TRANSLATION ELONGATION FACTOR EEF1A2 IS ESSENTIAL FOR POST-
WEANING SURVIVAL IN MICE.

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Running title: Translation elongation factor eEF1A2

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Translation elongation factor eEF1A1, formerly known as EF-1α, exists as two variant forms: eEF1A1, which is almost ubiquitously expressed, and eEF1A2, whose expression is restricted to muscle and brain at the level of whole tissues. Expression analysis of these genes has been complicated by a general lack of availability of antibodies that specifically recognise each variant form. Wasted mice (wst/wst) have a 15.8 kb deletion that abolishes activity of eEF1A2, but prior to this study it was unknown whether the deletion also affected neighbouring genes. We have generated a panel of anti-peptide antibodies and used them to show that eEF1A2 is expressed at high levels in specific cell types in tissues previously thought not to express this variant, such as pancreatic islet cells and enteroendocrine cells in colon crypts. Expression of eEF1A1 and eEF1A2 is shown to be generally mutually exclusive, and we relate the expression pattern of eEF1A2 to the phenotype seen in wasted mice. We then carried out a series of transgenic experiments to establish whether the expression of other gene(s) are affected by the deletion in wasted mice. We show that aspects of the phenotype such as motor neuron degeneration relate precisely to the relative expression of eEF1A1 and eEF1A2, whereas the immune system abnormalities are likely to result from a stress response. We conclude that loss of eEF1A2 function is solely responsible for the abnormalities seen in these mice.

Introduction
Translation elongation factor eEF1A1, formerly known as EF-1α, is the second most abundant protein in the cell; it is responsible for delivering aminoacylated tRNAs to the A site of the ribosome in a GTP dependent reaction. Unusually for a translation factors, eEF1A1 exists as two variant forms: eEF1A1, which is almost ubiquitously expressed, and eEF1A2, whose expression is much more restricted (1,2). The two variant forms are encoded by distinct genes on different chromosomes - EEF1A1 is on 6q13 in humans and chromosome 9 in mice, and EEF1A2 is on 20q13.3 in humans and distal chromosome 2 in mice (3). The encoded proteins are 92% identical and 98% similar, and eEF1A2 has been found in all mammals so far investigated, in chickens and in Xenopus (Newbery et al, submitted), but not in Drosophila or other lower organisms, suggesting that eEF1A2 is specific to vertebrates. eEF1A1 and eEF1A2 appear to function with equivalent properties in terms of their activity in an in vitro translation assay; the only measurable difference is seen in the GDP dissociation rate, which is 7 fold higher for eEF1A1 than for eEF1A2 (4). It has been found by yeast two hybrid analysis that eEF1A2, unlike eEF1A1, shows little or no affinity for the eEF1B guanine nucleotide exchange factors (5). It is well-established that eEF1A1, at least, has multiple non-canonical (or “moonlighting”) roles in addition to its role in translation. These non-canonical functions range from cytoskeletal modification (6), through targeting proteins...
for degradation (7), to involvement in the heat shock response (8). It is as yet unclear to what extent eEF1A2 shares these non-canonical functions; indeed, it has been shown in cultured myotubes that whilst eEF1A1 is pro-apoptotic, eEF1A2 has anti-apoptotic activity (9), suggesting that the two forms may have complementary non-canonical roles.

The two mammalian eEF1A variants have distinct and largely non-overlapping expression patterns. eEF1A1 is almost ubiquitously expressed. It is present throughout embryonic development, but is down-regulated in neonatal muscle and ultimately shut down in mouse muscle by 21 days after birth (10-12). In the brain eEF1A1 has been shown to be expressed in glial cells and is thought not to be expressed in neurons, although this has not been categorically demonstrated (11). eEF1A2, on the other hand, has only been found to be expressed in skeletal muscle, heart and neurons (1,2,4,13). In muscle from mice of 21 days eEF1A2 has completely replaced expression of eEF1A1 (10,11). It is not clear whether such a developmental switch exists in neurons, as extracts of whole brain show the presence of both isoforms (11). In NIH3T3 cells, in which eEF1A2 is not normally expressed, serum deprivation induces the expression of the gene (14). The expression of eEF1A2 thus seems to be associated with terminal differentiation and quiescence, but its precise role is unclear, nor is the reason for such tightly controlled switching between variants in muscle.

In recent years, two lines of evidence have implicated eEF1A2 in disease, suggesting that there may indeed be subtly different functions for the two forms of eEF1A. Firstly, EEF1A2 has been shown to be a potential oncogene (15); it is overexpressed in a proportion of ovarian tumours but is not expressed in normal ovary (15) and is similarly overexpressed in two-thirds of breast tumours but not normal breast tissue (16). In all these cases, eEF1A1 was also expressed in the tumours studied (and the tissue in which they arose), and in cell lines (16). We have shown that loss of activity of eEF1A2 is implicated in the phenotype of wasted mice (10). Wasted (wst) is a spontaneous autosomal recessive mutation of the mouse that arose in 1972 (17). Homozygous wasted mice are characterised by weight loss, tremors, gait abnormalities and spleen and thymus atrophy. All of these abnormalities arise after weaning, usually at 21 days. The mice then deteriorate rapidly and die by about 28 days (17); this timing is unaffected by genetic background or environmental influences (18). The spinal cords of wasted mice show vacuolar degeneration of motor neurons (19,20) and the loss of body weight is primarily accounted for by loss of muscle bulk (20,21). Thymuses and spleens from wasted mice show extensive, profound apoptosis (22); the spleen:body weight ratio of a wasted mouse is less than half that of a normal littermate by day 28 (17). We found through a positional candidate cloning approach that the genetic lesion in wasted mice is a 15.8 kb deletion (10). This deletion starts in the first intron of the gene encoding eEF1A2 (locus symbol Eef1a2), and then continues 5′, removing the first (non-coding) exon and all promoter elements. The other end of the deletion falls within a repetitive element. Sequencing of the 15.8 kb region deleted in the mice did not reveal the presence of any other gene. The loss of eEF1A2 activity fits with the muscular abnormalities: the onset of the abnormal phenotype coincides with the point at which eEF1A1 is no longer detectable in muscle (10), but no expression analysis of eEF1A1 and eEF1A2 has yet been carried out in spinal cord, the site of the major lesion in wasted mice. Since the primary genetic defect in wasted mice is a deletion, and as the only aspect of the phenotype that could readily be explained by known expression patterns is the muscle loss, we set out to establish the precise involvement of eEF1A2 in the wasted phenotype. We also wanted to evaluate the expression patterns of eEF1A1 and eEF1A2 at the level of immunohistochemistry, to see how well the expression patterns correlated with the wasted phenotype, and to assess the implications for eEF1A function.

In this study, we address three questions. Are there cells in normal tissues that co-express eEF1A1 and eEF1A2 or is their expression mutually exclusive? If certain cells do co-express, what is the effect...
of deleting eEF1A2? Is loss of eEF1A2 solely responsible for the wasted phenotype? In order to answer these questions, we developed a panel of antibodies that, unlike commercially available antibodies, specifically recognise the eEF1A1 and eEF1A2 variants. We describe the construction and characterisation of these antibodies, demonstrating their specificity. We use the antibodies to show that there are sites of expression of eEF1A2 in both human and mouse that have not previously been identified. We have constructed a series of transgenic mice designed to establish which aspects of the wasted phenotype were attributable to loss of eEF1A2 and which could be caused by aberrant activity of neighbouring gene(s). We used three different constructs: a human PAC, a mouse BAC, and the same mouse BAC which had been engineered to create a deletion within the eEF1A2 gene. Whilst both the human PAC and mouse intact BAC rescued all aspects of the wasted phenotype, the deleted BAC failed to rescue any aspect, including the spleen and thymus atrophy.

Finally, we show that expression of eEF1A variants correlates precisely with the phenotype of wasted mice. We conclude that loss of eEF1A2 expression underlies all aspects of the wasted phenotype, and that eEF1A2 is essential for post-weaning survival of mice.

Experimental Procedures

Antibody Generation and Purification
Peptides were generated and conjugated to KLH by Zinsser Analytic (Maidenhead, Berkshire, UK). Antiserum was generated by the Scottish National Blood Transfusion Service (Penicuik, Midlothian, UK). Sheep were injected monthly with 0.5mg KLH-conjugated peptide, and serum was withdrawn one week after the second, third and fourth injections. Rabbits were injected with 0.25 mg conjugated peptide. The immunoglobulin component of the serum was purified using ammonium sulphate precipitation, as follows. Serum was mixed with 0.5 volumes saturated ammonium sulphate for 6 hours or overnight at 4°C. After centrifugation at 6000 x g for 30 minutes, the supernatant was stirred as above with an additional 0.5 volumes ammonium sulphate. After further centrifugation, the pellet was dissolved in 0.3 volumes PBS, and dialysed versus PBS overnight. The specific antibodies were purified from the ammonium sulphate-precipitated immunoglobulin component by immunoaffinity purification, using the peptides against which the sera were raised. This was carried out using a Sulfolink kit (Pierce, Cramlington, Northumberland, UK), according to the manufacturer’s instructions.

Immunofluorescence
Slides were deparaffinised, rehydrated and subjected to antigen retrieval as described for immunohistochemistry, above. Slides were blocked with donkey serum, and then incubated with anti-eEF1A antibodies, diluted 1 in 10 in PBS, for 30 minutes, and with fluorescently labelled secondary antibodies (Alexaflour 488 anti-rabbit for eEF1A2; Alexaflour 594 anti-sheep for eEF1A1; Molecular Probes, Invitrogen, Paisley, UK) diluted 1 in 1000 for 30 minutes, mounted in Vectashield (Vector, Peterborough, UK) and visualised on a Zeiss Axioskop 2 using Smartcapture software.

Western blots
Protein lysates from cell lines were prepared using previously published protocols (23). Western blot analyses were carried out using standard protocols. The blots were incubated with primary anti-eEF1A2 rabbit antibody and primary anti-eEF1A1 sheep antibody diluted 1:200 in blocking solution, as well as primary anti-glyceraldehyde-3-phosphate dehydrogenase polyclonal mouse antibody (Chemicon International, Hampshire, UK) diluted 1:10000. Blots were then incubated in the appropriate horse radish peroxidase conjugated secondary antibody (Dako Cytomation, Cambridgeshire, UK) at 1:500. Detection was performed using enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK).

Immunohistochemistry
Formalin fixed, paraffin embedded sections of human and mouse tissue were deparaffinized with xylene, rehydrated, treated with picric acid and microwaved in citric acid pH6. They were then treated in 3% hydrogen peroxide for 10 mins.
Slides were blocked in a 1:5 dilution of sheep serum for 30 minutes at room temperature. Primary anti-eEF1A2 rabbit antibodies were used at a concentration of 1:10 diluted in PBS, for 40 minutes at room temperature and secondary goat anti-rabbit IgG biotin conjugated antibody (Dako Cytomation, Cambridgeshire, UK) was used at 1:200 at room temperature for 30 minutes. Slides were incubated with StreptABC complex/HRP (Dako Cytomation, Cambridgeshire, UK) at room temperature for 30 minutes and in diaminobenzidene (Sigma Fast DAB, Sigma, Dorset, UK) for 2 minutes at room temperature. Finally slides were counterstained in haematoxylin, dehydrated and mounted in pertex.

Bioinformatics

PIP analysis was carried out using Pipmaker at http://nog.cse.psu.edu/pipmaker/

Generation of transgenic mice

The human PAC (h697K14) was isolated from a chromosome 20 PAC library by Dr. Panos Deloukas at the Sanger Centre. The mouse BAC, m219F24, was described previously (10). The modification of the BAC was achieved using the pGETrec system described by Orford et al (24); the plasmid was a kind gift from Dr. Pannos Ioannou. Modified clones were detected on the basis of having the right combination of antibiotic resistance in the first instance and then by PCR and sequencing across the site of modification, and restriction mapping of the whole construct. Transgenic mice were made using standard pronuclear injection methods into oocytes derived from (C57BL/6 X CBA)F1 mice. Founder mice that carried an intact transgene (checked by PCR and expression analysis) were then crossed to +/wst mice and then intercrossed to derive transgene-positive wst/wst mice.

Real-time RT-PCR

Real-Time PCR was performed using MyCycler™ Thermal Cycler (Bio-Rad) in a final volume of 20µl, containing 10µl of 2X iQ™ SYBR Green Supermix (Bio-Rad), 0.8µl of 5µM each primer, 5.5µl of the template and 2.9µl of dH2O, under the following conditions: 95°C 3 min, followed by 50 cycles each of 95°C 30 sec, 63°C 30 sec, and 72°C 30 sec. The melting curve analysis was performed from 55°C to 95°C and 18S rRNA was used as an internal standard. A standard curve was generated by plotting the log10 of control template on the X axis against the Ct value from serial dilutions of target DNA on the Y axis. The standard curve was linear over 5 logs (10^1 to 10^5 dilutions) with a correlation coefficient R^2 greater than 0.99. Primer sequences were as follows: 18S rRNA f 5’ GTAAACCGTTGAAACCCAT 3’, 18s rRNA r 5’ CCATCAAATCGGTAGTGAGCG 3’, Clwd f 5’ GACTACTATCGGCACGGCTG 3’, Clwd r 5’ GATTCGTCCGAGCGCTCAGGA 3’.

Results

An antibody panel specific for each eEF1A variant

We developed a series of antibodies that would specifically recognise eEF1A1 and, separately, eEF1A2. Peptide sequences were designed to maximise differences between the two variants whilst still falling in regions of high predicted antigenicity; these sequences are shown in table 1. KLH-conjugated peptides were used to generate polyclonal antibodies in either sheep or rabbits. After affinity purification the antibodies all recognised a single band on Western blots of approximately 50kDa for both eEF1A1 and eEF1A2 (representative blots are shown in figure 1). In every case, the antibodies are specific for the correct eEF1A isoform: anti-eEF1A2 antibodies recognise a band in extracts from brain and muscle, but not from liver (which has been shown not to express eEF1A2 at the RNA level) or brain from wasted (wst/wst) mice, which contains eEF1A1 but not eEF1A2 because of the deletion in the mice. No null-animals exist for eEF1A1, but muscle acts as a negative control as it has been shown to express only eEF1A2 by RT-PCR; the eEF1A1 antibodies recognise a band in all tissues except adult muscle, consistent with this. The antibodies work equally well for human and mouse tissue, as expected given the almost complete conservation of sequence at the amino acid level.
Expression analysis of eEF1A1 and eEF1A2 by immunohistochemistry in wild-type mice

We used the antibodies for immunohistochemical analysis of tissue sections, in order to establish the precise cell types within tissues that express each variant. No evidence for expression of eEF1A2 in spleen and thymus has been found using a variety of whole-tissue techniques, including sensitive assays such as RT-PCR and RNase protection. Immunohistochemistry for eEF1A2 on spleen and thymus sections revealed a small number of cells that stained positive with this antibody but only in a few sections. These cells seem likely to be macrophages. There was no convincing evidence for expression of eEF1A2 in either spleen or thymus by any technique.

We then went on to examine expression of eEF1A1 and eEF1A2 in spinal cord, the site of major pathological changes in wasted mice. The eEF1A2 antibodies showed very strong cytoplasmic expression in motor neurons of +/+wst mice, but not in wst/wst mice, again demonstrating that the antibodies are completely specific (figure 2A). Furthermore, the expression pattern in spinal cord fits perfectly with the known phenotype of wasted mice: motor neurons do not express eEF1A1 in the cytoplasm (figure 2A), so that in wasted mice there will be no translation elongation factor activity; this goes a long way towards explaining the particular vulnerability of these cells in wasted mice. Unexpectedly, we do see some nuclear staining of eEF1A1 in motor neurons- it is unclear what role this would play, or how eEF1A1, which is usually almost entirely cytoplasmic, and which is normally actively exported from the nucleus by exportin 5 (25,26), would become entrapped in this way. Importantly, the expression patterns of the two variants in the spinal cord is clearly mutually exclusive, with eEF1A1 antibodies staining white matter and glial cells, and eEF1A2 being confined to motor neurons.

We next examined expression patterns within the brain. In sections of cerebellum, the mutually exclusive staining pattern of eEF1A1 and eEF1A2 can again clearly be seen (figure 2B); as before, eEF1A1 is present in the white matter whereas eEF1A2 is absent. Strong staining of eEF1A2, but not of eEF1A1, is observed in Purkinje cells; again this ties in with the phenotype of wasted mice, which have been reported to show signs of ataxia and Purkinje cell loss (17). We went on to use two-colour immunofluorescence to show that eEF1A1 and eEF1A2 are genuinely mutually exclusively expressed in brain (figure 2C), with eEF1A1 only seen in glial cells (and some small neuronal cells) and sole expression of eEF1A2 in most neurons. This is consistent with, but extends the observations of, Pan et al (27), who showed expression of eEF1A1 in glial cells and eEF1A2 in neuronal cells, but did not directly demonstrate reciprocity of expression.

Immunohistochemistry reveals eEF1A2 expression in previously unsuspected locations

We then carried out immunohistochemistry of eEF1A2 in panels of normal tissues from human and mouse and found specific cell types expressing eEF1A2 in tissues that do not have detectable expression of eEF1A2 by Western blotting. Notably, eEF1A2 was expressed in specific cells in the pancreas (figure 3B-D), colon (figure 3E-H), lung (figure 3I-J) and stomach (figure 3K). The eEF1A2-expressing cells in pancreas are predominantly glucagon-producing islet cells, and those in the stomach are neuroendocrine cells (we have only been able to analyse these cells in mouse). Staining of pancreas sections with the anti-eEF1A1 antibody (figure 3A) reveals a near reciprocal, but not entirely mutually exclusive, pattern of expression. Whereas eEF1A2 staining is confined to islets, eEF1A1 expression is widespread; although staining is stronger in the exocrine areas of the pancreas, there does appear to be weak staining in islet cells. Figure 3 also shows a pancreatic islet from wild-type mouse (3C), from a wst/wst mouse showing specificity of staining (3D) and a human islet showing identical staining pattern in both species (3B). In gut tissue, the cells expressing eEF1A2 are found towards the base of the crypts; no more than two (and usually zero or one) are ever seen in any single crypt in a given section. The eEF1A2-positive cells have a displaced nucleus, suggesting that
they are enteroendocrine cells. In all cases with the possible exception of lung where staining was variable, the same cell types were seen to express eEF1A2 in both human and mouse (3E-3H), showing that this expression pattern is conserved, and suggesting a functional role for eEF1A2 in these cells. No staining with the anti-eEF1A2 antibodies was seen in other cell types within these tissues, and no obvious expression of eEF1A2 was detected in tissue sections of liver or kidney (data not shown). Wasted mice of 24 days were subjected to a detailed pathological analysis, but no changes were seen in the pancreas or gut of these mice compared to their normal littermates, potentially because even a low level of expression of eEF1A1 seen in these tissues is sufficient to protect the mice from overt damage, at least in the short term.

**Bioinformatic analysis reveals the presence of a novel gene 15kb from the wasted deletion**

We then went on to establish whether the phenotype of wasted mice is completely due to loss of eEF1A2 function, or whether other genes could be affected by the presence of the deletion in wasted mice. We first carried out Percentage Identity Plot (PIP) analysis of the human and mouse regions surrounding the gene encoding eEF1A2; the results are shown in Supplementary online figure 1. This analysis was based on the region contained in human PAC clone h697K14 as this was to be the starting point for our transgenic experiments. No significant region of human/mouse homology was detected other than within known genes, with the exception of a 100bp region upstream of the gene encoding eEF1A2 which showed 70% homology between human and mouse. There is no feature of this sequence which suggests that it might be coding DNA; it seems most likely to be an enhancer. Within the region deleted in wasted mice (shown in green on figure 4), no mouse/human homology could be detected beyond the putative eEF1A2 promoter region. The mouse BAC (m219F24) we had isolated previously (10) contains only two complete genes, Ef1a2 and the mouse ortholog of C20orf149, a small gene encoding a novel protein. This BAC also contains the coding sequence of Ptk6 (28) but ends within the 5'UTR of the gene and thus contains no Ptk6 promoter sequence (figure 4). The human PAC spans the whole of the EEF1A2, C20ORF149 and PTK6 genes, and also contains intact genes for CHRNA4, KCNQ2 and SRMS. The features of these genes are shown in table 2. The only gene which appeared to be a possible candidate for the immune system abnormalities in wasted mice, based on expression pattern and proximity to the wasted deletion, is C20orf149, but this needed to be tested directly.

**Transgenic analysis shows eEF1A2 is the only gene responsible for the wasted phenotype**

We next made transgenic mice carrying the human PAC which contains the complete sequence for the CHRNA4, KCNQ2, EEF1A2, C20ORF149, PTK6 and SRMS genes. A total of three founder mice were obtained, all of which transmitted the transgene in Mendelian proportions to their offspring. Transgene-positive mice from two of these lines (called A238 and A239) were crossed to +/wst heterozygous mice and resulting +/wst transgene-positive mice were backcrossed to +/wst mice. The resulting litters were analysed and a number of mice were identified that were genotypically wst/wst homozygotes and that also carried the transgene. Without exception, these mice grew and developed normally, had no evidence of tremor or weight loss (average weight at 24 days 13.8g compared with 13.5g for non-transgenic wild-type and 7.5g for non-transgenic wst/wst; figure 5C), had normal spleen: and thymus: body weight ratios (figure 5B and 5D), were fertile and had a normal lifespan (data not shown). We therefore concluded that the sequences present on the human PAC were sufficient to correct the wasted phenotype, that one or more of the 6 genes present on the PAC must be responsible for all aspects of the wasted phenotype, and further that there is functional equivalence of the gene(s) between mouse and human. It is also worth noting that mice carrying this transgene failed to develop tumours, in spite of the fact that at least two of the genes (EEF1A2 and PTK6) on the PAC are known oncogenes.
We then made transgenic mice using a mouse BAC which contains only two intact genes together with their promoter regions, those encoding eEF1A2 and C20orf149. The presence of complete coding sequence of Ptk6 meant that we could not rule out the possibility that this gene would become activated depending on the insertion site, but we reasoned that this was unlikely to occur in more than one independently-derived line. We obtained four independent founders which again transmitted the transgene in Mendelian proportions. The analysis of these mice was more complicated than in the PAC experiment, because there was no simple molecular assay that would distinguish between the genotype of +/-wst mice, and of wst/wst mice that also carried a transgene (the presence of the transgene itself could be identified at the DNA level by PCR of vector sequences). Furthermore, we were unable to identify any coding sequence polymorphisms that could be used to evaluate transgene-specific expression. We therefore set up crosses as before but simply assessed the proportion of phenotypically wasted mice that were obtained; a figure of 25% would show that the transgene had failed to correct the wasted phenotype whereas a figure of 12.5% would suggest that the sequences encoded by the transgene were sufficient for correction. Similar results were obtained from each of the founders; in total only 12 out of 104 offspring analysed had the phenotype characteristics of wasted mice (11.5%). This gives a p value of 0.0003 at 0.25 and 0.12 at 0.125 (where 0.125 is equivalent to a 12.5% figure, indicating that correction had occurred). Furthermore, none of the phenotypically wasted mice carried the transgene. All other mice survived and bred normally and on dissection had normal spleen and thymus to body weight ratios (data not shown). It is clear, therefore, that the sequences present on the BAC were sufficient to compensate for those lost in the wasted deletion. Since the same results were obtained from all four lines, any correction was highly unlikely to result from activation of the Ptk6 coding sequences by the insertion site, so we concluded that Eef1a2 and C20orf149 were the only remaining likely candidates for involvement in the wasted phenotype.

In order to establish whether eEF1A2 alone was responsible for all aspects of the wasted phenotype we then engineered a deletion into the same BAC that had been used for the transgenic experiments in order to specifically inactivate eEF1A2. This was carried out using a version of ET recombination in E.coli (29) and resulted in the replacement of exons 2 to 4 of the Eef1a2 gene with zeocin (figure 4). This design was chosen for two reasons; firstly exons 2, 3 and 4 of Eef1a2 contain the three GTP binding sites which would be predicted to be essential for eEF1A2 to function as a translation elongation factor, and secondly the resulting deletion within the Eef1a2 coding sequence is completely non-overlapping with the original deletion in wasted mice. Thus, in the unlikely event that the wasted deletion spans a hitherto unidentified small gene, the BAC would still retain it as an intact sequence. When this construct was used to make transgenic mice four independent founders were obtained. Unfortunately, only one of these founders transmitted the transgene (which was present in a single copy) so the resulting crosses to wasted mice were carried out using this line (called A240 or ΔBAC) only. Given that all the founders with wild-type BACs transmitted successfully it seems possible that the presence of the deleted BAC was in some way toxic to germ cells at higher copy numbers, possibly via the expression of zeocin. This ΔBAC transgene failed to correct any aspect of the wasted phenotype. Nineteen out of eighty eight animals (21.6%) born to wst/+ transgene-positive by wst/+ matings had a characteristic wasted phenotype including spleen and thymus atrophy, and died by 28 days (see figure 5). Half of these mice carried the transgene, but it made no difference to the phenotype. These mice were shown by Western blotting to have no expression of eEF1A2, as predicted (figure 6). All previous transgenic lines tested (intact BAC and intact PAC) showed position-independent expression of eEF1A2 (figure 6 and data not shown), but it still remained a formal possibility that the mutant transgene was not being expressed in this line. The only intact gene on the BAC aside from Eef1a2, which had been mutated, was C20orf149, so the only way to test whether the mutant BAC was being...
expressed in the transgenic mice was to establish whether C20orf149 was over-expressed. As expected given that the transgene was present in a single copy (data not shown), the level of expression of C20orf149 in the spleens and brains of transgenic mice, as measured by real-time RT-PCR, was on average one-two Ct values higher than in non-transgenic litter-mates, suggesting both that the transgene was being expressed and that C20orf149 over-expression was not sufficient to correct the wasted phenotype. Furthermore, C20orf149 expression occurs at essentially the same level in wst/wst and wild-type mice in all tissues examined (data not shown). We therefore conclude from this series of experiments that loss of eEF1A2 function is solely responsible for all aspects of the wasted phenotype.

Discussion

In terms of eEF1A expression, cells in the body clearly fall into one of three categories: those expressing eEF1A1 only (the vast majority of cell types), those expressing eEF1A2 only (muscle, neurons) and those co-expressing both forms (notably in certain tumours and cell lines). It has been suggested (30) that eEF1A2 expression is associated with terminal differentiation, but there are other cell types (keratinocytes, for example) which are terminally differentiated but which do not express eEF1A2. Those cells expressing eEF1A2 but not eEF1A1 tend to be cells with a large cytoplasmic volume and a stable shape, as well as being terminally differentiated. It is an attractive hypothesis that such cells replace eEF1A1 with eEF1A2 in order to prevent or modify the cytoskeletal interacting properties of eEF1A1, but this has yet to be proven. It is also possible that these properties may be associated not with eEF1A2 expression per se, but with the withdrawal of expression of eEF1A1.

Those cells that express eEF1A2 in the context of tissues that predominantly express eEF1A1 often have a secretory function, but have no obvious structural similarities to each other. It is clearly of interest to know whether only those cells that express just eEF1A2 are affected in wasted mice, or whether those cell types that co-express eEF1A1 and eEF1A2 are affected. We have shown that the cells that do not express eEF1A1 are affected in wasted mice—there is dramatic loss of muscle bulk during the period after eEF1A1 expression is shut down and clear degeneration of motor neurons (20). Similarly, there have been reports of Purkinje cell loss in wasted mice, and Purkinje cells very clearly express eEF1A2 but not eEF1A1. It might be expected that those cells expressing both eEF1A variants would be spared the effects of loss of eEF1A2 (at least in the short term) because of a protective effect of the presence of eEF1A1, in terms of protein synthetic capacity. Again, those cell types expressing eEF1A2, whether or not they also express eEF1A1, could be said to be cells that need to be long-lasting, and the role of eEF1A2 as an anti-apoptotic agent has clear relevance for this (9,31) In the light of the expression of eEF1A2 in pancreatic islet cells it is worth noting that a diabetes susceptibility locus has been mapped to 20q13.3 (32). There is no sign of pancreatic dysfunction in wasted mice at the level of gross pathology, but any such dysfunction could of course take longer than 28 days (the lifespan of wasted mice) to manifest itself. Alternatively, the co-expression of eEF1A1, even at an apparently low level, could confer some protection on these cells.

The lack of expression of eEF1A1 in some cell types is worth noting in the context of the use of “ubiquitous” promoters. The eEF1A1 (or EF1α) promoter is commonly used in transgenic and gene therapy contexts, but caution should be exercised with regard to its use in multiple tissue and cell types.

We have shown that the phenotype of wasted mice is highly likely to result entirely from the loss of expression of eEF1A2. Bioinformatic analysis and the generation of transgenic lines carrying a human PAC and a mouse BAC allowed us to narrow down the list of genes sufficient to correct the wasted phenotype from the 6 genes on the human PAC (EEF1A2, C20orf149, PTK6, CHRNA4, KCNQ2 and SRMS), to the 2 intact genes on the mouse BAC (Eef1a2, C20orf149), and thence, by showing that a BAC with an inactivating mutation of Eef1a2 fails to correct any aspect of the wasted
phenotype, to Eef1a2 alone. The observation that the human PAC was able to completely rescue the wasted phenotype also establishes the functional equivalence of the mouse and human genes (perhaps not surprisingly, since the resulting protein would be predicted to be identical, but this does show that the control of expression of the gene is functionally equivalent). We then narrowed down the region containing the gene(s) that corrected the wasted phenotype to an area containing only Eef1a2 and C20orf149, as a mouse BAC containing only these sequences was able to completely complement the wasted deletion. There were two potential confounding factors in this analysis. Firstly, the mouse BAC transgenes also contained the coding region of an additional gene, Ptk6. The BAC sequence terminates in the 5’UTR of this gene, so none of the cognate promoter elements were carried on the BAC. Since all the coding sequence was present there was always a possibility that the gene would be expressed if the transgene became integrated adjacent to an active promoter. However, we generated and analysed a total of four transgenic lines containing this sequence on the wild-type mouse BAC, all of which corrected the wasted phenotype, and it seems highly unlikely that the Ptk6 gene would have been activated to an appropriate level in every line, as the integration site will likely be different in each case. We therefore argue that Ptk6 is not affected by the wasted deletion, as predicted also by its distance from the deleted region (40 kb from the closest end). The other confounding factor is that of the four transgenic founders generated with the BAC that contained an engineered mutation in Eef1a2, only one transmitted the transgene. The final piece of evidence that Eef1a2 is the only gene implicated in the wasted phenotype thus relies on this transgene being expressed in this line. As C20orf149 is the only intact gene on the construct, expression analysis of this gene was used to show that the transgene was indeed being transcribed, and thus that increased expression of C20orf149 is insufficient to correct the wasted phenotype. When this evidence is taken together with that showing that C20orf149 is expressed at normal levels in wasted mice, it is clear that C20orf149 is highly unlikely to play a role in the abnormalities seen in wst/wst mice. The motor neuron degeneration, muscle wasting, and other abnormalities are thus the result of loss of function of eEF1A2. Atrophy of the thymus and spleen in conjunction with extensive apoptosis is a common finding in mice exhibiting clinically severe phenotypes. Activation of the hypothalamic-pituitary-adrenal axis, increased corticosterone production and corticosterone-induced apoptosis has been proposed as the mechanism responsible for atrophy of thymus and spleen in some of these conditions (33-39). It is therefore entirely possible that these aspects of the phenotype are non-specific and result from the known state of stress and failure to eat of wasted mice (17,40).

It would clearly be of great interest to rescue specific aspects of the wasted phenotype, by carrying out tissue-specific transgenic rescue, or conditional knockouts, in order to study the effects of loss of eEF1A2 on cells in the pancreas or colon, for example, over a longer time-scale than 28 days.

Since co-expression of eEF1A1 and eEF1A2 is rarely if ever seen at high levels in normal cells, it would be of interest to know how co-expression relates to tumourigenesis. For example, is it necessary to have both isoforms present to develop malignancy, or is it simply the appearance of eEF1A2 in tissues where it is normally switched off that contributes to tumourigenesis? It is also worth considering why co-expression might be deleterious. Although it is possible that this is simply because the translation rate could be increased, leading to misincorporations (41) this seems unlikely since eEF1A1 is already in excess over other translation factors. There could be competition between eEF1A1 and eEF1A2 for crucial binding sites on other molecules, or it could be that eEF1A2 has non-canonical properties that confer an advantage on tumour cells; the increasing evidence for an anti-apoptotic role for eEF1A2 makes this an attractive possibility (9,31). Transgenic experiments in which the eEF1A1 coding sequence is knocked into the eEF1A2 locus would shed considerable light on the functional equivalence of the two genes and illuminate their relative roles in disease processes.
References

Figure legends

Figure 1

Representative Western blots with two anti-eEF1A1 and two anti-eEF1A2 antibodies. The anti-eEF1A1 antibodies detect a band of ~50kDa in brain and liver but not muscle, consistent with the known expression of eEF1A1. The anti-eEF1A2 antibodies detect a band in muscle and brain from wild-type mice but not brain from wst/wst mice, or liver, both of which do express eEF1A1. The anti-eEF1A1 antibodies do not give a band in muscle samples. On the GAPDH loading controls for eEF1A1, the original eEF1A1 band can also be seen (arrowed).

Figure 2

Figure 2A Immunohistochemistry of eEF1A1 and eEF1A2 in mouse spinal cord showing reciprocal expression patterns. eEF1A2 is very strongly expressed in motor neurons in wild-type mice but shows no staining in wasted homozygous mice.

Figure 2B Immunohistochemistry of eEF1A1 and eEF1A2 in mouse brain; eEF1A1 is strongly expressed in the white matter but is barely detectable in Purkinje cells; eEF1A2 is strongly expressed in Purkinje cells but is not expressed in white matter.

Figure 2C Immunofluorescence of eEF1A1 (red) and eEF1A2 (green) in mouse brain, showing complete reciprocity of expression.

Figure 3

Figure 3A eEF1A1 immunohistochemistry in human pancreas x40 showing higher expression in the exocrine pancreas than in islets.

Figure 3B eEF1A2 immunohistochemistry in human pancreas x40 showing strong, specific staining in islet cells.

Figure 3C eEF1A2 expression in a mouse pancreatic islet x100; the cells expressing the highest levels are at the periphery of the islet, consistent with them being glucagon-producing cell types.

Figure 3D eEF1A2 immunohistochemistry in pancreas from a wasted mouse demonstrating specificity of staining.

Figure 3E eEF1A2 expression in specific cells in the human gut in longitudinal section.

Figure 3F eEF1A2 expression in specific cells in the human gut in cross section.

Figure 3G eEF1A2 expression in specific cells in the mouse gut in longitudinal section.

Figure 3H eEF1A2 expression in specific cells in the mouse gut in cross section.

Figure 3I eEF1A2 expression in the specific cells in the human lung.

Figure 3J weak expression of eEF1A2 in some cells of the mouse lung.

Figure 3K eEF1A2 expression in neuroendocrine cells of the mouse stomach.

Figure 4

Schematic of the constructs used to generate transgenic mice, showing the positions and direction of transcription of the genes, the deletion engineered into the mouse BAC, and the deletion in wasted mice.

Figure 5

Figure 5A shows littermates at 24 days, both of the wst/wst genotype but whereas the one on the left is transgenic for the human PAC, the one on the right is non-transgenic and is considerably smaller and hunched. Figure 5B shows spleens removed from 24 day old littermates- the two on the right are from non-transgenic wst/wst mice and are very small, the
one on the left is from a wild-type non-transgenic mouse and the second from left from a
\textit{wst/wst} transgenic mouse, showing correction of spleen size. Figure 5C shows average body
weights for wild-type, transgenic \textit{wst/wst} and non-transgenic \textit{wst/wst} mice. Mice were
weighed at P24 by placement on a digital weighing scale, accurate to 0.1g. The average
weight for each genotype was calculated from the number of mice indicated by n. A238 and
A239 are human PAC transgenic lines and A240 is the deleted mouse BAC transgenic line.
Figure 5D shows average organ weights (brain, heart, spleen and thymus) for wild-type,
transgenic \textit{wst/wst} and non-transgenic \textit{wst/wst} mice. Mice were weighed at P24 by placement
on a digital weighing scale, accurate to 0.0001g. The average wet weight for each genotype
was calculated from the number of mice indicated by n. A238 and A239 are human PAC
transgenic lines and A240 is the deleted mouse BAC transgenic line. The weights for \textit{wst/wst}
and A240 \textit{wst/wst} mice are indistinguishable.

Figure 6

Figure 6 shows Western blots for eEF1A2 and an alpha tubulin control showing lack of
eEF1A2 expression in non-transgenic \textit{wst/wst} mice and those carrying the \( \Delta \)BAC transgene.
M is the marker track. There is clear evidence of eEF1A2 expression in the \textit{wst/wst} PAC
transgenic samples (with differing levels depending on the line), showing that the transgene is
expressed in this line.

Supplementary online figure
Percentage identity plot (PIP) of the region surrounding the wasted locus. The nucleotide
positions for the mouse sequence are shown on the x axis, and the percent identity with the
corresponding human sequence is shown on the y axis (50-100%). Genes are labelled
according to known cDNA sequences. The locations of mouse repeats were determined using
RepeatMasker. The region corresponding to the wasted deletion is marked on the plot in light
green shading. A short segment of 80% identity (boxed in red) between mouse and human
within the sequence deleted in \textit{wst} (at ~158.5kb) is a potential regulatory element.

Table 1
Peptide sequences used to generate antibodies

\begin{tabular}{ll}
\textbf{eEF1A1-1} & KAVDKKAAGAGKVT\textbf{C} \\
\textbf{eEF1A2-1} & KNVEKKSAGGAGKVT\textbf{C} \\
\textbf{eEF1A1-2} & CPYSQKRYEEIVKEV\textbf{ST} \\
\textbf{eEF1A2-2} & CAYSEKRYDEIVKEV\textbf{SA} \\
\textbf{eEF1A1-3} & TRKDGHASGTTLEALDC \\
\textbf{eEF1A2-3} & CERKEGASNOSLLEALDT \\
\end{tabular}

Table 2

\begin{tabular}{lll}
\textbf{Gene symbol} & \textbf{Expression sites} & \textbf{Comments} \\
\textit{Kcnq2} & Brain-specific (42) & \textit{-/-} die at birth (43) \\
\textit{Eef1a2} & Muscle, neurons (refs herein) & \\
\textit{C20orf149} & Ubiquitous (ESTs) & \\
\textit{Ptck149} & Skin, gut (28) & \\
\textit{Srms} & Ubiquitous (44) & \textit{-/-} have no phenotype (44) \\
\end{tabular}
Acknowledgements
We thank Brendan Doe for his help and advice with the construction of the transgenic lines, Panos Deloukas for help with the isolation of the human PAC, Alexey Larionov for advice on real-time RT-PCR and Simon Cooper for help with the figures.
Figure A: Images showing different mouse genotypes.

Figure B: Images illustrating the weight differences in various organs.

Figure C: Bar graph showing body weight comparison between different genotypes. The graph includes wild-type, A238, A239, A240, and wst/wst mice.

Figure D: Bar graph illustrating the weight of different organs (Brain, Heart, Spleen, Thymus) in wild-type and wst/wst mice. The graph includes wild-type, A238, A239, A240, and wst/wst mice.