



CAPNS1 REGULATES USP1 STABILITY AND STEM CELLS MAINTENANCE

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Abstract — Calpains are a family of calcium-related cysteine-proteases that are involved in a wide number of cellular processes. The ubiquitous calpains, micro- and milli-calpain, are heterodimers composed of catalytic subunits and a common regulatory subunit, encoded by CAPNS1. We identified USP1 deubiquitinase as a CAPNS1-interacting protein. USP1 is a key modulator of DNA repair, partly through deubiquitination of its known targets FANCD2 and PCNA. Usp1 knockout mice have a severe phenotype and die soon after birth. Usp1^{-/-} cells are defective in FANCD2 focus formation and are hypersensitive to DNA damage. PCNA ubiquitination is higher in USP1-depleted cells than in control cells, thus leading to recruitment of error-prone, translesion DNA synthesis (TLS) polymerases and the consequent increase in mutation rate. USP1 promotes inhibitor of DNA binding (ID) protein stability and stem cell-like characteristics in osteosarcoma and is required for normal skeletogenesis. We found that the ubiquitinated form of the USP1 substrate PCNA is stabilized in CAPNS1-depleted U2OS cells and mouse embryonic fibroblasts (MEFs), favoring polymerase- η loading on chromatin and increased mutagenesis. USP1 degradation directed by the cell cycle regulator APC/C^{cdh1}, which marks USP1 for destruction in the G1 phase, is upregulated in CAPNS1-depleted cells. USP1 stability can be rescued upon forced expression of calpain-activated Cdk5/p25, previously reported as a cdh1 repressor. Our data suggest a connection between the calpain system and the ubiquitin pathway in the regulation of DNA damage response and place calpain at the interface between cell cycle modulation and DNA repair.

Index Terms — Calpain, USP1, PCNA, ID proteins, APC/C^{cdh1}, breast cancer

1 BACKGROUND

Calpains regulate a wide spectrum of biological functions crucial for cancer development, including migration, adhesion, apoptosis, and autophagy, through the modulating cleavage of specific substrates. Ubiquitous microcalpain (μ -calpain) and millicalpain (m-calpain) are heterodimers composed of

catalytic subunits encoded, respectively, by CAPN1 and CAPN2 and a regulatory subunit encoded by CAPNS1. We have shown that calpain is required for the stability of the deubiquitinating enzyme USP1 in several cell lines. USP1 modulates DNA replication polymerase choice and repair by deubiquitinating PCNA. Moreover, USP1 promotes inhibitor of DNA binding (ID) protein stability and stem cell-like characteristics in osteosarcoma and is required for normal skeletogenesis. USP1 is a “druggable” protein for differentiation therapy. Indeed, USP1 knockdown in osteosarcoma cells determined ID2 protein destabilization, cell cycle arrest, and osteogenic differentiation.

2 OBJECTIVES

- Characterization of calpain regulation of USP1, mediated by a direct cleavage and by the regulation of APC/Ccdh1 complex
- Study of the role of calpains in breast cancer, and in particular their involvement in stemness regulation
- Development of novel therapeutic strategies based on USP1 and/or calpain modulation.

3 APPROACH & METHODS

General approach

We used a biochemical approach to characterize the study of USP1 cleavages mediated by calpain, and a molecular approach to dissect the pathway of USP1 proteasomal degradation mediated by APC/Ccdh1 complex.

Mammospheres formation assay was performed to understand the role of calpain in neoplastic progression in breast cancer.

Methods

1. CAPNS1 silencing to deplete calpain expression and activity; 2. Cell cycle synchronization; 3. Cycloheximide assay; 4. Mammospheres formation assay

4 RESULTS

We investigated the effect of calpain depletion on USP1 protein stability in human osteosarcoma U2OS cells using siRNA transfection. We studied the effect of calpain depletion both under basal conditions and upon UV light irradiation, inducing USP1 proteasomal turnover. After siRNA transfection, the cells were left untreated, or exposed to 30 J/m² UV light, and lysates collected at different time points after irradiation. CAPNS1 depletion is coupled to a reduction in USP1 protein level, in both untreated and irradiated cells (Fig. 1a). Similar results were obtained using other osteosarcoma cell lines (Fig. 1b and 1c).

USP1 is a target of APC/Ccdh1 which marks it for proteasomal degradation in the G1 phase of the cell cycle. Therefore we investigated whether the instability of USP1 occurring in CAPNS1 depleted cells could be linked to an increase in APC/Ccdh1 activity toward USP1. To this end we knocked down cdh1 in CAPNS1 depleted cells and found that Cdh1 silencing rescues USP1 stability, both in an asynchronous population and in late G1 enriched cells, collected 16 hours after mimosine addition (Fig. 2a). Calpain stabilizes USP1 specifically in late G1, indeed USP1 is not affected by CAPNS1 depletion in G2/M arrested cells, obtained by incubation with nocodazol for 16 hrs (Fig. 2b). Altogether these results indicate that calpain specifically acts as a brake for USP1 degradation at the G1/S border.

USP1 has been described as a druggable protein for differentiation therapy. Indeed, USP1 knockdown in osteosarcoma cells determined ID2 protein destabilization, cell-cycle arrest, and osteogenic differentiation.

We found that CAPNS1 depletion in osteosarcoma cells is linked to USP1 destabilization in a *cdh1*/Cdk5 dependent manner. As expected, CAPNS1 silencing, just as USP1 depletion, is coupled to ID2 protein decrease, and subsequent p21 accumulation (Fig. 3).

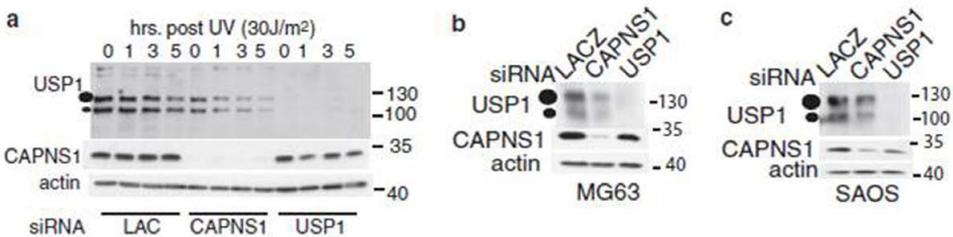


Figure 1: Calpain is required for USP1 stability in different cell lines. Panel a. U2OS cells; Panel b. MG63 cells; Panel c. SAOS cells.

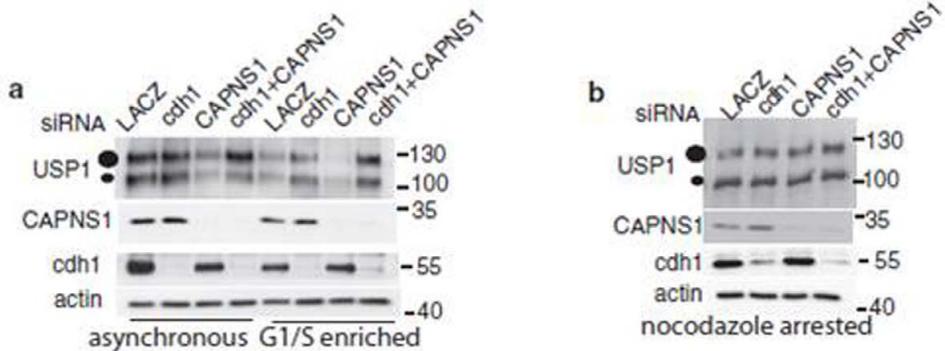


Figure 2: CAPNS1 modulates USP1 protein via APC/C^{*cdh1*} complex in G1/S phase. Panel a. U2OS cells, asynchronous or arrested after mimosine treatment; Panel b. U2OS cells arrested in M phase after nocodazole treatment.

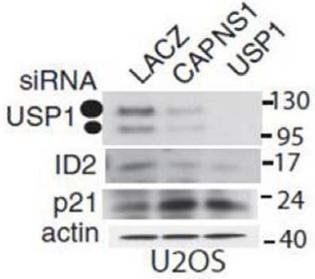


Figure 3: CAPNS1 silencing is coupled to ID2 protein decrease and subsequent p21 accumulation.

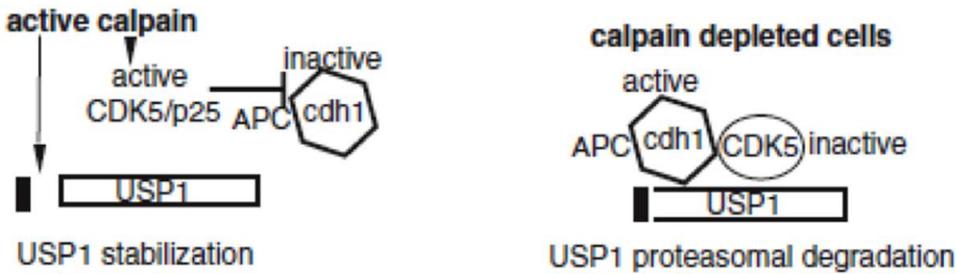


Figure 4: Working model. Active calpain can cleave USP1 at the very N-terminus. This cleavage product is less susceptible to APC/C^{cdh1} interaction or ubiquitination. Calpain may activate Cdk5/p25, which in turn inhibits APC/C^{cdh1}, further preserving USP1 stability.

Altogether the results presented here, support a model schematized in Fig. 4. Calpain cleaves USP1 at the very N-terminus resulting in its stabilization. In addition, calpain activates Cdk5/p25 that acts as a brake for APC/C^{cdh1} dependent ubiquitination of USP1, further stabilizing the protein. In the absence of the stabilizing cleavage exerted by calpain, USP1 is more prone to ubiquitination by APC/C^{cdh1}.

5 POTENTIAL NEW PRODUCTS & SERVICES

Since we found that calpain inhibition is coupled to USP1 instability, we may speculate that calpain inhibitors may represent convenient drugs for specific cancers where the calpain/Cdk5/cdh1/USP1 axis is active. We plan to investigate the feasibility of a therapeutic approach for osteosarcoma based on calpain inhibition. In addition, since USP1 inhibitors have been proposed as potential drugs for non-small-cell lung carcinoma, also in this type of cancers calpain inhibitors might be considered for combination therapy trials.

Moreover, the studies performed in human mammary epithelial cell lines point to find new drugs that, interfering with calpain function, can disrupt tumour progression in breast cancer, acting on the survival of cancer stem cells population.

6 CURRENT COLLABORATIONS

6.1 With other researchers

Dr. Paola Storici - Elettra - Sincrotrone Trieste S.C.p.A., Italy

Dr. Ario de Marco – University of Nova Gorica, Slovenija

Dr. Jan Mavri – Center of Excellence for Biosensors, Instrumentation and Process Control (COBIK), Ajdovščina, Slovenija

Prof. Claudio Brancolini – Department of Medical and Biological Sciences – University of Udine, Italy

7 CONTACT OR COLLABORATIONS NEEDED

To apply the results we obtained, we plan to collaborate with a company or other researchers that have developed a strategy to convey drugs that modulate calpain activity to specific cancer cells in vivo, for example calpain or USP1 inhibitors in osteosarcoma cells.

Moreover, we plan to screen a great number of chemical compounds that interfere with calpain activity (for example, calpain activator thapsigargin) and perform in vivo analysis to test if they perturb tumour growth and progression in mice.

Furthermore, a collaboration with clinicians could be useful to evaluate alterations in calpain and USP1 protein levels in tumour samples.

8 COMMUNICATION TOOLS

PUBLICATIONS:

- CAPNS1 regulates USP1 stability and maintenance of genome integrity. Cataldo F, Peche LY, Klaric E, Brancolini C, Myers MP, Demarchi F, Schneider C. - Mol Cell Biol. 2013 Jun;33(12):2485-96. doi: 10.1128/MCB.01406-12. Epub 2013 Apr 15.
- DNA damage response links calpain to cellular senescence. Demarchi F, Cataldo F, Bertoli C, Schneider C. - Cell Cycle. 2010 Feb 15;9(4):755-60. Epub 2010 Feb 16.
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9 FUNDS NEEDED

9.1 For basic research (investigation of biological mechanisms): 100.000 €

9.2 For applied research (solutions for real-world problems): 50.000 €

10 CONCLUSION

In this study we have unveiled a calpain/cdh1/USP1 axis, and we have found a stabilizing role of calpain for USP1 deubiquitinating enzyme in physiological conditions. Further studies are ongoing to dissect the CAPNS1-USP1-cdh1 interaction and reciprocal regulation under pathological conditions linked to APC/Cdh1 impairment and/or calcium overload. These studies will deepen our understanding of the molecular basis by which the calpain system can enhance cancer cell survival and may be instrumental for the design of novel drugs for specific diseases. Moreover we found a role of calpain in the regulation of stemness maintenance in a breast cancer cell model, that is worthy to be further investigated.

ACKNOWLEDGEMENT

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REFERENCES

[1] CAPNS1 regulates USP1 stability and the maintenance of genome integrity. Cataldo F, Peche L, Klaric E, Brancolini C, Myers M, Demarchi F and Schneider C. Molecular and Cellular Biology; 33(12):2485-96; June 2013