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# Determining Synthesis Rates of Individual Proteins in Zebrafish (*Danio rerio*) with Low Levels of a Stable Isotope Labelled Amino Acid

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**Abstract**

The zebrafish is a powerful model organism for the analysis of human cardiovascular development and disease. Further, zebrafish are able to fully regenerate their hearts following injury. Understanding these processes at the protein level not only requires changes in protein concentration to be determined but also the rate at which these changes occur on a protein-by-protein basis. The ability to measure protein synthesis and degradation rates on a proteome-wide scale, using stable isotope labelling in conjunction with mass spectrometry is now a well-established experimental approach. With the advent of more selective and sensitive mass spectrometers, it is possible to accurately measure lower levels of stable isotope incorporation, even when sample is limited. In order to challenge the sensitivity of this approach, we successfully determined the synthesis rates of over 600 proteins from the cardiac muscle of the zebrafish using a diet where either 30% or 50% of the L-leucine was replaced with a stable isotope labelled analogue ([$^{2}H_{7}$]L-leucine]. It was possible to extract sufficient protein from individual zebrafish hearts to determine the incorporation rate of the label into hundreds of proteins simultaneously, with the two labelling regimens showing a good correlation of synthesis rates.

**Significance of the Study**

This manuscript is the first to describe the calculation of the absolute rates of protein synthesis on a proteome-wide scale in the zebrafish myocardium; a popular model for cardiac disease and regeneration. Moreover, this has been achieved by feeding the zebrafish a diet containing low-levels of a stable isotope labelled amino acid.

## 1 Introduction

The zebrafish (*Danio rerio*) is a recognised model organism that is widely used to investigate physiological processes and disease states [1-5]. The genome of the zebrafish is fully-sequenced and well annotated [6] and mutants and genetically modified variants for the investigation of many biological processes are available [1, 7]. The zebrafish heart is of particular interest as it has a substantial regenerative capacity, indeed full regeneration of cardiac tissue is possible after a loss of 20% of ventricular mass [8]. Damaged tissue is replaced by *de novo* synthesis of new heart tissue, with accompanying proliferation of existing cardiomyocytes [9]. Evidence also exists for a similar, albeit slower, process in human hearts [10]. Understanding the molecular events of cardiac regeneration in the zebrafish model may provide insights into an important physiological process for human health and disease.

Proteomics is a key technology used to probe the biology of living systems and seeks to provide a functional link between expressed genes and phenotypic outcomes. Increasingly, proteomic analyses in zebrafish are being used to understand fundamental biological processes [11-13]. Over the past decade there has been considerable effort made to define the temporal proteome. This not only encompasses changes at the post-translational level, such as cellular localization, phosphorylation or ubiquitination, but also includes intracellular protein stability. This often takes the approach of a SILAC approach, elegantly developed by Mann and colleagues [14-16]. However, whilst this has significantly advanced the field of proteomics and has brought the discipline into the mainstream of cell biology, it still only yields a series of ‘snap-shots’ of the proteome at defined points or experimental stages. To fully integrate the proteome and understand how a cell or tissue transitions from one state to

another, it is necessary to understand the flux of the system and, specifically, when and how new proteins are synthesised and existing proteins are degraded.

We have previously described a modification to the SILAC approach, termed 'dynamic SILAC', which allows the rates of synthesis and/or degradation of individual proteins to be determined on a proteome-wide scale [17]. By sampling the proteome along the labelling time-course, it has been possible to determine protein turnover in yeast [18], algae [19], human cell lines [17], chicken [20], mice [21, 22] and fish, specifically common carp [23, 24]. It is relatively easy to manipulate the amount of stable isotope label available in cellular systems as the medium can be controlled so that the cell is exclusively exposed to a labelled amino acid. However, in higher organisms, the situation is more complex as the stable isotope labelled pool is diluted by unlabelled amino acid derived from pre-existing protein. Further, to ensure physiological integrity, it is expedient to deliver the stable isotope labelled amino acid via the diet. It is impossible to substitute 100% of a given amino acid in a standard laboratory diet with a crystalline stable isotope label as amino acids are obtained from many protein sources. In addition, a synthetic diet containing only pure amino acids leads to issues of palatability (and hence low incorporation) and decreased physical robustness of the diet. In this study we have determined absolute rates of protein synthesis in the cardiac muscle of zebrafish. We have also extended the experimental strategy and compared the use of different concentrations of a stable isotope labelled amino acid in the diet to measure the synthesis rates of individual proteins in zebrafish myocardium.

## 2 Materials and Methods

### 2.1 Animal Husbandry and Sample Collection

Zebrafish were maintained in the University of Liverpool aquarium at 28°C +/-1°C (at pH 7.6) on a 14h light:10h dark photoperiod throughout the study. Zebrafish were housed in identical 25 cm x 11 cm x 15 cm zebrafish tanks (Aquatic Habitats, now Pentair Aquatic Ecosystems, Manchester, UK). Daily checks were carried out for pH, ammonia, nitrite and nitrate levels, with desired levels being pH 7.6, 0 mg/L, 0 mg/L and <20 mg/L respectively. Weekly water changes replaced at least 20% of the water of the system.

For turnover experiments zebrafish were fed an experimental diet, in which either 30% or 50% of the L-leucine in the diet (that proportion added as crystalline amino acid) was replaced with [$^{2}H_{7}$]L-leucine (98% purity) (Cambridge Isotope Laboratories, Tewksbury, MA, USA). Throughout the feeding regime, the fish were observed at least three times daily, at morning and afternoon feeds then at approximately 5pm. Fish were sampled at various time-points (n = 3 per time-point) over an 8 week period. Fish were killed in accordance with UK Home Office Schedule One regulations. Immediately after sacrifice, fish were weighed and the heart of each fish was swiftly dissected. Whole hearts were then stored in individual 0.5 ml Eppendorf tubes at -80°C until analysed. The heart samples (approximately 1 mg wet weight of tissue) were mechanically homogenized in 500 µl of 1X phosphate buffered saline (PBS, Invitrogen, Carlsbad, United States) containing Complete Protease Inhibitors (Roche, Lewes, UK). The homogenate was centrifuged at 19,000 x *g* at 4°C for 45 min and the supernatant collected. The protein concentration of the supernatant was determined using the Coomassie Plus Protein Assay (Pierce Biotechnology, Rockford, IL, USA).

## 2.2 1-D SDS-PAGE

The soluble proteins (20 μg) from zebrafish heart were separated by 1-D SDS-PAGE using a Mini-Protean Tetra system (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Samples were electrophoresed at a constant potential of 200V through a 15% w/v polyacrylamide resolving gel with a 4% w/v stacking gel. Samples were incubated at 95°C for 5 min in a reducing buffer (125 mM Tris-HCl; 140 mM SDS; 20% v/v glycerol; 200 mM DTT and 30 mM bromophenol blue) prior to loading. Gels were stained with Coomassie Blue (Bio-Rad).

2.3 In-Gel Digestion

Gel lanes were cut into 12 slices and each slice placed in distilled deionized water (50 μl). The water was then removed and the gel piece was treated with destain solution (10 μl of ACN/100 mM ammonium bicarbonate 1:1 v/v). The protein disulphide bonds were reduced by the addition of dithiothreitol (20 μl of 10 mM for 30 min) and alkylated by iodoacetamide (20 μl of 55mM for 30 min incubation in the dark). Each gel slice was dehydrated in acetonitrile. Trypsin (Roche) (0.2 μg/μl in 50 mM acetic acid) was added at a ratio of protein:trypsin 50:1 and the digestion allowed to proceed overnight at 37°C. The peptides were then extracted from the gel by addition of acetonitrile and then dried under vacuum in a MiVac concentrator (Genevac, Ipswich, United Kingdom) prior to resuspension in 50% methanol.

2.4 Mass Spectrometry

Peptide analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed in positive ion mode using a Thermo LTQ-Orbitrap XL LC-$MS^n$ mass spectrometer equipped with a nanospray source and interfaced to a Waters nanoAcquity ultra performance liquid chromatography (UPLC) system. The samples (5 μl) were initially desalted and concentrated on a 5μm Symmetry C18 180 μm x 20 mm trapping column

(Waters, Milford, MA, USA). The peptides were then separated on a BEH C18 nanocolumn (1.7 μm, 75 μm x 250 mm, Waters). Mobile phase A comprised 0.1% formic acid in water and mobile phase B comprised 0.1% formic acid in acetonitrile (all Fisher Scientific, Loughborough, UK). A gradient of 10-40% acetonitrile over 120 min was employed with a flow rate of 400 nl/min. Peptides were ionised using a PicoTip emitter (New Objective, Woburn, MA) at 3.5 kV source voltage. Acquisition was in data-dependent mode over the range m/z 300-2000 with the top 10 ions being fragmented using the lock mass setting for increased accuracy and comparability. Dynamic exclusion settings allowed a single repeat with a duration of 30 s, keeping a list of 500 ions. Charge state screening was enabled, rejecting unassigned and single positive charge states.

2.5 Data Analysis

Data were analysed using MaxQUANT with the Andromeda search engine [25-27]. The initial search parameters allowed for two trypsin missed cleavages, carbamidomethyl modification of cysteine residues, oxidation of methionine and acetylation of N-terminal peptides and a FDR of 0.01. A mass tolerance of 20 ppm for the precursor ion first search and a tolerance of 6ppm for main search were allowed along with a fragment mass tolerance of ± 0.5 Da. A maximum 1% false discovery rate was used for both protein and peptide identification. Protein identifications were made from a minimum of two peptides per protein including at least one unique peptide. Identified contaminants were removed. An additional parameter, coded as a pseudo post-translational modification was included to search for peptides containing [$^{2}H_{7}$] L-leucine.

2.6 Calculation of Protein Turnover Rates

All data pertaining to calculation of protein turnover, including calculation of the relative isotope abundance (RIA), was derived from the same LC-MS/MS analyses used to identify the proteins. The RIA of the precursor pool of cardiac tissue was determined using peptides containing two leucine residues from a range of different proteins. These proteins were taken from the list of identified proteins and covered a range of molecular weights. Our previous work has shown good agreement between data derived from di- and tri-leucine peptides. As the S/N for the di-leucine peptides is in general, more favourable, we have focussed solely on these peptides for determination of RIA. Once the precursor RIA was calculated it was used to deconvolute the peptide ion intensity from mono-leucine peptides to pre-existing 'old' protein and newly synthesised protein. This relative partition of intensity over time was plotted over time, allowing the rates of synthesis and degradation of each protein to be determined using the data generated from both labelling strategies. From these data, the appearance of labelled peptide was analysed by non-linear curve fitting to derive the rate of incorporation of label from the protein pool. Although the use of a deuterated amino acid can cause a small shift in the retention time of the labelled peptides, this is accounted for using the MaxQuant software and was verified by manual interrogation of the data.

2.7 Gene Ontology

To assign a functional category to each of the proteins, PANTHER Classification system (http://pantherdb.org, version 10) was used. This classifies proteins according to protein class cellular location, class, function and biological process. Enrichment of functional classes of proteins was performed using the PANTHER over-representation test as was pathway enrichment analysis [28]. The identified proteins were compared to a background derived

from the annotated zebrafish reference proteome, with data Bonferroni corrected for multiple testing.

## 3 Results

### 3.1 Calculating Absolute Rates of Protein Synthesis in Zebrafish Myocardium

The zebrafish were maintained for eight weeks on the experimental diet containing 30% of its leucine as the labelled form, hearts excised and proteins separated by 1D-SDS-PAGE. There were no fatalities during the trial, all the fish ate well and remained active and alert throughout. There was no evidence of abnormality in the fish. For the 50% labelled fish, there was no significant difference in the Hepatosomatic index, Splenohepatic Index or Intestinal Somatic index.

Following LC-MS/MS analysis, peptides containing multiple leucine residues were identified and the relative isotope abundance determined using the equations derived in our previous studies (Equation 1) [20]. The RIA of the precursor pool at each time-point was determined and plotted (Figure 1).

$$r = 2\frac{I_{H_2}}{I_{HL}} \Big/ \left(1 + 2\frac{I_{H_2}}{I_{HL}}\right) \qquad \text{[Equation 1]}$$

Where r is the relative isotope abundance; $I_{HL}$ is the intensity of the di-leucine peptide that contains both heavy and light leucine; $I_{H2}$ is the intensity of the di-leucine peptide that is all heavy.

Data were fitted to a monoexponential, which rapidly plateaued to give a RIA of 0.31 ± 0.01 when the 30% diet was introduced and 0.46 ± 0.01 using the 50% labelled diet. This indicates that virtually all of the amino acid in the diet was bioavailable and could be used for protein

synthesis. Once the RIA is calculated it is then possible to determine the amount of newly synthesised protein at each time-point (Equation 2).

$$F_N = \frac{I_H}{(I_L + I_H).r} \qquad \text{[Equation 2]}$$

Where $F_N$ is the fraction of newly synthesized protein; $I_L$ is the intensity of the mono-leucine peptide that is light; $I_H$ is the intensity of the mono-leucine peptide that is heavy.

By plotting this surrogate of $fraction_{new}$ and $fraction_{old}$ protein at each time-point over the experimental period, the rate of incorporation of label, and hence protein synthesis can be calculated for each protein (Supplemental Table 1). Data were fitted to a single exponential growth curve allowing calculation of $k_{synthesis}$ (± SE). In Figure 2 exemplar plots are shown for two proteins, pyruvate kinase and 4-alpha-glucanotransferase. Rates of protein synthesis were determined for 661 proteins (Figure 3) ranging from 7.6 x $10^{-4}$ $week^{-1}$ for gelsolin, an actin binding protein, to 0.63 $week^{-1}$ for Upb1 protein 1a, which has sequence homology to beta-ureidopropionase. This protein is a hydrolase, catalysing the conversion of N-carbamoyl-beta-alanine to beta-alanine. There was an overlap of 207 proteins for which synthesis rates were calculated between the two dietary interventions. The agreement between the rates was good, with a correlation of 0.77 ± 0.1, $p < 0.0001$. This further indicates that the level of labelled amino acid present in the diet has no significant effect of the synthesis rates of the proteins. Indeed, the limiting factor may be the limit of detection and quantification of the labelled pairs in the mass spectrum. However, using the 30% diet, it was possible to extract data from even those proteins with relatively low rates of synthesis with good S/N (Supplemental Figures 1 and 2). The calculated CV between biological replicates (both individual fish and different peptides) showed an average of 9.95%. In

addition, the CV between the same proteins from the 30% and 50% labelled datasets was low (typically less than 1%), showing that the approach is robust and reproducible.

3.2 Gene Ontology and Comparison of Protein Cohorts

The proteins for which synthesis rates were determined covered a range of functions and were found in various cellular locations (Figure 4). A large number of proteins (42.3%) were found to be involved in metabolic processes, with oxidation-reduction processes (14.1%) comprising the next most abundant process. Other proteins were allied to lipid transport and localisation (3.8%), regulation (9.9%) and cellular component biogenesis (5.8%). Specific cardiac functions were also represented including heart contraction (2.1%, >5 fold-enrichment, $p = 2.49 \times 10^{-7}$), circulatory system process (2.1%, >5 fold-enrichment, $p = 4.70 \times 10^{-3}$) and striated muscle cell differentiation and development (2.25%, 4.88 fold-enrichment, $p = 8.29 \times 10^{-3}$ and 2.1%, >5 fold-enrichment, $p = 3.69 \times 10^{-3}$ respectively).

The enrichment analysis found 52 clusters of proteins in terms of biological process, of which 37 were enriched ($p < 0.05$) compared to the complete *Danio rerio* database. Those clusters with the greatest enrichment included proteins involved generation of precursor metabolites and energy, fatty acid metabolic process and respiratory electron transport chain. Interestingly, muscle contraction was found to be 4.2-fold increased ($p = 8.82 \times 10^{-8}$) and muscle organ development 2.93-fold enriched ($p = 1.5 \times 10^{-3}$). The most represented molecular function was catalytic activity (42.8%), which included hydrolase, peptidase, lyase, isomerase and transferase activity followed by oxidoreductase activity (16.3%), structural molecule activity (13.2%) and cytoskeletal protein binding (3.5%). Data analysis (33 clusters of which 25 were significantly enriched) also indicated that specific functions including oxidoreductase, isomerase and transferase activity were specifically enriched along

with lipid transport and antioxidant activity. The proteins were predominantly intracellular (84%) as opposed to extracellular (16%) and were located in the cytoplasm (25.7%), mitochondrion (8.1%), extracellular region (4.98%) and associated with organelles (11.73%). A number of proteins were also described as being associated with complexes including the proteasome, myosin complex and ribosome. In particular, there was enrichment (41 clusters, 35 significantly enriched) observed for mitochondrial envelope and proton-transporting ATP synthase complex proteins along with mitochondrial proteins and those involved in respiratory chains. A total of nine pathways were identified in the dataset, which were all significantly (>5-fold) enriched in the zebrafish heart compared to the whole proteome. These included glycolysis, the TCA cycle, ATP synthesis, blood coagulation and the plasminogen activating cascade. These data are perhaps unsurprising as they represent known functions of the heart but reinforce the lack of bias in the sampling of the proteome in this approach.

Data were then analysed to determine if there was a bias in the gene ontology of both rapidly synthesised proteins and those that were synthesised at the slowest rates (Figure 5). There was some difference in terms of biological process with reference to the general distribution of the proteins. Proteins with lower synthesis rates were found to be more involved in catabolic processes, nitrogen compound metabolic processes, generation of precursor metabolites and energy and both general transport and transmembrane transport. The rapidly synthesised proteins were more represented in anatomical structure development, cellular protein modification processes, protein complex assembly and protein maturation. Proteins with lower rates of synthesis were more highly represented in the molecular functions of including ATPase activity, DNA binding, isomerase activity and ligase activity whereas the most rapidly synthesised proteins were found to have enzyme regulator activity, kinase activity and lipid binding functions. The cellular locations of the proteins were broadly

similar with the proteins with the slowest rates of synthesis associated with organelles and protein complexes.

The proteins with the lowest synthesis rates were enriched for antioxidant, isomerase, lyase and peroxidase activity whereas the highest synthetic rates were associated with hydrolase, motor and structural molecule activity. Catalytic and oxidoreductase activity was found to be enriched in both cohorts of proteins. The major differences between the protein cohorts included anatomical structure morphogenesis, cytokinesis and homeostatic processes, which were represented in the rapidly synthesised proteins exclusively and fatty acid metabolic processes, glycolysis and respiratory electron transport chain, which were found to correlate only with the slower rates of synthesis. The substantive difference in protein location was primarily between intracellular (enriched for proteins with a higher rate of synthesis, 2.13-fold, $p = 2.5 \times 10^{-2}$) and specifically mitochondrion (enriched for proteins with a lower rate of synthesis, >5-fold, $p = 1.4 \times 10^{-4}$). No pathways were found to be significantly enriched for the rapidly synthesised protein cohort. However, in the proteins with lower rates of synthesis glycolysis and blood coagulation were both found to be enriched along with the methylmalonyl pathway.

## 4 Discussion

### 4.1 Protein Turnover in Complex Organisms

The ability to determine protein turnover rates on a proteome-wide scale is an important step in the integration of proteomics data into a wider systems understanding of cellular response and adaptation. There have been a number of approaches developed in uni- and multi-cellular organisms that, in general, use the incorporation of a stable-isotope labelled amino acid (or alternative precursor) into proteins as a surrogate for protein synthesis [18-24]. The loss of label in a ‘pulse-chase’ approach similarly permits the calculation of protein degradation.

Some studies have reported an adaptation of this approach for zebrafish. For example, Westman-Brinkmalm and colleagues introduced [$^{13}C_6$]-lysine into zebrafish by feeding a commercially available stable isotope labelled diet designed for mice [29]. The incorporation of the label was measured by a simple ratio of labelled:unlabelled peptides but neglected to determine the relative isotope abundance (RIA) of the precursor pool. The data presented, whilst useful in comparing different stages of tissue regeneration, was not a true determination of protein synthesis as absolute rates were not determined. Moreover, as the zebrafish were maintained on a non-physiological diet, there was an observed loss of fertility after four months of feeding. Similarly, other studies in which a SILAC mouse diet combined with labelled murine tissues was administered to zebrafish [30] have been reported to lead to substantially higher levels of mortality compared to zebrafish maintained on standard aquarium diets. In our studies, none of our fish died and they remained active and displayed normal physiology and growth profiles.

4.2 Protein Synthesis in Zebrafish Heart with 30% and 50% [$^{2}H_7$] L-Leucine

In the present study, we have used a diet designed for zebrafish that contains deuterated leucine at a level of either 30% or 50% of the total leucine in the diet. This diet provided sufficient nutrients to allow the zebrafish to grow at normal rates. Leucine was selected as it is an essential amino acid for the zebrafish and abundant in the zebrafish proteome. A total substitution (100% replacement) of free amino acid in the standard aquarium diets with crystalline leucine was not possible as this was not palatable to the zebrafish.

As the labelled amino acid is a considerable cost in these types of experiments, we decided to determine whether low levels of stable isotope amino acid enrichment would permit calculation of synthesis rates of proteins from zebrafish myocardium. The results show that it

is possible to calculate synthesis rates of hundreds of proteins simultaneously using [$^{2}H_{7}$]L-leucine supplementation of only 30%. These data are supported by a high correlation of the rates of protein synthesis derived from the 30% and 50% diets. This is a departure from the current emphasis on incorporating the maximum amount of label into an organism and minimizes any cost associated with the formulation of the diet. Moreover, it has been possible to use these baseline data to probe the functional relationship between rapidly synthesised proteins and those where protein doubling is in line with the growth of the fish. Rates of synthesis were determined from proteins with various functions and cellular location and it was found that there was a tendency for a greater number of rapidly synthesised proteins to be intracellular proteins. Rapid protein synthesis was also more associated with cytokinesis and anatomical structure morphogenesis activity with slower protein synthesis linked to glycolysis and respiration.

It was possible to observe the dynamics of components of protein complexes. Eleven proteins were identified as being part of the ribosome protein complex and a further eleven proteins were identified as being part of the proteasomal complex. Previous study into the rates of degradation of the ribosome complex in HeLa cells indicated that the degradation rates of these highly similar proteins could cover two orders of magnitude with similar values calculated for proteasomal proteins [17]. In the present study, we have shown that the rate of synthesis of ribosomal proteins (both 40S and 60S) in zebrafish cardiac muscle range from 0.02 week$^{-1}$ to 0.09 week$^{-1}$ and proteasomal subunits from 0.016 week$^{-1}$ to 0.079 week$^{-1}$. Interestingly, it was also observed that there were differential rates of synthesis of core histone proteins. The majority of the observed histone proteins had calculated $k_{syn}$ equal to 0.01 to 0.04 week$^{-1}$. However, there were outliers to these date including histone H3.3 ($k_{syn}$ = 0.004 ± 0.003) and core histone macro-H2A ($k_{syn}$ = 0.126 ± 0.01). The molecular processes

controlling histone synthesis and degradation are not fully understood [31]. Histones are thought to be semi-conservatively replicated during DNA replication, and were once considered to be non-degradable [32] but it is has been proposed that they are in a highly dynamic equilibrium, with an excess of histones inhibiting transcription [33]. An extension of the work outlined in this paper focussed on histones could offer additional insights into the regulation of their synthesis and recycling.

## 5 Conclusion

In this study, we have demonstrated that it is possible to determine the absolute rates of protein synthesis in zebrafish cardiac muscle using a stable isotope labelling in conjunction with high resolution mass spectrometry. In order to challenge the sensitivity of this approach, we designed a diet where only 30% of the chosen label, [$^{2}H_{7}$]-leucine, was replaced in the diet. This was then compared with a diet where 50% of the label had been replaced. Despite this low level of label, it was possible to accurately determine the precursor pool amino acid availability and show that the supplemented leucine is rapidly available for protein synthesis and that there is little loss of label in the aquatic environment. Further, we were able to extract sufficient protein from individual zebrafish hearts to determine the incorporation rate of the label into hundreds of proteins simultaneously. This is an important step towards integrating genotype-phenotype data and will be used in further studies to explore the effect of physiological stimuli and dietary change on the zebrafish.

## 6 References

[1] Bai, Q., Garver, J. A., Hukriede, N. A., Burton, E. A., Generation of a transgenic zebrafish model of Tauopathy using a novel promoter element derived from the zebrafish eno2 gene. *Nucleic acids research* 2007, *35*, 6501-6516.

[2] Keller, E. T., Murtha, J. M., The use of mature zebrafish (Danio rerio) as a model for human aging and disease. *Comp Biochem Physiol C Toxicol Pharmacol* 2004, *138*, 335-341.

[3] Konzer, A., Ruhs, A., Braun, H., Jungblut, B., *et al.*, Stable isotope labelling in zebrafish allows in vivo monitoring of cardiac morphogenesis. *Mol Cell Proteomics* 2013.

[4] Lieschke, G. J., Currie, P. D., Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 2007, *8*, 353-367.

[5] Sager, J. J., Bai, Q., Burton, E. A., Transgenic zebrafish models of neurodegenerative diseases. *Brain Struct Funct* 2010, *214*, 285-302.

[6] Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., *et al.*, The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 2013, *496*, 498-503.

[7] Flinn, L., Mortiboys, H., Volkmann, K., Koster, R. W., *et al.*, Complex I deficiency and dopaminergic neuronal cell loss in parkin-deficient zebrafish (Danio rerio). *Brain* 2009, *132*, 1613-1623.

[8] Poss, K. D., Wilson, L. G., Keating, M. T., Heart regeneration in zebrafish. *Science* 2002, *298*, 2188-2190.

[9] Laflamme, M. A., Murry, C. E., Heart regeneration. *Nature* 2011, *473*, 326-335.

[10] Bergmann, O., Bhardwaj, R. D., Bernard, S., Zdunek, S., *et al.*, Evidence for cardiomyocyte renewal in humans. *Science* 2009, *324*, 98-102.

[11] Biales, A. D., Bencic, D. C., Villeneuve, D. L., Ankley, G. T., Lattier, D. L., Proteomic analysis of zebrafish brain tissue following exposure to the pesticide prochloraz. *Aquat Toxicol* 2011, *105*, 618-628.

[12] Mendelsohn, B. A., Malone, J. P., Townsend, R. R., Gitlin, J. D., Proteomic analysis of anoxia tolerance in the developing zebrafish embryo. *Comparative biochemistry and physiology. Part D, Genomics & proteomics* 2009, *4*, 21-31.

[13] Tay, T. L., Lin, Q., Seow, T. K., Tan, K. H., *et al.*, Proteomic analysis of protein profiles during early development of the zebrafish, Danio rerio. *Proteomics* 2006, *6*, 3176-3188.

[14] Andersen, J. S., Lam, Y. W., Leung, A. K., Ong, S. E., *et al.*, Nucleolar proteome dynamics. *Nature* 2005, *433*, 77-83.

[15] Geiger, T., Velic, A., Macek, B., Lundberg, E., *et al.*, Initial quantitative proteomic map of twenty-eight mouse tissues using the SILAC mouse. *Mol Cell Proteomics* 2013.

[16] Zanivan, S., Krueger, M., Mann, M., In vivo quantitative proteomics: the SILAC mouse. *Methods Mol Biol* 2012, *757*, 435-450.

[17] Doherty, M. K., Hammond, D. E., Clague, M. J., Gaskell, S. J., Beynon, R. J., Turnover of the human proteome: determination of protein intracellular stability by dynamic SILAC. *Journal of proteome research* 2009, *8*, 104-112.

[18] Pratt, J. M., Petty, J., Riba-Garcia, I., Robertson, D. H., *et al.*, Dynamics of protein turnover, a missing dimension in proteomics. *Mol Cell Proteomics* 2002, *1*, 579-591.

[19] Martin, S. F., Munagapati, V. S., Salvo-Chirnside, E., Kerr, L. E., Le Bihan, T., Proteome turnover in the green alga Ostreococcus tauri by time course 15N metabolic labeling mass spectrometry. *J Proteome Res* 2012, *11*, 476-486.

[20] Doherty, M. K., Whitehead, C., McCormack, H., Gaskell, S. J., Beynon, R. J., Proteome dynamics in complex organisms: using stable isotopes to monitor individual protein turnover rates. *Proteomics* 2005, *5*, 522-533.

[21] Claydon, A. J., Ramm, S. A., Pennington, A., Hurst, J. L., *et al.*, Heterogenous turnover of sperm and seminal vesicle proteins in the mouse revealed by dynamic metabolic labeling. *Mol Cell Proteomics* 2012, *11*, M111 014993.

[22] Price, J. C., Guan, S., Burlingame, A., Prusiner, S. B., Ghaemmaghami, S., Analysis of proteome dynamics in the mouse brain. *Proc Natl Acad Sci U S A* 2010, *107*, 14508-14513.

[23] Doherty, M. K., Brownridge, P., Owen, M. A. G., Davies, S. J., *et al.*, A proteomics strategy for determining the synthesis and degradation rates of individual proteins in fish. *J Proteomics* 2012, *75*, 4471-4477.

[24] Doherty, M. K., Owen, M. A., Davies, S. J., Young, I. S., Whitfield, P. D., Assessment of global proteome dynamics in carp: a model for investigating environmental stress. *J Proteome Res* 2013, *12*, 5246-5252.

[25] Cox, J., Mann, M., MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature biotechnology* 2008, *26*, 1367-1372.

[26] Cox, J., Matic, I., Hilger, M., Nagaraj, N., *et al.*, A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nature protocols* 2009, *4*, 698-705.

[27] Cox, J., Neuhauser, N., Michalski, A., Scheltema, R. A., *et al.*, Andromeda: a peptide search engine integrated into the MaxQuant environment. *Journal of proteome research* 2011, *10*, 1794-1805.

[28] Mi, H., Muruganujan, A., Thomas, P. D., PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. *Nucleic acids research* 2013, *41*, D377-386.

[29] Westman-Brinkmalm, A., Abramsson, A., Pannee, J., Gang, C., *et al.*, SILAC zebrafish for quantitative analysis of protein turnover and tissue regeneration. *J Proteomics* 2011, *75*, 425-434.

[30] Nolte, H., Holper, S., Housley, M. P., Islam, S., *et al.*, Dynamics of zebrafish fin regeneration using a pulsed SILAC approach. *Proteomics* 2015, *15*, 739-751.

[31] Chen, Y.-S., Qiu, X.-B., Transcription-Coupled Replacement of Histones: Degradation or Recycling? *Journal of Genetics and Genomics* 2012, *39*, 575-580.

[32] Seale, R. L., Conservation of non-histone chromatin proteins during growth in HeLa cells. *Biochemical and biophysical research communications* 1975, *63*, 140-148.

[33] Singh, R. K., Kabbaj, M. H., Paik, J., Gunjan, A., Histone levels are regulated by phosphorylation and ubiquitylation-dependent proteolysis. *Nature cell biology* 2009, *11*, 925-933.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Supporting Information**

Supporting Table 1 contains the calculated rates of synthesis of the proteins determined in this study.

Supplemental Figures 1 and 2 detail the incorporation of labelled amino acid into specific peptides.

**Figure Legends**

**Figure 1: Calculation of RIA from multi-leucine peptides**

The relative abundance of the dietary-supplemented amino acid [$^{2}H_{7}$]L-leucine in the precursor pool for protein synthesis was determined experimentally. Data was obtained from both di- and tri-leucine containing peptides from multiple proteins across the time-course of the experiment. The relative isotope abundance of labelled leucine in cardiac muscle was determined to be 0.31 ± 0.01 using the 30% diet and 0.46 ± 0.01 for the 50% diet.

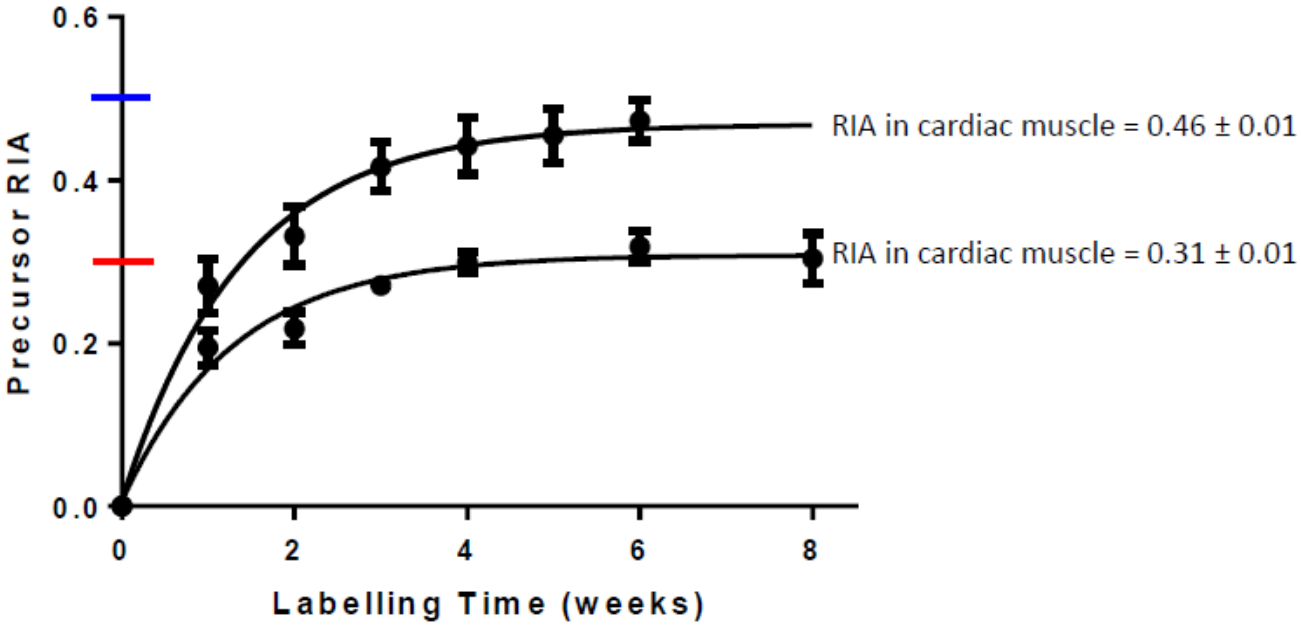


**Figure 2: Exemplar incorporation plots**

The amount of newly synthesized protein was measured at each time-point for individual proteins. The rate of synthesis for each protein was determined by fitting the data to a single exponential growth curve. Plots are shown for two proteins, pyruvate kinase and 4-alpha-glucanotransferase. Data from 30% labelled fish are denoted by ●, with 50% labelled proteins denoted by ●.

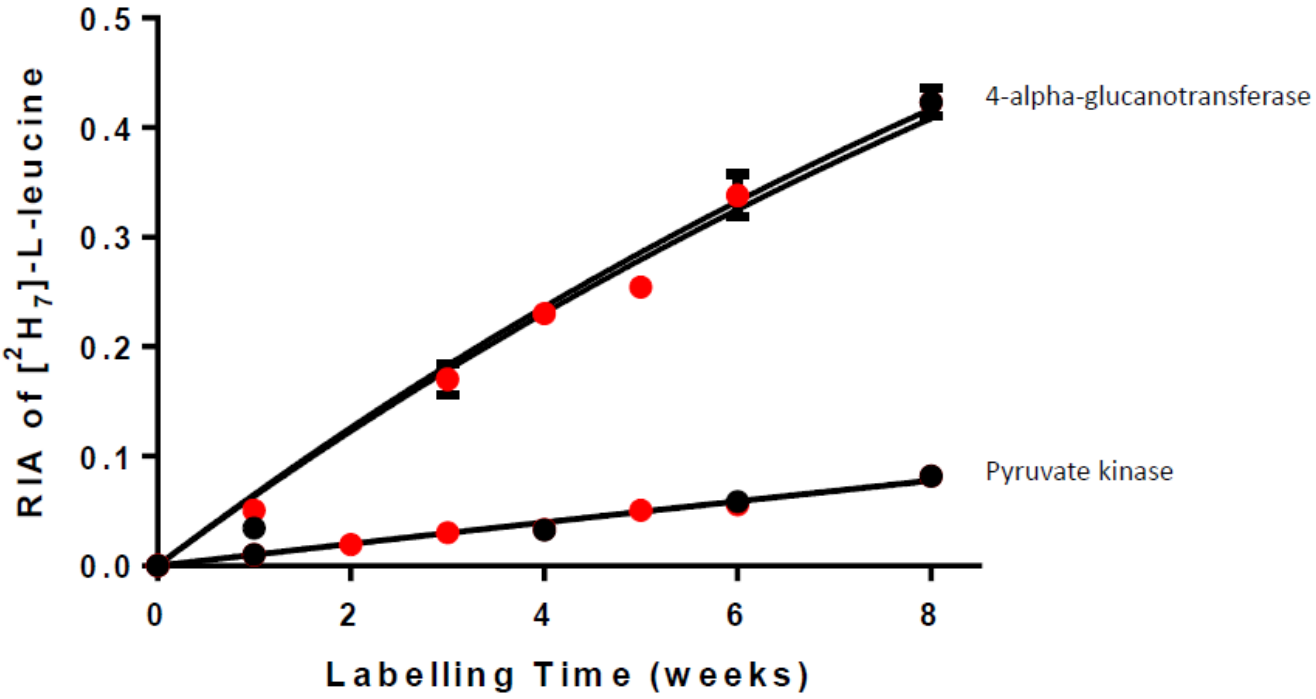


**Figure 3:**

**Range of protein synthesis rates**

First order rates of protein synthesis were calculated for over 660 hundred proteins simultaneously. Rate constants covered over four orders of magnitude.

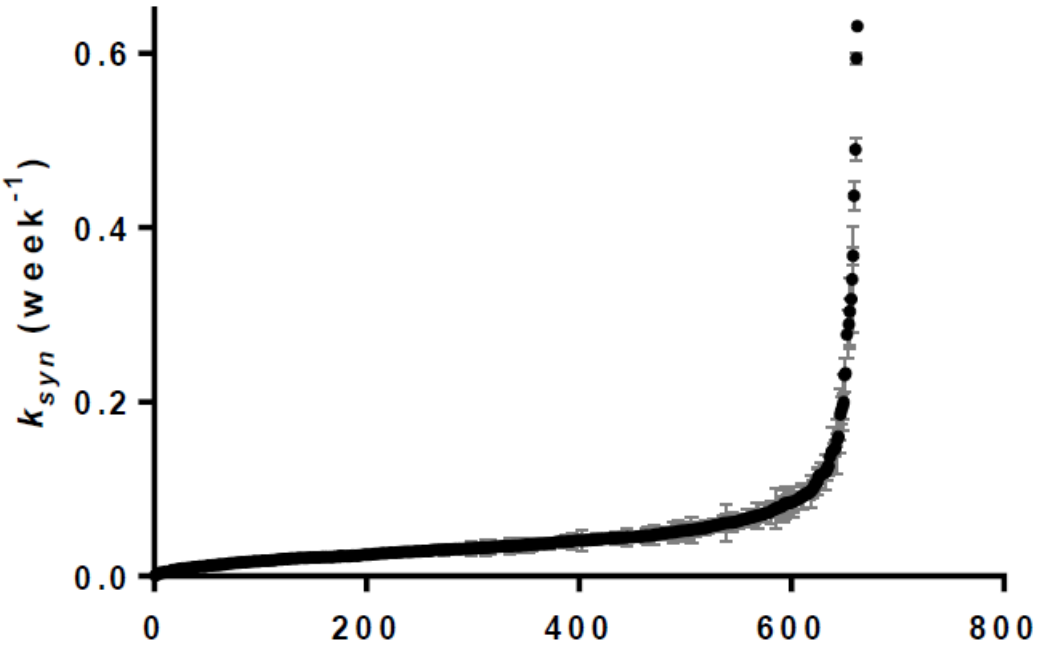


**Figure 4: Gene ontology analysis**

Gene ontology was performed on all zebrafish proteins for which rates of synthesis were determined. The proteins were classified in terms of biological process, molecular function, cellular component and pathway.

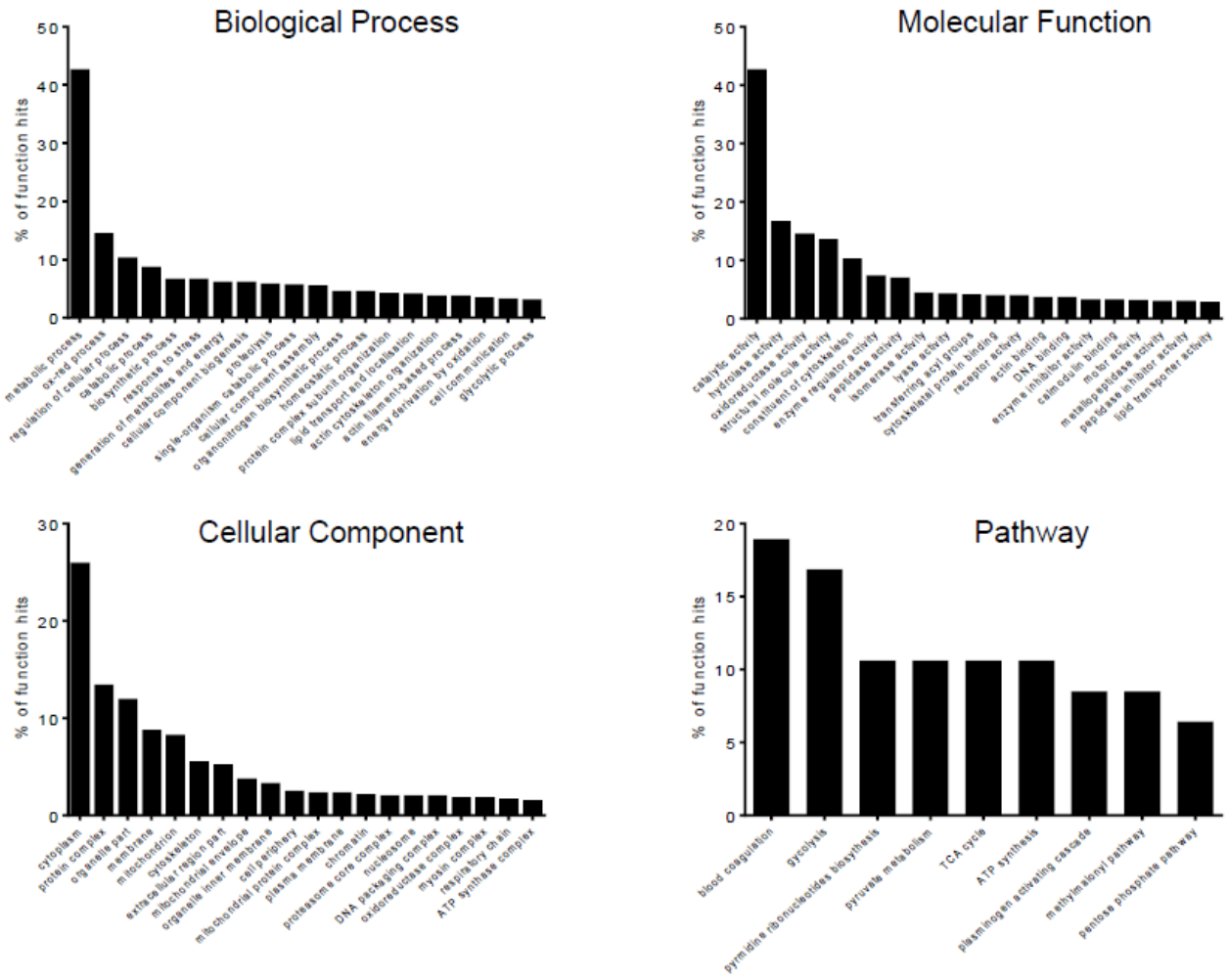


**Figure 5: Gene ontology analysis of slow and rapidly synthesised proteins**

Gene ontology of the 100 most and least rapidly synthesised zebrafish proteins were determined. The proteins were classified in terms of biological process, molecular function and cellular component. The 100 fastest rates of synthesis are shown as white bars, the 100 slowest with blue bars.