論文の内容の要旨

Transcriptional Regulation of the Mammalian 26S Proteasome – A Genome-wide Perspective

（哺乳類の２６Ｓプロテアソームの転写調節機構の研究）

コグレク　ラリッサ

[Introduction]

The mammalian 26S proteasome (Figure 1) is a large ATP-dependent protease complex responsible for the targeted intracellular degradation of ubiquitin tagged proteins, thereby playing a vital role in regulating the cell cycle as well as maintaining cellular homeostasis. Malfunctions in the proteasome pathway have been associated with neurodegenerative diseases like Huntington's and Parkinson's, and several types of cancer, making the proteasome a very attractive therapeutic target. So far therapeutic development has been focused on functional impairment using drugs such as bortezomib, but recently interest has grown in exploiting other parts of the proteasome pathway, such as directly manipulating proteasomal gene expression.

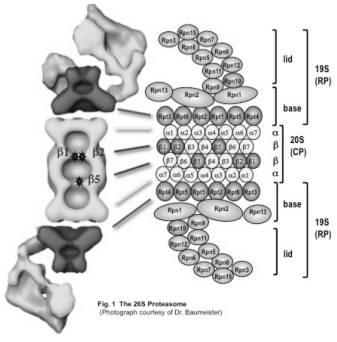


Figure 1 The 26S Proteasome
Four stacked hepta-heteromeric rings (α1-7 and β1-7) make up the 20S core which is capped by the 19S regulatory particle on either end. Catalytic activity is conveyed by β1, β2 and β5.

At present, little is known about the transcriptional regulation of proteasome subunit genes in mammals. The most prominent general transcription factor candidate to date is Nuclear Factor (Erythroid-Derived 2)-Like 1 Protein 1 (NFE2L1, also known as Nrf1). The Nrf pathway appears to be involved in the transcriptional regulation of proteasomal gene expression when proteasome function is impaired and under cellular stress, initiating the 'bounce-back response', which results in a drastic increase of proteasome subunits. However, its effect on the basal expression of proteasomal subunit genes appears limited, suggesting that other yet unknown transcription factors are required for regulating the basal gene expression of the 26S proteasome. To date, the molecular mechanism governing proteasomal gene expression in normal cells remains unresolved.

To address this issue, I tried to identify potential transcriptional modulators of the mammalian 26S proteasome on a genome-wide basis by applying a novel approach using an in vitro luciferase reporter assay. Screening a human siRNA library consisting of almost 20,000 genes, I have been able to select a pool of potential basal transcription modulators. From amongst them, I have been able to show that GC-rich promoter binding protein 1-like 1 (GPBP1L1), a paralog of the GPBP1 transcription factor, is the most promising transcriptional modulator necessary for the regulation of basal expression of the mammalian 26S proteasome.

[Results]

1. A Human Genome wide screen yielded GPBP1L1 as the strongest basal transcriptional modulator candidate for the mammalian 26S proteasome.

Luciferase assays are a proven method to characterise transcriptional modulators due to their exquisite sensitivity, enabling detection of even the slightest effects on gene expression. However, previously luciferase assays have been used only on a small scale, due to their relative labour intensiveness, whereas genome-wide screens have so far used other methods, trading sensitivity for labour efficiency. This study is the first to employ an in vitro firefly luciferase reporter assay system to screen a human genomic siRNA library containing 20,000 genes in order to identify potential transcription modulator candidates regulating the basal expression of the 26S proteasome in mammals. To achieve this, I measured the effect of RNAi of each sample on luminescence levels in cells stably expressing *Psmb5* (subunit β5, chymotrypsin-like catalytic activity) promoter driven luciferase (*luc2*) during multiple screenings. Combined with data of cell viability assays and statistic analyses I was able to identify 48 potential transcriptional modulator candidates. Based on information about their known or potential functions, domain structures and pathways, a final 23 were considered for further analysis (Figure 2 A&B). Amongst these 23 candidates, qPCR analysis revealed that GPBP1L1 had the strongest and most consistent effect on PSMB5 mRNA levels (Figure 2A). This effect was repeatedly shown in both HeLa and 293T cells and was independent of the siRNA construct.

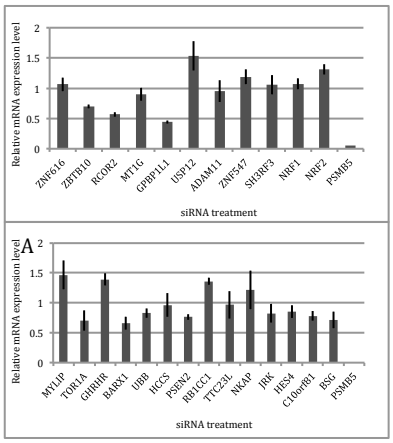


Figure 2 Transcriptional modulator candidates affect relative PSMB5 mRNA expression levels
qPCR data are represented for each siRNA sample from an Ambion Custom pooled siRNA library, consisting of the 23 transcriptional modulator candidates selected during previous screens. Columns represent average ±SD of three independent measurements normalised to the negative control. PSMB5 siRNA was used as a positive control and scrambled siRNA as a negative control. Nrf1 and Nrf2 siRNA was used as an independent control. All data were measured against GUSB. **(A)** 293T cells **(B)** HeLa cells

2. Only GPBP1L1 but not its paralog GPBP1 affects mRNA expression levels of both 20S and 19S subunit genes.

In order to confirm whether the effect of GPBP1L1 on proteasomal gene expression extends to other proteasomal subunit genes besides PSMB5, I analysed proteasome subunit genes in both α- and β-rings of the

20S core particle as well as those of the base and lid complexes of the 19S regulatory particle (Figure 3, left). qPCR analysis showed that the knockdown of GPBP1L1 has a consistent effect on subunits from all proteasome components. Moreover, to determine whether this effect is specific to GPBP1L1, I investigated whether its more widely known paralog vasculin (GPBP1) affected proteasomal gene expression levels in a similar way. qPCR analysis revealed that only RNAi of GPBP1L1 but not GPBP1 affects mRNA expression levels of proteasome subunit genes (Figure 3, centre and right). These findings suggest that GPBP1L1 may indeed be a transcriptional modulator of proteasomal gene expression, a function it does not share with other members of the same protein family.

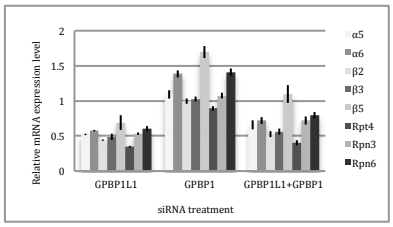
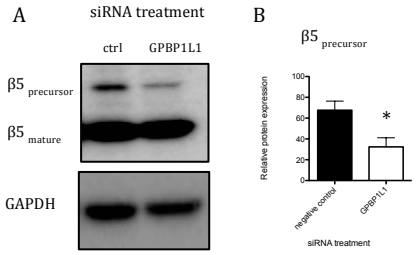


Figure 3 RNAi of GPBP1L1 but not GPBP1 affects expression levels of PSM subunit genes
Relative PSM subunit mRNA expression levels in HeLa cells after 52hrs single or double siRNA treatment. qPCR data represent average ±SD of three independent measurements normalised to the negative control. All data were measured against GUSB.

3. GPBP1L1 affects the novel synthesis of β5.

To assess whether GPBP1L1 also affects the actual protein levels of proteasome subunits, I performed Western Blot analyses. Knockdown of GPBP1L1 significantly decreased protein levels of newly synthesised premature β5 (Figure 4). This finding supports the hypothesis that GPBP1L1 plays a role in the regulatory mechanism of the mammalian 26S proteasome, directly affecting not only transcription levels of the different subunit genes, but also the rate of novel protein synthesis.



*p-value of Student's t-test <0.05

Figure 4 RNAi of GPBP1L1 affects protein expression levels of newly synthesised β5
(A) Western Blot analysis. Whole-cell extracts of 293T cells treated with GPBP1L1 or negative control siRNA were subjected to Western Blot analysis. GAPDH acts as loading control. Reproducibility was confirmed in separate experiment **(B)** Band intensity quantification of $\beta5_{precursor}$. Band intensity data represent the average ±SD of two independent experiments.

4. GPBP1L1 affects the catalytic activity of both the 20S and the 26S proteasome.

To find out whether GPBP1L1 has further functional implications on the proteasome I investigated the effect of GPBP1L1 knockdown on the catalytic activity of the 20S and 26S proteasome. Assaying chymotrypsin-like activity in whole-cell extracts of 293T cells treated with either GPBP1L1 siRNA or negative control siRNA, I revealed that GPBP1L1 knockdown strongly decreases catalytic function of both the 20S as well as the 26S proteasome compared to the negative control (Figure 5). These findings hint at the possibility that the knockdown of GPBP1L1 results in a halt in the novel synthesis of proteasomes, leading to this subsequently observed lower overall catalytic activity compared to the negative control under basal conditions.

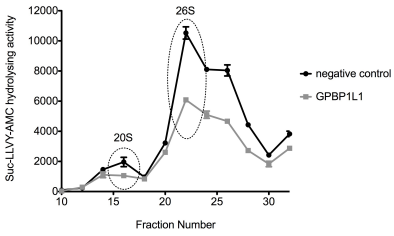


Figure 5 RNAi of GPBP1L1 affects 20S and 26S proteasomal catalytic activity
Whole-cell extracts of 293T cells treated with GPBP1L1 or negative control siRNA were subjected to glycerol gradient centrifugation and assayed for Suc-LLVY-AMC hydrolysing activity. Data represent the average ±SD of two independent experiments.

5. GPBP1L1 only affects basal but not induced expression levels of proteasomal genes.

To further characterise the relationship between GPBP1L1 and the proteasomal subunits, I investigated whether GPBP1L1 also has an effect on induced expression levels caused by cellular stress responses upon functional inhibition of the proteasome. To this end, I performed a comparative analysis between GPBP1L1 and Nrf1. qPCR analysis showed that under basal conditions, both GPBP1L1 and Nrf1 RNAi showed a similar effect on proteasomal expression levels (Figure 6A, left and centre). Knockdown of both GPBP1L1 and Nrf1 simultaneously resulted in an even more pronounced decrease of proteasomal gene expression (Figure 6A, right),

giving the first suggestion that the two genes might lie on separate regulatory pathways, each of which is necessary for basal expressional regulation. Upon treatment with the proteasome inhibitor MG132, as expected, control cells showed an increase in proteasomal gene expression as part of the bounce back response (Figure 6B, right). This increase, again expectedly, was completely abolished when the cells were treated with Nrf1 siRNA (Figure 6B, centre left). However, when the cells were treated with GPBP1L1 siRNA, no effect was observed (Figure 6B, left). This result, taken together with the fact that knockdown of both genes showed the same effect as a single Nrf1 knockdown (Figure 6B, centre right), suggests that only Nrf1, but not GPBP1L1 is involved in the up-regulated transcriptional stress response of the proteasome subunits. Taken together, this finding gives further strength to the hypothesis that GPBP1L1 may be a necessary factor involved exclusively in the basal regulation of proteasomal gene expression.

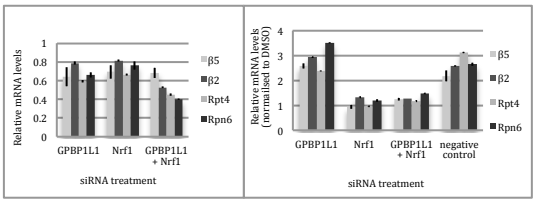


Figure 6 Effect of GPBP1L1 and Nrf1 on basal and induced proteasomal gene expression levels
Relative proteasome subunit mRNA expression levels in HeLa cells after 52hrs single or double siRNA treatment and 10hrs inhibitor/control treatment. qPCR data represent the average ±SD of three independent measurements. DMSO treated negative control was set to 1. All data were measured against GUSB. **(A)** Treatment with DMSO **(B)** Treatment with 0.5 µM MG132, data normalized to DMSO negative control set to 1.

[Discussion & Summary]

Transcriptional regulation of the proteasome has long been an unsolved mystery. Whereas RPN4 is the universal transcription factor of the proteasome in yeast, there appear to be no homologs in higher organisms. Given the evolving complexity of the proteasome as one moves further up the evolutionary ladder it stands to reason that together with more complex functions a more complex mechanism regulating both basal and induced expression is required as well. Whilst the Nrf pathway seems to control induced proteasomal expression in response to cellular stress, it appears that other regulatory pathways exclusive to higher organisms are required to ensure that the proteasome can successfully carry out its various functions. Based on the data above, GPBP1L1, a gene exclusive to vertebrates, is potentially a good choice for the regulation of basal proteasomal expression in mammals. Overall, this study represents a first step towards understanding the molecular mechanism governing basal proteasomal gene expression, showing that in particular research focusing on GPBP1L1 and its involvement in proteasomal regulation warrants further exploration.