constant (KD) of 7.207E-8 M. On the other hand, even though NusA alone is able to interact with LC3 (Figure 5), it displays a lower affinity than NusA-DOR, with a Ka of 5.492E+4 $M^{-1}s^{-1}$ and a KD of 1.237E-7 M (Table II). These results indicate that DOR alone is able to form a complex with LC3.

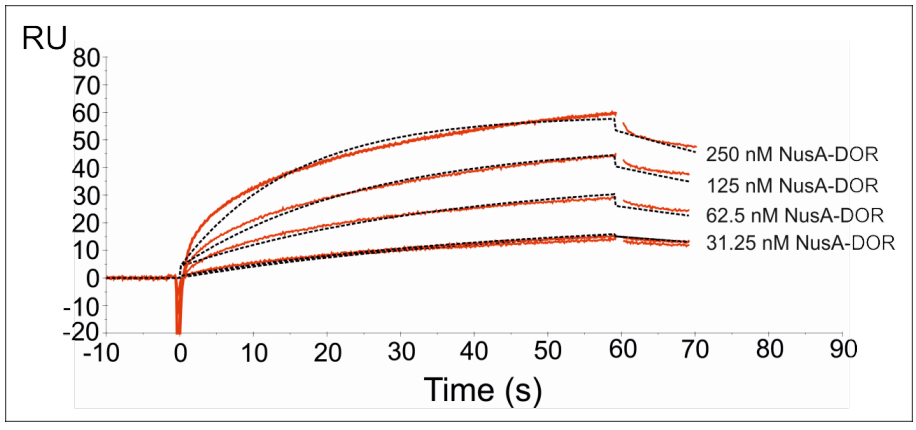


Figure 5- Interaction between LC3 (Ligand) and different concentrations of NusA-DOR (analyte). Representation of the interaction with LC3 on Channel 1 (300 RUs). Red lines represent the experimental interactions measured in a Biacore T100. Dotted lines correspond to the curves used for fitting the data.

Table I - **Kinetic parameters for the interaction between LC3 and NusA-DOR. Ka, affinity constant; Kd, dissociation rate constant; KD, dissociation constant; Rmax, response at saturation; Flow, flow rate; Chi^2, Chi2-test that shows information about the goodness of fit (typically <10).**

Ka (1/Ms)	Kd (1/s)	KD (M)	Rmax (RU)	Flow (ul/min)	Chi^2 (RU^2)
2.02E+5	0.01457	7.207E-8	70.4	30	1.26

Table II - Kinetic parameters for the interaction between LC3 and NusA, used as negative control. Ka, affinity constant; Kd, dissociation rate constant; KD, dissociation constant; Rmax, response at saturation; Flow, flow rate; Chi2, Chi2-test that shows information about the goodness of fit (typically <10).

Ka (1/Ms)	Kd (1/s)	KD (M)	Rmax (RU)	Flow (ul/min)	Chi^2 (RU^2)
5.492E+4	**0.006792**	**1.237E-7**	**18.9**	**30**	**0.765**

4.4. CRYSTALLIZATION OF LC3 IN COMPLEX WITH A DOR PEPTIDE

The analysis of the NusA-DOR sequence revealed the presence of a region with similarity to the LIR (LC3-interacting) motif of p62 and other LC3-interacting proteins (Ichimura *et al.*, 2008). Mutation of the strictly conserved residues W35 and I38 (Figure 6) significantly reduced the interaction with LC3 (Figure 7). These results show that DOR is directly interacting with LC3, through the LIR region of the former.

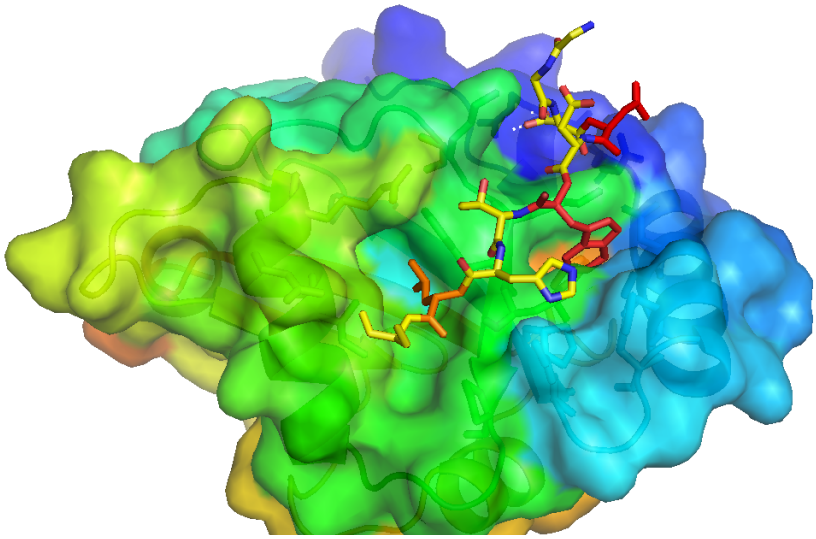


Figure 6– Surface representation of LC3 in complex with a LIR peptide belonging to the LC3-interacting region of p62. The peptide is represented as sticks with strictly conserved residues in DOR shown in red and conserved residues shown in orange. Notice the conserved Trp (equivalent to Trp35 in DOR) and Leu (Ile38 in DOR) tightly fitting in hydrophobic pockets at the suface of LC3.

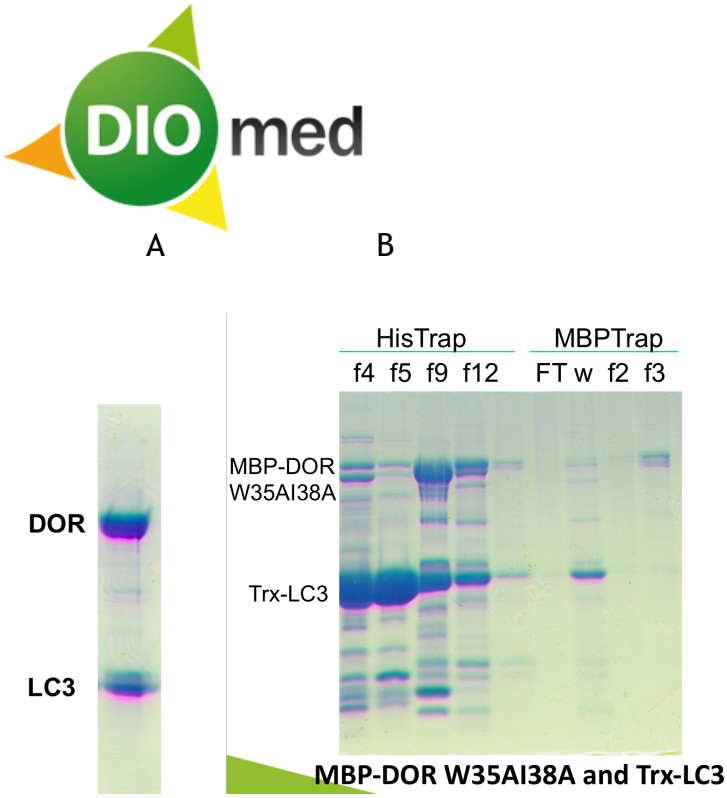
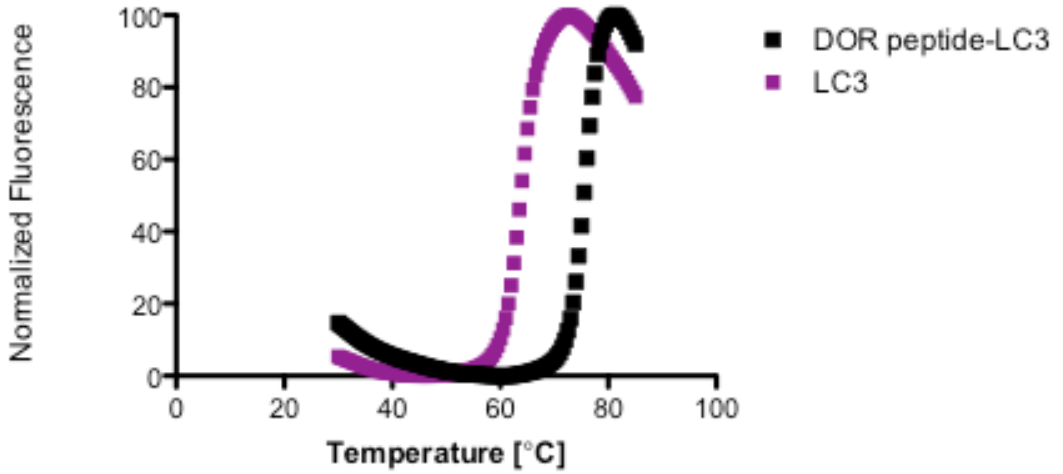


Figure 7- A) Final fraction after chitin-agarose affinity chromatography of co-purified MBP-DOR/LC3 (as reported in deliverable 3.2-1). B) Co-purification of MBP-DOR(W35A/I38A)/LC3 shows that the mutation drastically reduces the interaction.

For co-crystallization experiments, the peptide *SEEDEVDGWLIIDLPDSYAA*, representing the LIR region of DOR was synthesized and incubated with LC3. The strength of the interaction between LC3 and the peptide was clear from the increase in melting temperature (ΔTm=12°C) of unfolding seen by DSF (Figure 8). Crystals for this complex were obtained by sitting drop vapour diffusion using 2-methyl-2,4-pentanediol as precipitant (Figure 9). Crystals appeared after 8 weeks and will be used for X-ray diffraction analysis.



with increasing concentration of DOR peptide. (Tm LC3=63.5ºC; Tm peptide+LC3(1:1)=75.5ºC). All assays were done in 20mM HEPES, pH7.5, 1mM EDTA, 100mM NaCl.

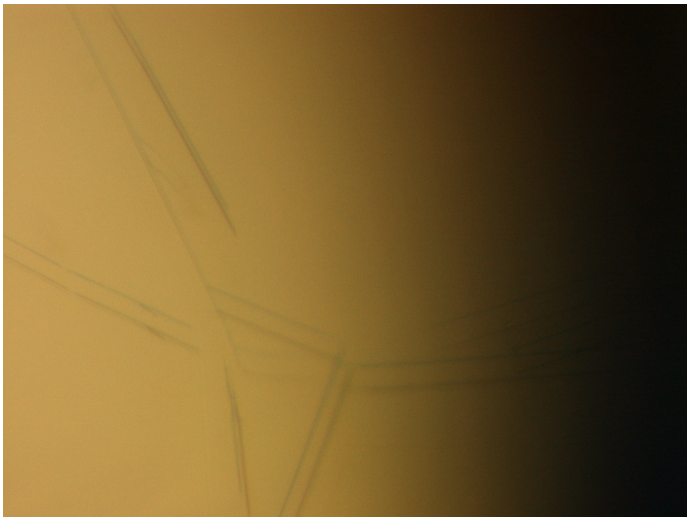


Figure 9- Needle-like crystals from the LC3-DOR peptide complex (1:1).

4.5. MAIN CONCLUSIONS

The detailed structural characterization of the NusA-DOR showed that DOR is mainly unstructured, partly explaining the difficulty in obtaining diffraction-quality crystals of this protein. The protein interacts with LC3, as shown by SPR, and this interaction is largely mediated by the LIR motif identified in DOR (*SEEDEVDGWLIIDLPDSYAA*). This peptide strongly interacts with LC3, stabilizing the protein. The information derived from the structural analysis of the LC3-DOR interaction, allowed to design crystallization experiments which yielded crystals of the complex between LC3 and a DOR peptide containing the LIR motif.

5. REFERENCES

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