The predicted NES in PBF appears to be functional *in vitro*

Poole VL, Smith VE, Ryan GA, Gilligan LC, Seed RI, Sharma N, Read ML, Boelaert K, and McCabe CJ

School of Clinical and Experimental Medicine, University of Birmingham, Birmingham, UK.

Background

Pituitary tumor-transforming gene (PTTG) binding factor (PBF) is a proto-oncogene which is frequently upregulated in endocrine cancers. It is a 22kDa protein, made up of 180 amino acids, that is highly conserved in a variety of species (73% homology to mouse, 67% frog, 60% chicken), and yet shares no homology with other human proteins[1].

PBF has previously been determined to contain several putative signal sequences within its 180 amino acids, and previous studies have shown the nuclear localisation signal (NLS) to be functional[2]. Prediction software (CBS Prediction Servers) has suggested PBF to have a nuclear export signal (NES) between amino acids 17-27; the signal is comprised of a run of hydrophobic leucines and fits the consensus sequence of LXXXLXXLXL, where "L" is a hydrophobic residue (often leucine) and "X" is any other amino acid. Although the software predicted the NES to be present, it is unknown whether the sequence is actually functional or under the regulation of exportin-1.

Exportin-1, often referred to as CRM1 or XPO1, is the main protein responsible for exporting proteins containing leucine rich NES from the nucleus. Structural studies have shown that CRM1 is a ring-shaped protein that binds the leucine-rich helix of substrates specifically into a hydrophobic groove along its central convex centre. The CRM1 specific inhibitor Leptomycin B has been shown to inhibit function by binding to cysteine 529 which is located in the hydrophobic groove, thereby blocking substrate binding[3].

Aim

To investigate whether the nuclear export signal (NES) in PBF is functional and under the regulation of exportin-1.

Methods

CRM1 was disrupted using two methods; specific siRNA knockdown and use of the inhibitor Leptomycin B.

Immunofluorescence was used to investigate protein subcellular localisation under different conditions. The peptide sequences were also entered into the CBS prediction server to see if they contained a predicted nuclear export sequence.

The sequences of PBF in four different species and the predicted sequences of PBF in another three species were obtained from the National Center for Biotechnology Information (NCBI) Homologene tool. The sequences were aligned to allow easy comparison using the NCBI Constraint-based Multiple Alignment Tool (COBALT).

Disruption of CRM1

siRNA

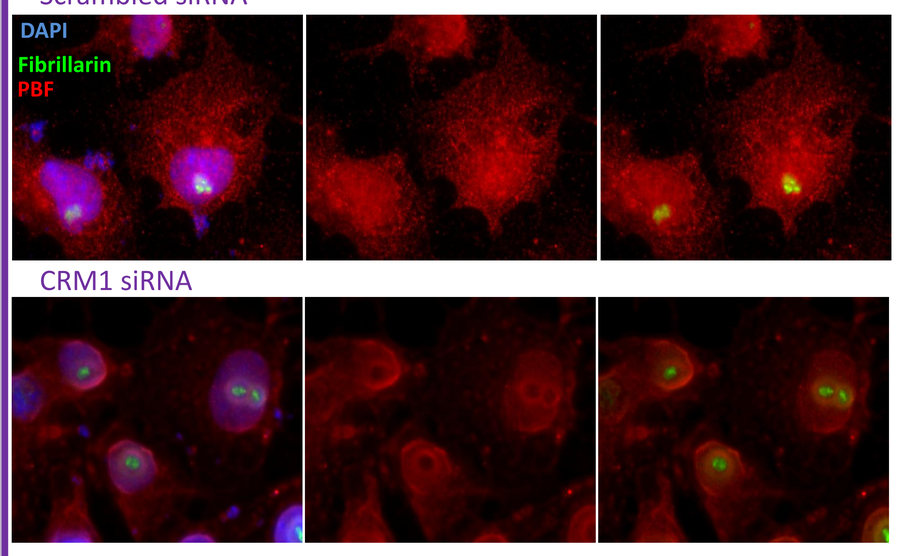


Figure 1 - Knockdown of CRM1 decreases cytoplasmic PBF – When CRM1 was knocked down there appeared to be less cytoplasmic and nucleolar PBF compared to control. Knockdown of CRM1 in COS7 cells led to reduced staining for PBF in the cytoplasm and also appeared to block PBF's entry to the nucleolus. With scrambled siRNA, there appeared to be colocalisation between PBF and fibrillarin (a nucleolus marker) depicted by the yellow staining. However when CRM1 levels were depleted there was no colocalisation and PBF appeared to form halo-like structures around the periphery of the nucleolus.

Leptomycin B

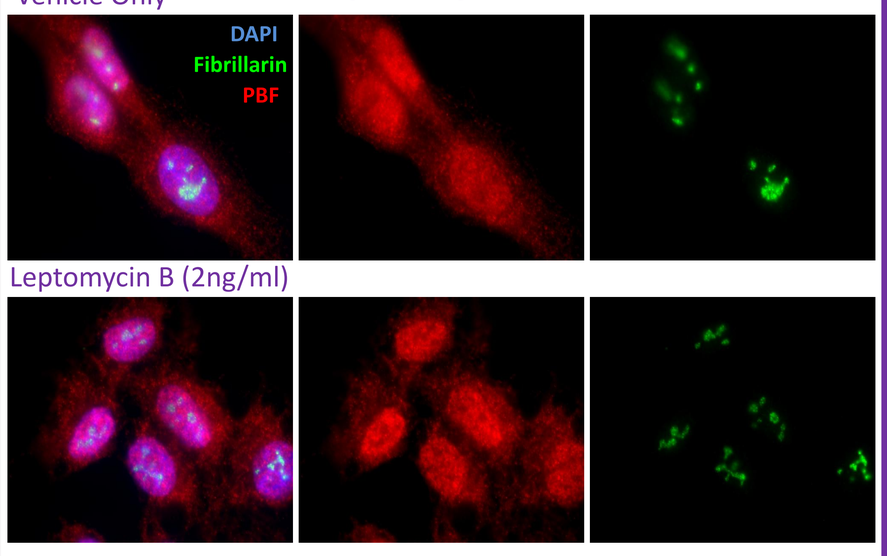
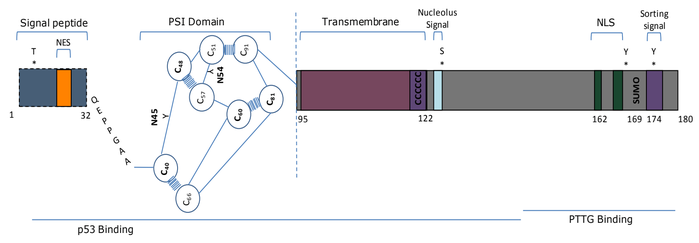


Figure 2 - Leptomycin B increased nuclear PBF staining and excluded PBF from the nucleolus – After treating K1 cells with Leptomycin B (2ng/ml) for 2 hours, there appeared to be more nuclear PBF along with a higher frequency of unstained regions that correspond with the nucleolus. However, unlike the siRNA treatment, there was no accumulation of PBF at the boundary of the nucleolus.

Homology

Figure 3 – Structure of PBF showing the location of the predicted NES



All of the species, except *Gallus gallus*, had sequences that contained an NES score that exceeded the threshold of 0.5 on the NES criterion[4]. The most resounding NES prediction was present in human PBF, with an average NES score of 0.69 over the 11 consensus NES residues. The positioning of the potentially involved amino acids shows the high level of homology between PBF sequences among different species. However, although most of the sequences contained amino acids predicted to participate in a nuclear export signal, most of the species contain only one or two residues expected to be involved, suggesting the signal is unlikely to be functional in most of the species.

Figure 4 – NES Prediction charts for PBF in 7 species

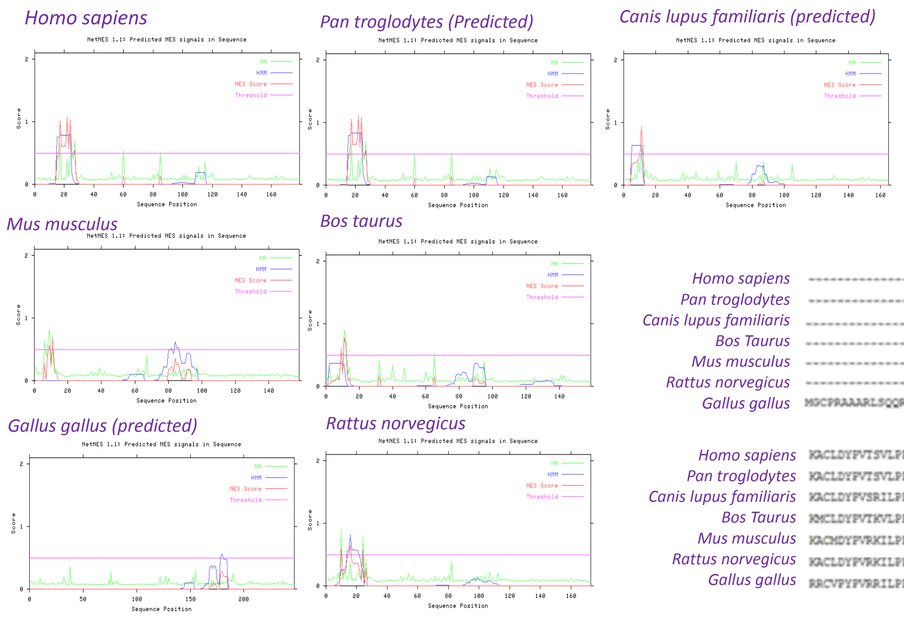
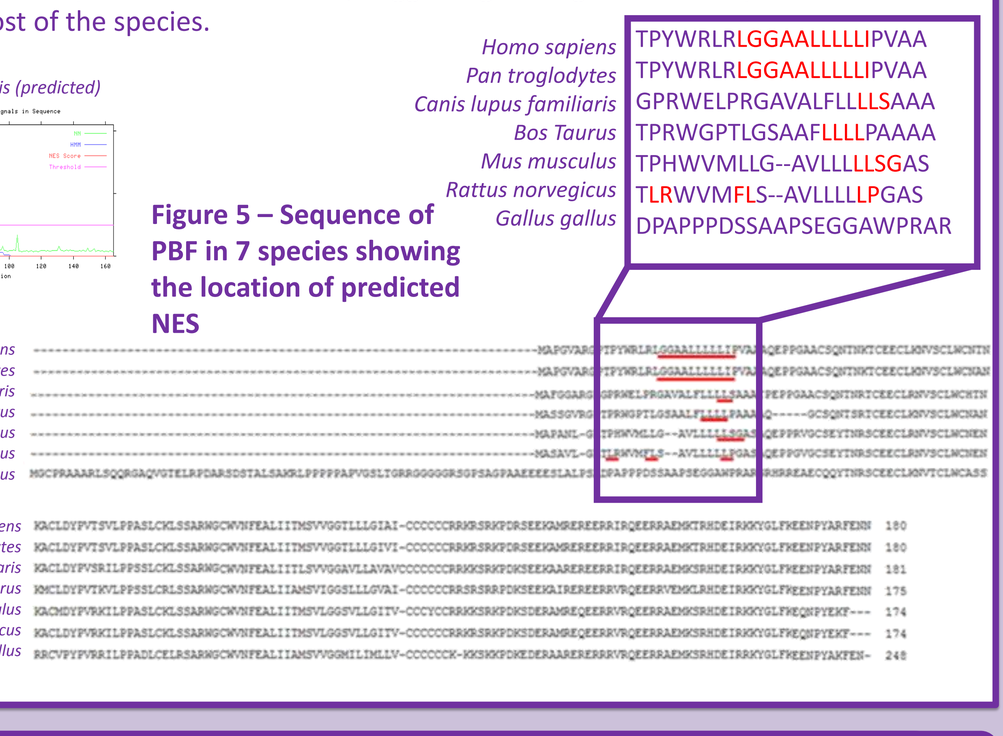


Figure 5 – Sequence of PBF in 7 species showing the location of predicted NES



Conclusions

Knockdown of CRM1 led to decreased cytoplasmic staining for PBF, and areas of PBF exclusion that correlated with the location of the nucleolus. Inhibition of CRM1 also resulted in similar staining , with increased nuclear staining and decreased nucleolar staining. These data suggest the NES of PBF is functional and under regulation of CRM1. CRM1 has previously been described to cluster in the nucleoli forming CNoBs (CRM1 Nucleolar Bodies) that can complex with other proteins[5]. If PBF were to complex with these CNoBs, this may explain the decreased nucleolar staining when CRM1 is depleted.

Although a putative NES is present in six of the seven species analysed, the homology of the NES among species is low suggesting the NES may not be functional in all species.

However, the prediction observed in *Homo Sapiens* along with the *in vitro* data suggest the NES of PBF may be functional and play an important role in the export of PBF from the nucleus and entry into the nucleolus. PBF's putative NES is located within the signal peptide, as this domain may be cleaved, it is predicted only the full length form of PBF would participate in nuclear trafficking.

Contact: Vikki Poole
E-mail: VLP840@bham.ac.uk

University of Birmingham CEDAM
Centre for Endocrinology, Diabetes and Metabolism

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MRC Medical Research Council