Atomic level investigation of Arkadia's selectivity and specificity towards E2 enzymes

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Introduction:

Ubiquitination and proteasome-dependent degradation of proteins is a major mechanism for the regulation of protein function in eukaryotic cells [1]. The covalent attachment of ubiquitin to protein targets occurs through the recruitment of three enzymes: an ubiquitin-activating enzyme E1, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase. The E2 and E3 enzymes are the key components in the ubiquitin pathway, as they are responsible for substrate recognition and ubiquitination [2]. In human there are a few E1s, around 30 E2s, hundreds of E3s and thousands of protein targets. Even though an E2 enzyme can recognize different E3s, an E3 enzyme can recognize only few E2s. This specific interaction of E2 with E3 enzymes determines the ubiquitination activity of E3, but binding between E2–E3 pairs is not always sufficient for function [3]. Arkadia and Arkadia2C proteins act as E3 Ubiquitin ligases via their C-terminal RING domains. Specifically Arkadia is a positive regulator of the Transforming Growth Factor-β pathway, while Arkadia2C is implicated in the Bone Morphogenetic Protein pathway [4, 5]. The enzymatic activity of both proteins depends on the interaction of the RING domain with the E2 enzyme UbcH5B [6, 7]. UbcH7 is an E2 enzyme, which exhibits small sequence similarity with UbcH5B [8]. Titrations experiments of Arkadia and Arkadia2C proteins with UbcH5B and UbcH7 revealed their interaction properties.

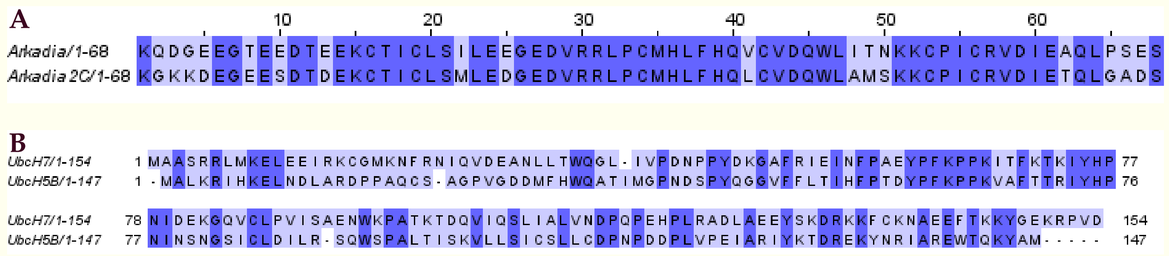

Figure 1: A: Sequence alignment of Arkadia (927-994 a.a.) and Arkadia2C (279-346 a.a.) RING domains, **B**. Sequence alignment of E2 enzymes, UbcH7 and UbcH5B.

The aim of this work:

In the present study, UbcH7 was expressed in high yield and its ability to interact with Arkadia and Arkadia2C was studied. NMR titrations experiments were performed, as well as biochemical tests, in order to determine the capability of this E2 enzyme to interact with Arkadia and Arkadia2C E3 enzymes. This study provides further insight into the selectivity and specificity of interactions between E2 and E3 enzymes in the ubiquitin pathway.

Methods:

All proteins were expressed in *E. coli* cells and purified by affinity chromatography. The interaction interface of RING domains and E2 enzymes (UbcH5B and UbcH7) was identified by NMR titration experiments of ^{15}N-labeled RING domains with unlabeled E2. According to established experimental procedures, after each addition of unlabeled protein a ^{1}H- ^{15}N HSQC spectrum was recorded. A final molar ratio of unlabeled/labeled protein of 1:2 was reached. Auto-ubiquitination assays (Boston Biochem – Ubiquitin Conjugation Kit K-960) were also performed, in order to study the functionality of each interaction. In these assays Arkadia2C long construct (237-346 a.a.) was used.

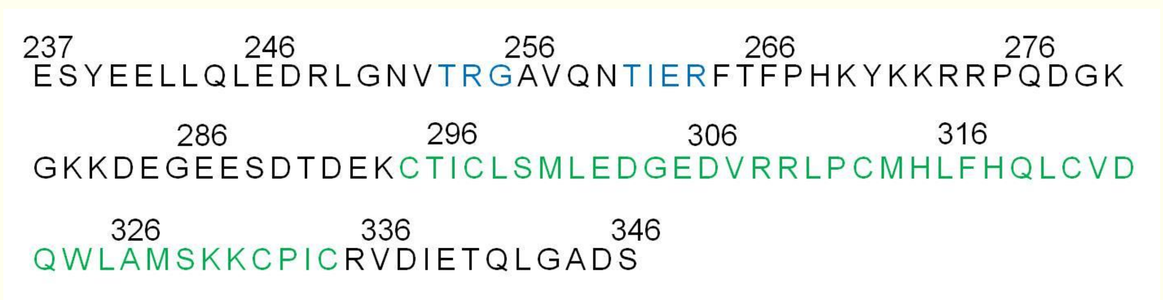

Figure 2: Sequence of Arkadia2C long construct (237-346 a.a.) : TRG-TIER domain (blue) and RING domain (green).

Results and Discussion:

The interaction interface of the RING domain of Arkadia and Arkadia2C with their E2 partner, the UbcH5b enzyme, was studied through chemical shift perturbations analysis in titration experiments [Fig. 3].

Chemical shift perturbations analysis of ^{15}N-labeled RING domains with unlabeled E2-UbcH7 shows that both Arkadia and Arkadia2C interact with E2-UbcH7 [Fig.4]. In addition, a comparison between the RING domains residues implicated in interaction with UbcH5B and UbcH7, shows that the interaction interface is similar in both cases. Consequently, both RING domains interact with the two E2 enzymes in a structurally similar way and with similar affinity.

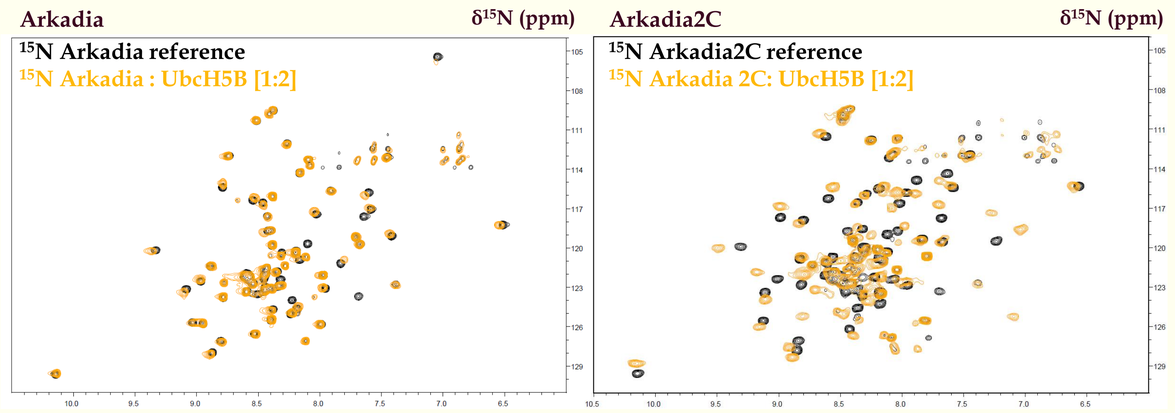

Figure 3: 2D [^{1}H-^{15}N] HSQC spectra of titration of Arkadia and Arkadia2C RING domain with E2-UbcH5B respectively.

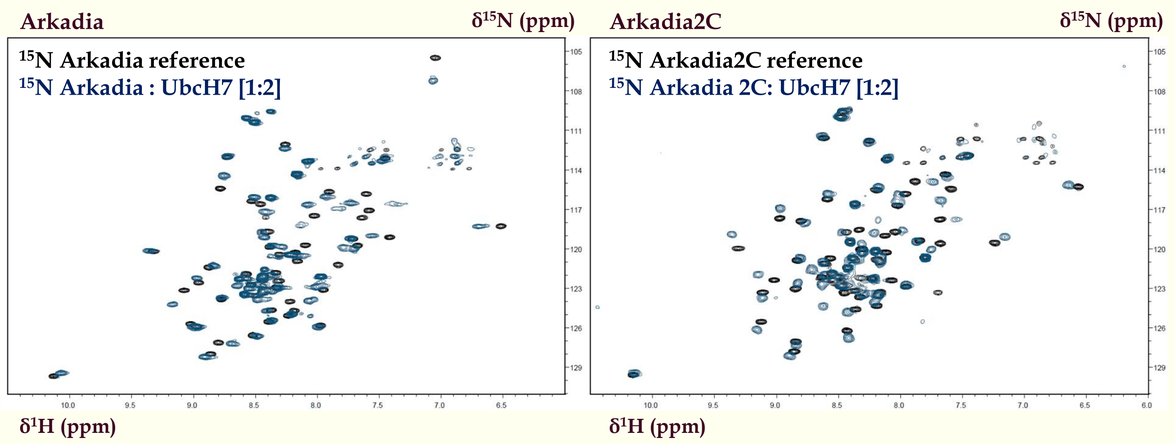

Figure 4: 2D [^{1}H-^{15}N] HSQC spectra of titration of Arkadia and Arkadia2C RING domain with E2-UbcH7 respectively.

Autoubiquitination assays show that UbcH5B can mediate the *in vitro* autoubiquitination of Arkadia2C but UbcH7 cannot, even though it interacts with its RING domain [Fig.5]. These results confirm that the E2-E3 interaction is not always sufficient for E3 ligase activity. E2-UbcH7 can also interact with E3 ligases c-Cbl and BRCA1/BARD1, but it was inactive in ubiquitination assays [3, 9]. Investigation of the mechanism underlying the E2-E3 interaction is still a challenge.

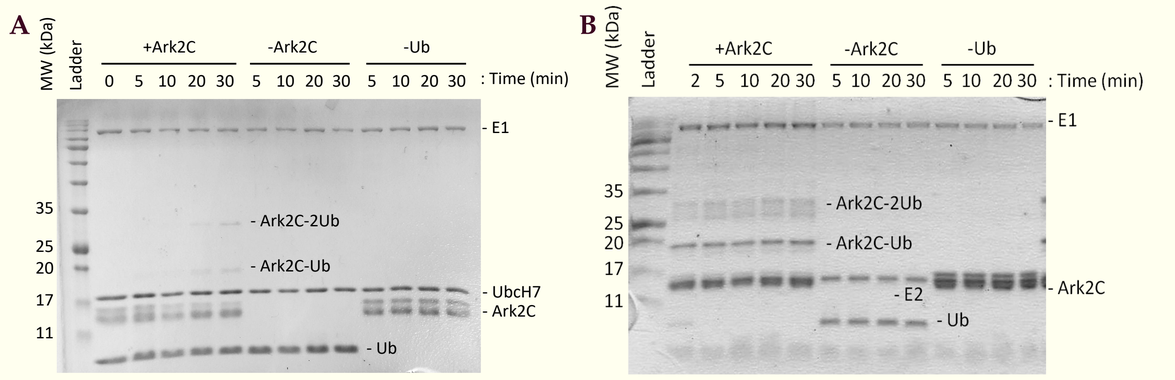

Figure 5: Autoubiquitination of Arkadia2C (237-346 a.a.) with UbcH7 **(A)** and UbcH5B **(B)**.

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