1. a) In one or two sentences, summarize the main research question addressed in this article. (4 marks)

How does the Hippo pathway (1) provide the building blocks for rapid growth (1), specifically, how do Yap target genes (1) contribute to rapid cell proliferation and increased organ size (1)?

b) Discuss whether the findings justify the use of ‘reprogram’ in the title. (4 marks)

Sensible discussion will be credited regarding the definition of reprogramming and how the authors are using it. Reprogramming may be overstating the findings given that they are only focusing on enhanced expression of Glul by Yap (1) and that essentially they are only showing increased production of glutamine that feeds into nucleotide biosynthesis rather than a rewiring of glutamine metabolism (2). However glutamine is an important precursor for nucleotide biosynthesis and the conditional requirement for GLUL suggests that it is activated under certain conditions so could argue that this constitutes reprogramming (1).

2. a) Discuss the advantages and disadvantages of using zebrafish in these studies rather than another model organism such as Drosophila or mouse, both of which have the Hippo pathway. (5 marks)

Zebrafish; vertebrate (1) and has a liver (unlike Drosophila) (1). Quicker and easier to care for than mice (1). Liver size can be assessed in a non-invasive manner (1) (transparent body is helpful). Fundamental metabolism question conserved across species however Zebrafish not a mammal so findings on therapeutic potential may not be applicable (1).

b) Suggest why the authors may have focused on liver. (4 marks)

Hippo pathway known to be involved in controlling organ growth (1). Liver provides a suitable organ to study as its size is regulated but it has an interesting ability to regenerate and repair and liver cancers are relatively common (2). Prior studies in mice (mentioned in the discussion) (1).

3. a) The paper makes use of a constitutively active mutant of YAP1, yap\textsuperscript{S87A}. What does this mutation suggest about how the Hippo kinase cascade regulates wild-type YAP1 activity. (2 marks)
That kinase activity negatively regulates YAP as the constitutively active form has serine 87 replaced by alanine that is not phosphorylatable.

b) To what extent can we be confident that the phenotypes associated with *lf:*Yap transgenics are due to Yap activation rather than Yap over-expression (4 marks). Design an experiment to address this issue (2 marks). (6 marks)

Comparisons are to wild-type siblings rather than non-activated Yap (1). Experiments expressing the non-mutated version are not shown and levels of the transgenic Yap are not determined (1). However this mutation is homologous to the human YAP1 constitutively active version so infer that the protein is active (1). Backed up by the gene expression data that is consistent with a mammalian Hippo signature (1). Could generate transgenics with the same transgene but with the wild-type sequence (1) measure protein levels (1).

c) In figure 1 data is shown regarding the impact of Yap activation on liver area, volume and liver/body mass ratio. Why was it important to include the liver/body mass ratio? (2 marks)

To assess whether the increased area and volume were a consequence/correlated with a general increase in body size of these fish.

4. The authors claim that Yap directly influences GLUL expression. Evaluate and critique the data shown in figure 3 in terms of which approach provides the strongest evidence to support their claim. (6 marks)

ChIP in Fig 3a (1) shows YAP to be present at the glula promoter and provides evidence that YAP is acting directly (rather than indirectly) (1). The use of controls (IgG and ctgfa and neg. region) adds weight to the data but there is no statistical analyses and the data is obtained from only two chromatin preps (2). The approaches in 3b, c & d show that YAP can influence GLUL levels but this could be indirect (i.e by affecting the expression of another transcription factor) (1) and no protein quantification is performed (1).

5. Identify the key similarities and differences between the $^{15}$N isotope incorporation experiments shown in Figures 4 and 7, and discuss the extent to which the findings are consistent. (5 marks)

Purine and pyrimidine biosynthesis use glutamine as a source of nitrogen atoms. In both approaches, the authors use mass spectrometry to detect the incorporation of $^{15}$N into nucleotides (1 mark). In Fig 4, a liver lysate is labelled acutely (20 min) with $^{15}$N-ammonium chloride, whereas Fig 7 uses dietary labelling with $^{15}$N-spirulina (algae) (1 mark) - this means
there will likely be a higher proportion of isotope incorporation in Fig 7 than Fig 4. In Fig 4 they detect free $^{15}$N GTP or CTP, whereas in Fig 7 they detect deoxynucleotides in genomic DNA (1 mark). In Fig 4 the only significant finding is that the Yap mutant lysate contains more M+3 guanosine than control. The effect of the GLUL inhibitor is not significant for guanosine or cytosine (1 mark). In Fig 7 they again find significant differences for the Yap mutant for M+2 15N incorporation into dG and dI but not dA and the effects of MSO are again not significant (1 mark). While the Yap mutant produces a small but significant effect in both approaches, either the design or lack of reproducibility means the authors cannot claim that the effect of Yap is via GLUL (1 mark).

6. a) The authors suggest that the involvement of GLUL is conditional. Summarize the evidence they are using to support this suggestion, comment on the strengths and weaknesses and discuss the implications for normal liver growth. (10 marks)

Figure 5a-c show that glula/glulb knockdown with morpholinos or MSO treatment had no effect on liver size or morphology in WT larvae (1). Liver area is measured using GFP fluorescence following treatments (1). Quantitative analysis is performed on multiple individuals (1) though according to the methods the experiments were not performed blind (1). The data is presented such that the differences are exaggerated (i.e broken axes). In figure 5f treatment with either MSO or verteporfin inhibits proliferation of Hep3B liver cancer cells but only when glutamine is absent from the media (1). Replicates were performed but no stats are described (1). Perhaps not surprising that GLUL would be required when glutamine is absent from the media - experiment could be considered to be of questionable physiological relevance (1). In figure 7f MSO treatment again had no impact in wild-type animals (this time looking at adults) but reduced the liver/body ratio in lf:Yap animals (1). The suggestion is that GLUL is not required in normal liver growth and development (though low levels of GLUL activity are measured in WT animals) and this is unchanged by MSO addition (1). Suggests that glutamine levels are sufficient to supply nucleotides during normal conditions (1).

b) Discuss the extent to which the authors can conclude that enhanced liver growth in lf:YAP transgenics is solely due to increased GLUL activity and nucleotide biosynthesis. (5 marks)

Evidence to support this. Liver size is reduced in lf::Yap when GLUL is inhibited via morpholino or MSO treatment (1) though sizes don’t look quite back to wild-type (1). MSO treatment is knockdown not knock-out and MSO treatment may not fully block GLUL activity (1). Comparing Fig 7c and 7f, MSO treatment reduces GLUL activity in liver back to levels similar to WT without treatment, but reduction in liver/body mass ratio is relatively small, suggesting GluL is not the only factor contributing to increased liver size (1). Can also reduce the liver area by MPA and 5FU which inhibits nucleotide biosynthesis but not back to wild-type levels and there is an additive effect of using MPA + MSO (1). MSO seems to be activating mTOR (in Fig 6) so this may complicate findings. They haven’t specifically over-expressed GLUL and asked whether that is sufficient to promote enhanced growth.
7. a) Comment on the authors’ claims for Figure 6a that “no significant differences were seen in WT and If:Yap liver homogenates” for p-Akt and p-S6 and that “MSO exposure led to a subtle increase in p-Akt and p-S6 levels”.

Without proper quantification it is hard to determine whether significant differences are present (1). By eye it looks like there is a slight increase in p-Akt and pS6 in If:Yap compared to WT but S6 controls are quite variable (1). The increase in p-Akt following MSO treatment is quite obvious so could argue that this goes beyond a subtle effect (1). Whether MSO affects p-S6 is hard to evaluate because of the variation in overall S6 levels on this blot (1).

b) Provide an explanation for why mTOR inhibition suppresses Yap-driven liver growth and why additive effects are not observed when rapamycin and MSO are used in combination.

mTOR has a role in stimulating nucleotide biosynthesis so inhibiting it using rapamycin is going to have a similar effect as inhibiting GLUL by MSO (1). This is consistent with a lack of additive effects, which also suggests that maximum inhibition had been achieved by either treatment alone (1).

8. a) In the abstract the authors state that fish expressing the activated form of Yap1 are prone to liver tumour formation. They also state that their findings demonstrate that Yap1 integrates the anabolic demands of tissue growth during tumorigenesis by reprogramming nitrogen metabolism. Discuss the extent to which the data support these claims.

Data in paper relating to tumorigenesis is limited. The If:Yap fish are prone to tumour formation but only when treated with the DMBA carcinogen (2). No -DMBA experiments are shown (1). The only other data relating to tumorigenesis is the Hep2/3 cell proliferation assay which shows that inhibiting GLUL or Yap reduces cell proliferation but only in the absence of added glutamine in the media (2). This is not demonstrating reprogramming of nitrogen metabolism during tumorigenesis and so this claim seems unsupported (1).

b) Discuss whether targeting Yap would be a better approach than targeting GLUL in terms

Data in the paper suggests targeting GLUL can reduce liver cancer cell proliferation and that the conditional requirement for GLUL is advantageous in terms of allowing a therapeutic ‘window’ for treatment (2). Targeting GLUL under conditions where Yap is not activated does not seem to have any negative consequences (at least in this zebrafish model) (1). However there are many changes in gene expression and metabolites in the If:Yap animals and so it is likely that other Yap targets play a role in facilitating rapid growth (1). Targeting Yap may be more effective than specifically targeting GLUL but possibly have a higher likelihood of negative side-effects (1). The Hep3B proliferation assay suggests that targeting GLUL may be more effective but lack of stats in figure 5f and details on effectiveness of the inhibitors is lacking (1).
9. Suggest a series of experiments that would allow you to investigate the involvement of YAP in a physiological setting such as normal organ growth during development, tissue regeneration following injury, cancer or another of your choice. You should include a proposed species and briefly justify your choice of both species and physiological setting.

(10 marks)

Various possibilities:

Eg. for regeneration of liver following injury. Liver has an interesting ability to regenerate following injury and findings could be relevant in the treatment of various diseases where liver regeneration is compromised by chronic liver disease (2). Proposed species: mouse as genetic manipulations possible and could surgically remove/injure part of the liver and assess consequences (2). Could monitor Yap activity before and after injury by assessing levels of phospho-Yap by western blot (1) and measuring gene expression of Yap targets (eg. some of the targets mentioned in this paper) (1). To understand whether Yap is required for liver regeneration could selectively knock-out Yap (create floxed alleles) in hepatocytes (2), surgically remove/injure the liver (1) and monitor the ability to regenerate compared to wild-type (1).