University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme

Summer Studentship

Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. **Student**

   Surname: Ritakari **Forename:** Tuula

   E-mail address: 2035874R@student.gla.ac.uk

2. **Supervisor:**

   Surname: Cobb **Forename:** Stuart

   E-mail address: stuart.cobb@glasgow.ac.uk

3. **Research Project Report**

   3.1 Project Title (maximum 20 words):

   Testing a recombinant protein therapy approach in Rett Syndrome

   3.2 Project Lay Summary (copied from application):

   The proposed project is to investigate whether a protein therapy is viable strategy in the neurological condition Rett Syndrome (RTT). Tuula would join an active project making recombinant forms of MeCP2 (the protein mutated in RTT), which have been modified in various ways to facilitate transport across the blood-brain barrier and into neurons. The objective of the experiment is to assess first in cultured neurons and then in

experimental mice, the efficiency by which therapeutic recombinant protein enters neurons/the brain and whether once inside cells, it assumes it correct position heterochromatin. The project will provide training in various cellular neuroscience techniques.

3.3 **Start Date:** 3.6.2014 **Finish Date:** 25.7.2014

3.4 Original project aims and objectives (100 words max):

The aim of this study is to test for the production of a secreted form of MeCP2 and its uptake by recipient target cells. This will give us information of use in subsequent design of gene/protein therapy vectors for use in Mecp2 KO mice in vivo.

3.5 Methodology: Summarise and include reference to training received in research methods etc. (250 words max):

3.5.1. *Production of recombinant protein*. Cells were transformed with an MeCP2/Flag/RFP/APOB construct using heat shock. Cells were subcultured and induced with IPTG to produce the protein at mid-log phase (confirmed by measuring the optical density at 600nm). Cells were lysed by a freeze-thaw method, and insoluble and soluble fractions were separated by centrifuging.

3.5.2. *SDS-PAGE gels*. Protein was quantified using a Bradford protein assay. Samples were electrophoresed on a pre-cast gel. Protein was either visualised with Coomassie staining or Western blotting.

3.5.3. *Western blotting*. Gels were transferred onto a polyvinylidene difluoride membrane. The membrane was and incubated overnight with anti-His antibodies. Next day, the membrane was washed and incubated with IRDye conjugated secondary antibodies. These were detected using Odyssey Sa infrared imaging system (Licor Biosciences).

3.5.5. *Protein purification*.

Protein was extracted from cell lysate using both His-purification and ion exchange chromatography. His purification was carried out with a nickel column using both uniform and graded salt concentrations. Cation exchange chromatography was done with an automated chromatography purification system.

3.5.6. *Mammalian cell cultures*

HEK-293 cells were split and $5x10^4$ cells were seeded onto coverslips coated with Poly-L Lysine. Protein was added to the wells and cells were incubated overnight. Cells were fixed and labelled with anti-MeCP2 antibodies. Coverslips were examined using a confocal microscope and images processed with ImageJ software.

3.6 Results: Summarise key findings (300 words max). Please include any relevant tables or images as an appendix to this report:

Bacteria were transformed with a plasmid containing a MeCP2/Flag/RFP/APOB construct. Strains of E. coli (BL21(DE3), BL21(DE3)CodonPlus-RIL (RIL) and BL21(DE3)CodonPlus-RIPL (RIPL)) were transformed with the plasmid, and successful bacteria were selected for using agar plates containing antibiotics. Two colonies of each strain were sub-cultured, one of which was induced to produce the protein and the other left as a control. A western blot comparing the cultures confirmed that the protein was expressed in induced cultures, although expression was leaky and protein fragments were also present (Figure 1).

Protein solubility was examined by lysing cells and separating the insoluble and soluble fractions. A western blot of lysis products indicated that the recombinant protein was partly soluble (Figure 2). At this point, it was decided to continue with only RIPL-strain cells.

The protein was then purified. Protein was extracted form the lysate using His-tag purification, first using a uniform and then graded salt concentration. SDS-PAGE electrophoresis confirmed that the purifications had been successful, and that efficiency was higher using a graded salt concentration (Figures 3 and 4). Cation exchange chromatography was then performed for a higher degree of purity. The elution profile indicated that the protein of interest was extracted efficiently (Figure 5). An SDS-PAGE gel run with the products of purification confirmed these findings (Figure 6).

Functionality of the protein was tested by culturing HEK-293T cells in its presence. Cells were seeded onto coverslips and grown overnight in growth medium with protein concentrations of 3 to 280 ug/ml. Confocal images of cells labelled with fluorescent anti-MeCP2 antibodies indicated that the protein was not taken up into cell nuclei as had been expected (Figure 7). Instead, highly fluorescent clusters could be detected in areas without cells, with clustering increasing with protein concentration.

3.7 Discussion (500 words max):

Protein replacement therapy remains a little studied approach of treating Rett syndrome. The present study has attempted to produce recombinant MeCP2 fused with an ApoB-motif for this purpose using a prokaryotic expression system. However, although protein expression in E. coli seemed to be successful, it remains unclear whether the manufactured protein is functional and suitable for therapeutic use.

Recombinant MeCP2 containing an ApoB motif was successfully manufactured using BL21(DE3) strain E. coli. The protein was found to be partly soluble, with smaller protein fragments being detectable in the lysate. Protein breakdown and aggregation is a relatively common problem with overexpression of recombinant protein in E. coli, as the

metabolic burden leads to protein misfolding and cleavage[1]. This could therefore be possibly improved by slowing down expression by methods such as induction at earlier stages and lower temperatures[2]. Alternatively, it may also be that the inefficiency in protein expression was due to a codon bias. Overcoming this would require methods such as codon optimisation or co-transformation with a plasmid coding for the rare codons[3,4]. Nevertheless, it is possible that prokaryotic expression is not well suitable for recombinant MeCP2, and that other expression systems should be looked into.

The protein was successfully purified using both His-tag purification and ion exchange chromatography. His-tag purification using a graded salt concentration was found to be a more efficient than a uniform salt concentration, although cation-exchange chromatography was still required to reach a high level of purity. Even after purifying the lysate twice much of the protein still remained in the flow through of the lysate; therefore, using a higher capacity column could be advisable.

However, it remains unclear whether the manufactured protein is suitable for therapeutic purposes. The protein was not taken up by HEK-293T cells grown in its presence; instead, the protein seemed to form clusters outside of cells, with the size and number of aggregates increasing with protein concentration. This could be due to a number of reasons; for instance, the protein may have been cleaved by proteases in the serum; this could be fixed by transferring cells into serum-free media before adding the protein. However, it may also be that the protein is not correctly folded, which could be either due to problems with the expression system or post-production stages.

[1] Baneyx, F., & Mujacic, M., 2004. Recombinant protein folding and misfolding in Escherichia coli. *Nature biotechnology*, *22*(11), pp.1399-1408.
[2] San-Miguel, T., Pérez-Bermúdez, P., & Gavidia, I., 2013. Production of soluble eukaryotic recombinant proteins in E. coli is favoured in early log-phase cultures induced at low temperature. *SpringerPlus*, *2*(1), pp. 1-4.
[3] Calderone, T. L., Stevens, R. D., & Oas, T. G., 1996. High-level Misincorporation of Lysine for Arginine at AGA Codons in a Fusion Protein Expressed in Escherichia coli. *Journal of molecular biology*, *262*(4), pp. 407-412.
[4] Dieci, G., Bottarelli, L., Ballabeni, A., & Ottonello, S., 2000. tRNA-assisted overproduction of eukaryotic ribosomal proteins. *Protein expression and purification*, *18*(3), pp. 346-354.

Performing tests for protein stability[5] and checking for correctly folded protein using monodispersity[6] before proceeding with purification could help identify the problem, after which appropriate measures in either expression or purification stages could be taken.

In conclusion, this study has attempted to manufacture a MeCP2-ApoB fusion protein in order to investigate the viability of a protein replacement therapy for treating Rett Syndrome. Although the protein seemed to be successfully expressed in E. coli, the produced protein was not taken up by mammalian cells, which suggests that the protein may be non-functional and unsuitable for therapeutic purposes. Different approaches to recombinant MeCP2 expression and purification should therefore be tried out in order to produce a recombinant protein suitable for treating Rett syndrome.

4. Reflection by the student on the experience and value of the studentship (300 words max):

This studentship has been an invaluable experience for me, and I am more than glad to have taken part in it. Having no prior experience in doing laboratorial work apart from my university course, this has taught me a lot about what is really involved in doing scientific research.

In terms of laboratorial techniques I have certainly come a long way since my first day, when even melting a jar of agar seemed a daunting task. During my placement, I learned a range of methods from Western blots to fixing cells; furthermore, as I became more familiar with these techniques I was allowed to work with relative independence, which has certainly boosted my confidence when it comes to doing practical work. I have no doubt these skills will prove very useful later in my studies.

[5] Mezzasalma, T. M., Kranz, J. K., Chan, W., Struble, G. T., Schalk-Hihi, C., Deckman, I. C., ... & Todd, M. J., 2007. Enhancing recombinant protein quality and yield by protein stability profiling. J*ournal of biomolecular screening, 12*(3), pp.418-428.

[6] Nominé, Y., Ristriani, T., Laurent, C., Lefèvre, J. F., Weiss, É., & Travé, G., 2001. A strategy for optimizing the monodispersity of fusion proteins: application to purification of recombinant HPV E6 oncoprotein. *Protein engineering*, *14*(4), pp. 297-305.

The placement has also taught me a lot about scientific research as a career. I now have a much better understanding of the laboratory as a workplace, and the nature of scientific research in general. In addition, having been able to see the kind of work that Master's and PhD students doing, as well as to ask them a few questions here and there, has helped me gain a much better picture of the path that one needs to take in order to establish a career in such a field.

Altogether, this studentship has been a very fun and educational experience for me, and I warmly recommend it to any undergraduate student with even the slightest interest in research. It has given me the kind of practical knowledge that I could hardly learn anywhere else, and I look very much forward to any future opportunities of working in a laboratory. And, perhaps most importantly, it has helped me to realise with certainty that a PhD and a career in academic research are what I want to pursue after I finish my undergraduate degree.

5. **Dissemination: (note any presentations/publications submitted/planned from the work):**

6. **Signatures:**

Supervisor	Date	Student	Date
	13/8/2014	Tuula Ritakari	13/8/2014

APPENDIX

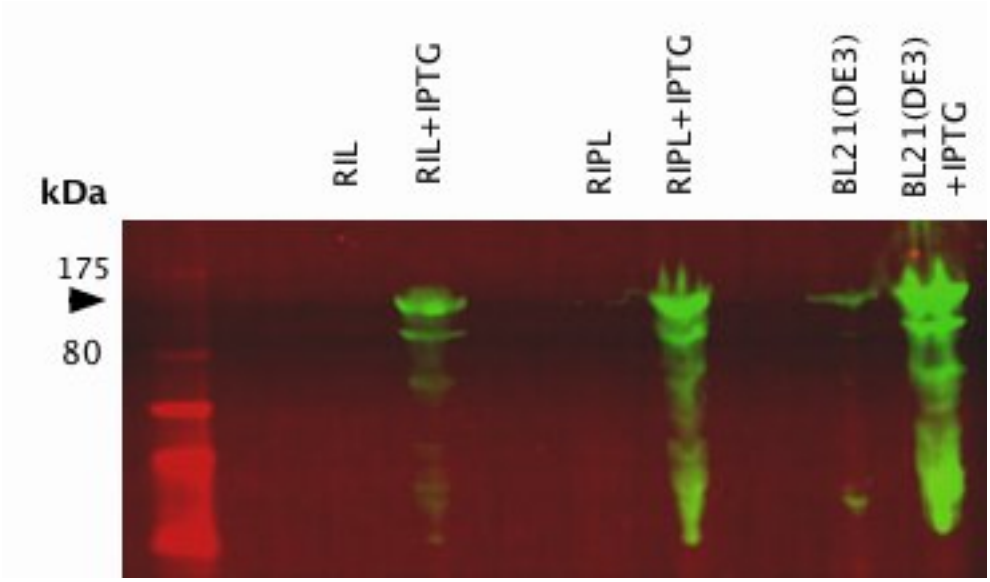


Figure 1 *MeCP2 production by bacterial cells.* Western blot comparing samples both from cultures induced to produce the recombinant protein and controls. The protein of interest, as indicated by the arrow, can be seen as strong bands in the range of 80-175 kDa. Weak bands at this level seen RIPL and BL21(DE3) control cultures indicate leaky expression of the protein. Additional bands below the protein of interest in all induced cultures indicate the presence of protein fragments.

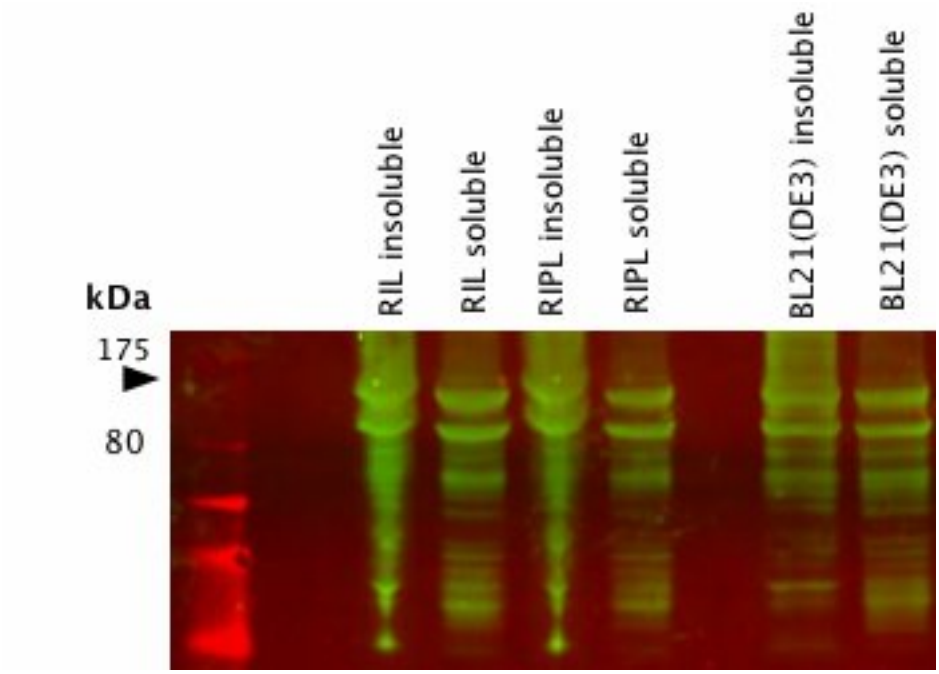


Figure 2 *A test for protein solubility.* A western blot comparing the soluble and insoluble fractions of cell lysates. Partial solubility is indicated by the presence of the protein of interest, indicated by the arrow, in all lanes. Additional bands below the band of interest indicate the presence of fragmented protein.

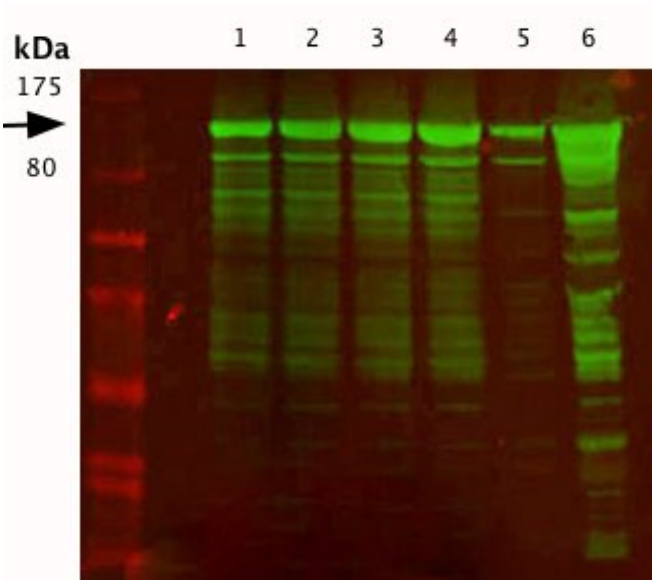


Figure 3 *His purification using a uniform salt gradient.* Western blot showing products of His purification. Lanes 1-4 are fractions of extracted protein, while lanes 5 and 6 contain the wash and lysate flow through, respectively. As indicated by the arrow, the protein of interest can be detected in all lanes, and in the flow through in particular. Additional bands below the band of interest indicate that the collected protein also contains smaller protein fragments.

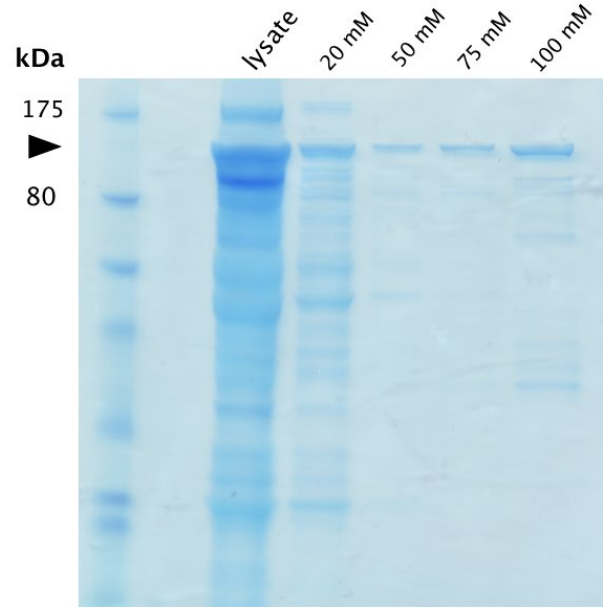
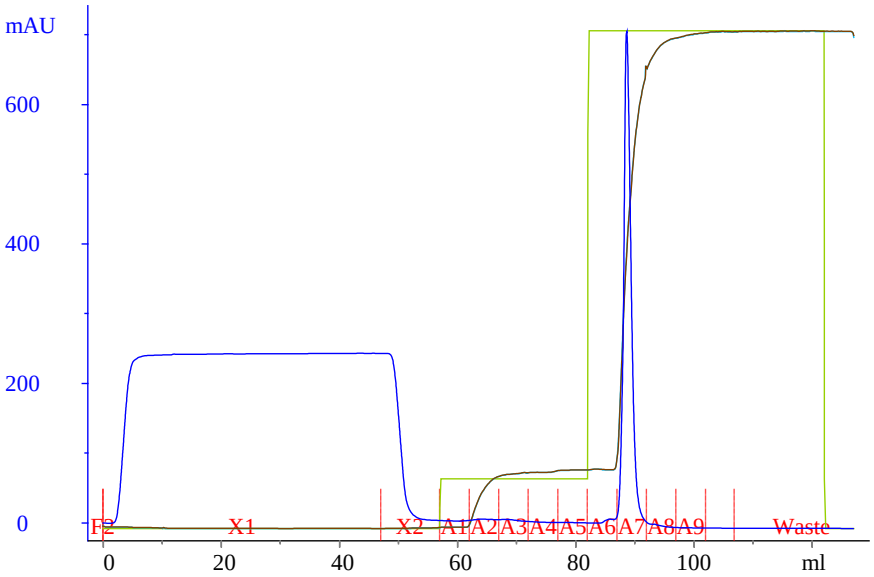


Figure 4 *His purification using a graded salt concentration*. Coomassie-stained SDS-PAGE gel showing the products of His purification using a graded salt concentration. The band of interest, indicated by the arrow, is found in all protein fractions, which indicates that the protein was successfully purified from the lysate. The diminished presence of additional bands at higher salt concentrations demonstrate increased purity of protein fractions.

a)



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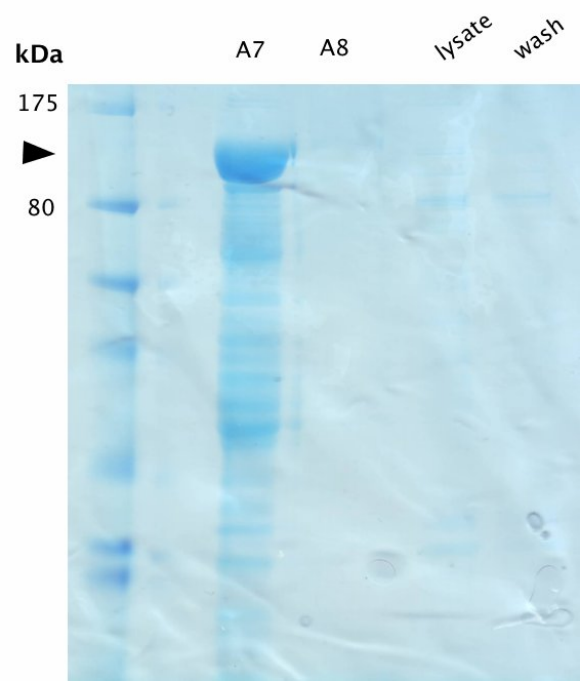
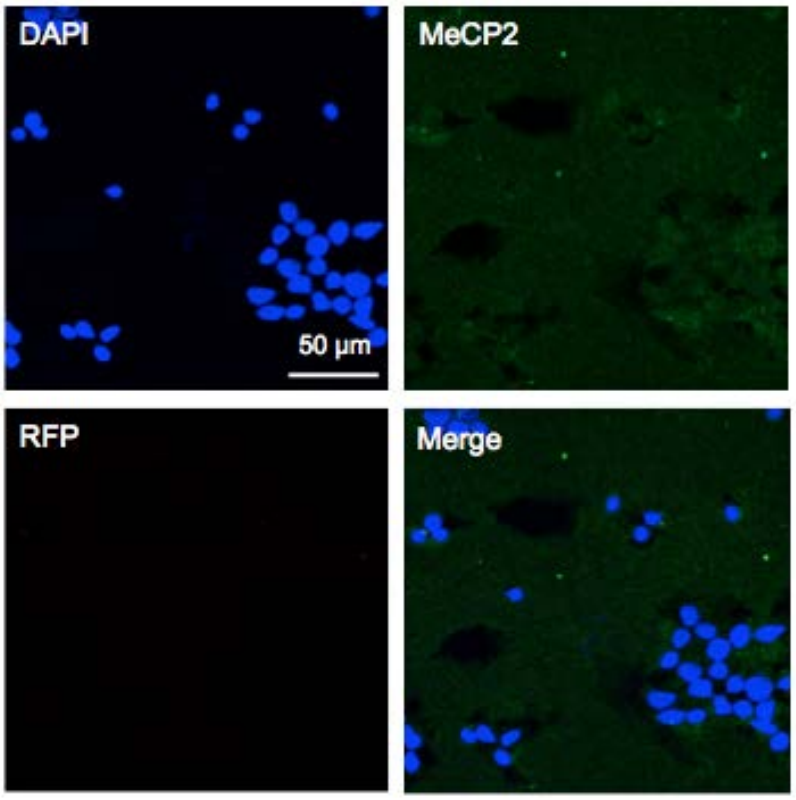


Figure 5 *Cation exchange chromatography of the purified protein*. **(a)** The elution profile of cation exchange chromatography. Blue line indicates absorbance of the sample, while green line is the concentration of elution buffer (10% from A1-A5, 100% from A6 onwards). The sharp, symmetrical peak at fraction A7 indicates that the protein of interest was purified form the sample with high efficiency. **(b)** A Coomassie-stained gel showing the results of the purification. The strong band in fraction A7 and its absence in other lanes indicate efficient purification. However, additional bands below the protein of interest suggest that fragmented protein is still present in the extract.

a)



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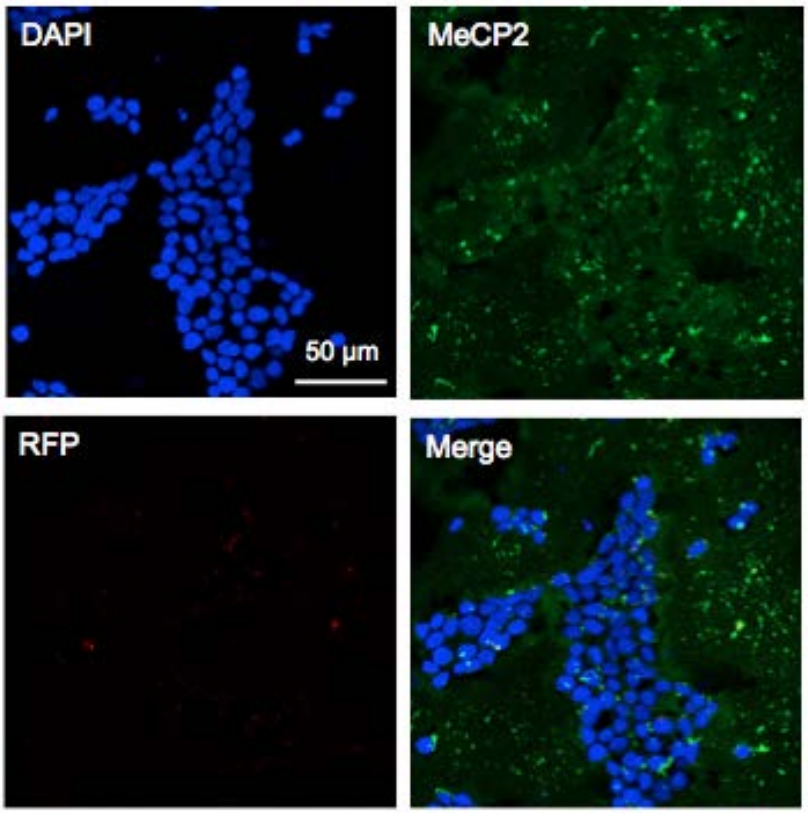

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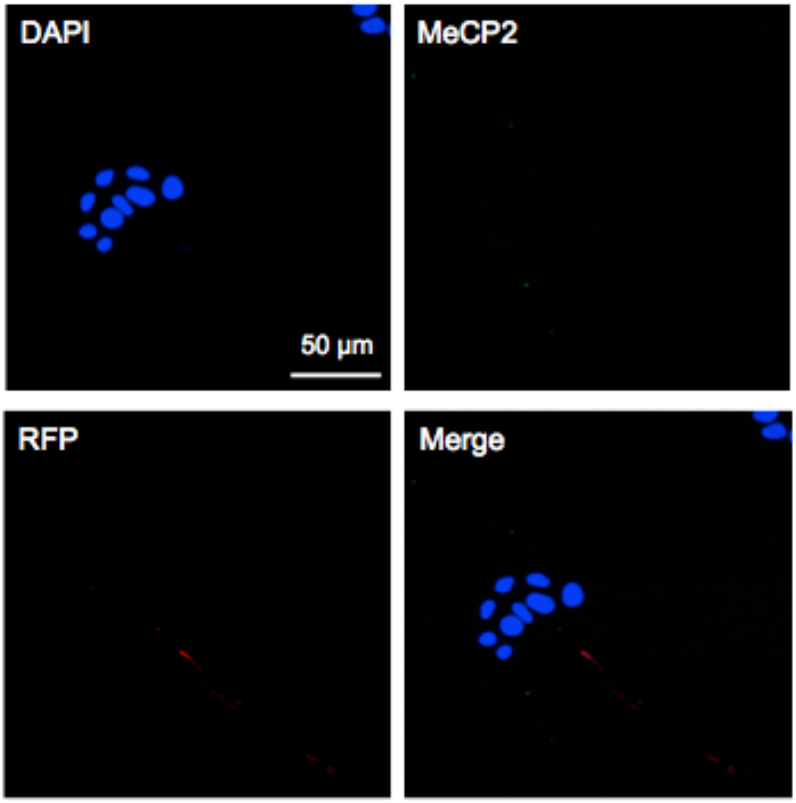

Figure 7 *Uptake of recombinant MeCP2 by HEK-293T cells*. Confocal images show HEK-293T cells cultured at protein concentrations of **(a)** 85ug/ml, **(b)** 200ug/ml, or **(c)** 0ug/ml. The location of green and red fluorescence indicates that the protein is not being taken up into cells. There is also an increase of clustered fluorescent areas outwith cells with increasing protein concentration.