Abstract no: [123]

Optimisation of methodologies for comprehensive screening of host proteins binding to Hepatitis B virus X protein

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Background

The hepatitis B virus (HBV) encodes for 7 main proteins including the hepatitis B virus X protein (HBx) protein. HBx consists of 154 amino acids and has important functions in viral replication and pathogenesis. As such, there are great merits in obtaining a comprehensive profile of host proteins binding with HBx. This could be achieved by introducing HBx as a "bait" along with a tag (common tags include flag,myc & biotin) into host cells. These bait-prey complex would then be sieved via pull-down assays and an interactome profile could then be obtained through mass-spectrometry (MS) using these complexes. Given that the post-processing of MS results is rather tedious and time-consuming, it is imperative that processes (for eg, maximizing the expressions of bait-tag construct) leading up to MS are optimised.

Objectives

Optimisation of two methodologies (affinity-based pull down & proximity labelling) for mass-spectrometry-based interactome study.

Methods

HepG2-NTCP cells were transfected using two different transfection reagents, X-tremeGENE™ 360 (Roche) & Lipofectamine™ 3000 (ThermoFisher Scientific) using different amounts (6ug & 12ug) of pXJ-flag-HBx plasmid per 6 cm dish. In addition, the cells were incubated with transfection mixtures at two different periods (24hrs and 48hrs). Transfection efficiencies of the conditions were then analysed using Western blot. Using ImageJ, the intensities of the bands were quantified, and the optimal condition was selected. Subsequently, HBx expressing cells were subjected to affinity purification-mass spectrometry (AP-MS) to identify host-interacting proteins. Gene ontology analysis was performed for these newly identified HBx interactors. Similarly, transfection was performed using plasmids encoding for two different biotin ligases, TurboID & miniTurbo (both fused with HBx). The TurboID is a highly active biotinylation enzyme (Branon et al. Efficient proximity labelling in living cells and organisms with TurboID. Nat Biotechnol. 2018;36(9):880–7) and may be more suitable for capturing weak or dynamic interactions involving HBx. As for the miniTurbo, it is a smaller variant of TurboID. 50uM of biotin were then supplemented into the transfected cells and allowed to incubate for three different timepoints (10mins, 1hr & 3hrs). The extent of the biotinylation achieved by these two ligases at different timepoints were then depicted through Western blot using Streptavidin conjugated-HRP. For both sets of optimisations, an empty vector was included as a negative control.

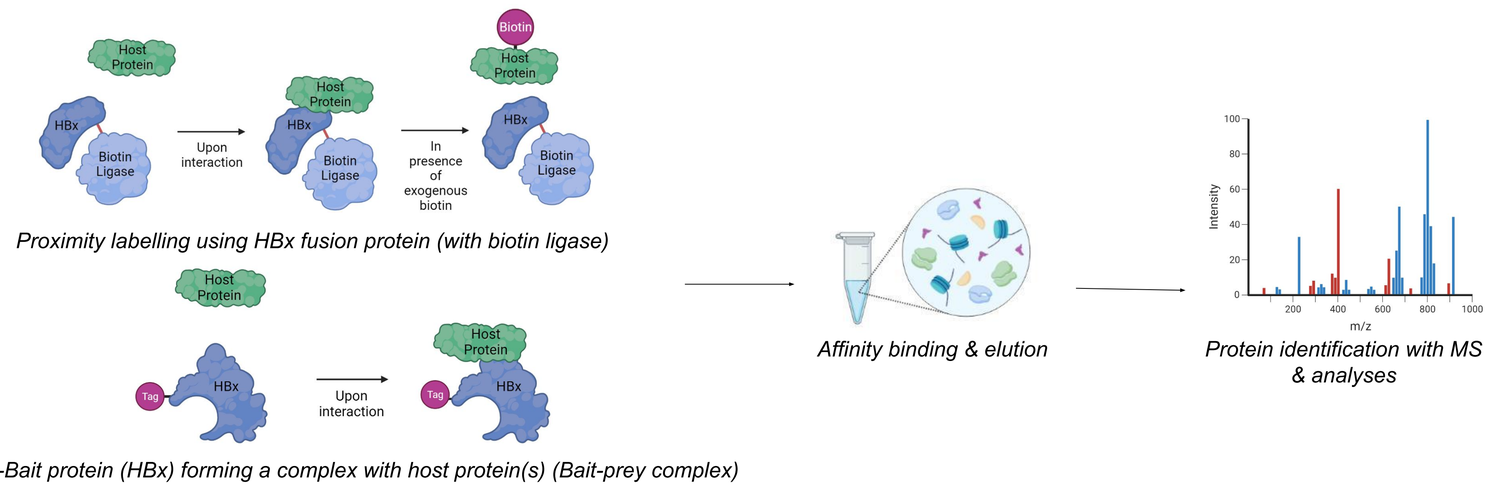

Fig.1. Schematic of our intended workflow for obtaining HBx interactome. Illustrations were created with BioRender.

Results

Maximizing the expression of HBx

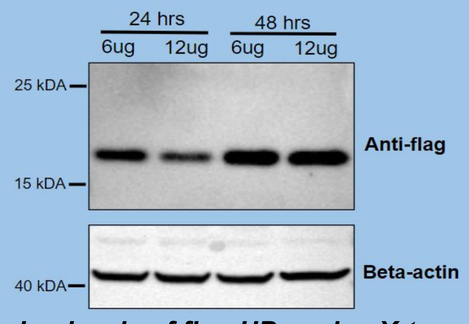

Fig 2a. Expression levels of flag-HBx using X-treme Gene and different amounts of plasmid & timepoints.

As shown in Fig 2a, the amount of recombinant HBx was the highest in the cell lysate 48 hrs after transfection and there is no significant increase in recombinant HBx when a higher amount of plasmid was used.

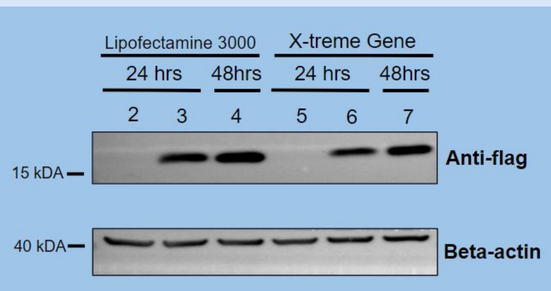

Fig 2b. Expression levels of flag-HBx using different transfection reagents & timepoints. Lanes 2 and 5 were transfected with empty vector while the rest were transfected with flag-HBx plasmid.

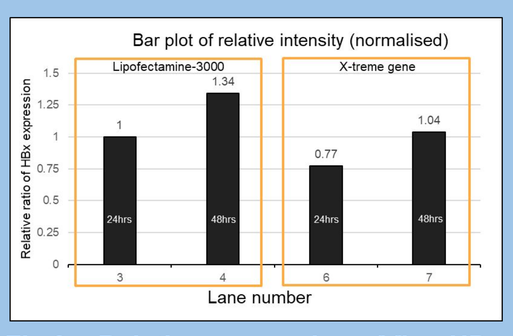

Fig 2c. Relative expression of flag-HBx

Based on Fig 2b and c, 48 hrs of incubation seems to yield a higher expression of HBx compared to 24 hrs. However, it is also worth noting that there was a higher cell death observed at longer incubations. Therefore, as to minimise cell death and at the same time not compromising the expression of HBx, the combination of using Lipofectamine 3000 and an incubation time of 24 hrs was chosen as the most optimal conditions for transfection.

Optimizing proximity labelling

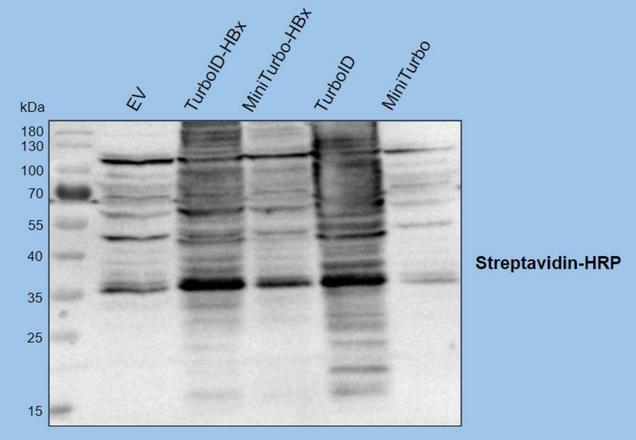

Fig 3a. Biotinylated protein profiles of different biotin ligase-fused & unfused when treated with biotin for 10 mins

Biotinylated profiles originating from TurboID with & without HBx were relatively comparable suggesting TurboID's promiscuity in proximity labelling. This leads us to believe that TurboID might not be an appropriate candidate for proximity labelling (PL), thus turning our attention to the alternative, miniTurbo.

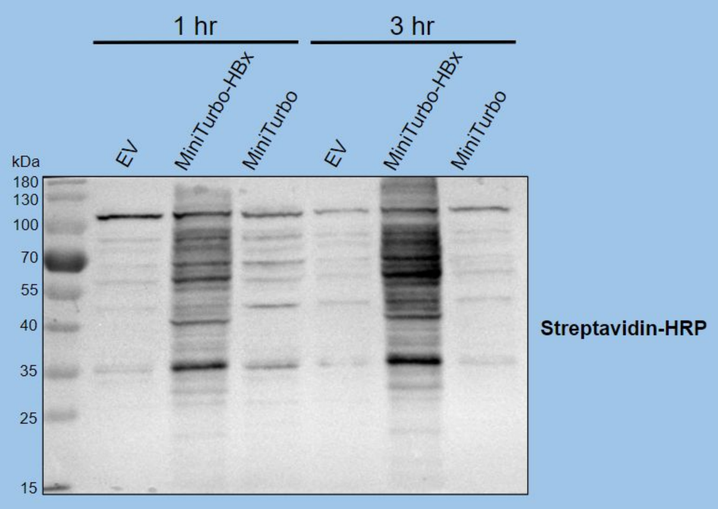

Fig 3b. Levels of biotinylated cellular proteins after treatment with biotin for 1 hr & 3 hrs

Based on fig 3a, a treatment of biotin for 10 mins was not sufficient to elucidate miniTurbo's activity visually when compared to the negative control. Additional time points were then included to establish the limits of treatment required. A stark difference in biotinylated profiles could be observed between miniTurbo-HBx & the negative control (fig 3b).

Gene ontology analysis

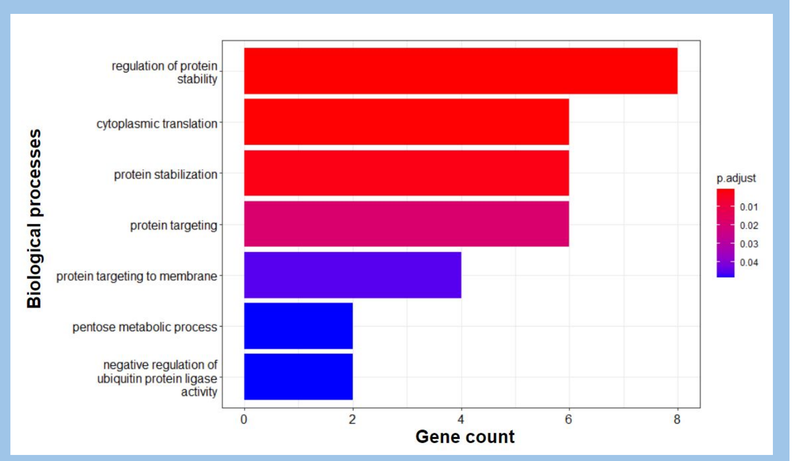

Fig 4. Gene ontology (GO) analysis (biological processes) of host proteins interacting with flag-HBx in HepG2-NTCP transfected cells

Lysates from HepG2-NTCP transfected with flag-HBx were subjected to affinity pull-down assay & HBx interactors were identified using MS. This set of identified proteins were then compared with another set of proteins that originated from cells transfected with the empty vector. Host proteins binding specifically to HBx were determined by comparing these two datasets and GO analysis was performed using "clusterProfiler", a package from Rstudio.

Conclusion & future works

From our experiments, we have managed to obtain the optimal conditions to maximize the amount of "bait", in this case HBx, to be expressed in the host cell. Using AP-MS, we identified ~40 host proteins interacting with HBx in transfected HepG2-NTCP cells. GO analysis reveals that most of these proteins are related to the regulation of protein stability or translation in the cytoplasm. Ongoing work is focused on determining if these host proteins are involved in regulating HBV replication or pathogenesis. In the future, we will also upscale our experiments and use proximity labelling to identify additional HBx interacting host factors.

Acknowledgements

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