

## Possible Minimum Pathways from RuBP\_h to Fum\_c

When deleting all reactions with no AT code there is no possible pathway for RuBP\_h to Fum\_c.

Here we added reactions without an AT code back into the model, one at a time, and determined the shortest possible paths from RuBP\_h to Fum\_c.

We found the following 17 possible shortest pathways (others exist but require more steps) and checked them against our flux sampling results (which are constrained according to the metabolite and proteomics data) to ensure that they are indeed feasible:

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**Pathway 1:** ['RuBP\_h', 'PGA\_h', '2PGA\_h', 'PEP\_h', 'Pyr\_h', 'AMP\_h', 'AMP\_c', 'IMP\_c', 'XMP\_c', 'Glu\_c', 'KG\_c', 'OAA\_c', 'Mal\_c', 'Fum\_c']

Reaction added: AMP\_h --> AMP\_c

Flux sampling concludes: This pathways is not feasible as there is not flux from PGA\_h to 2PGA\_h

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**Pathway 2:** ['RuBP\_h', 'PGA\_h', 'DPGA\_h', 'GAP\_h', 'R5P\_h', 'AMP\_h', 'AMP\_c', 'IMP\_c', 'XMP\_c', 'Glu\_c', 'KG\_c', 'OAA\_c', 'Mal\_c', 'Fum\_c']

Reaction added: AMP\_h --> AMP\_c

Flux sampling concludes: This pathway is unlikely to contribute substantially to Fum\_c production because the flux from AMP\_c to IMP\_c is too low (<0.4).

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**Pathway 3:** ['RuBP\_h', 'PGA\_h', '2PGA\_h', 'PEP\_h', 'Pyr\_h', 'AMP\_h', 'AMP\_c', 'IMP\_c', 'XMP\_c', 'Glu\_c', 'KG\_c', 'OAA\_c', 'Mal\_c', 'Fum\_c']

Reaction added: AMP\_h + NAD\_c <=> AMP\_c + NAD\_h

Flux sampling concludes: This pathway is not feasible, see Pathway 1.

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**Pathway 4:** ['RuBP\_h', 'PGA\_h', 'DPGA\_h', 'GAP\_h', 'R5P\_h', 'AMP\_h', 'AMP\_c', 'IMP\_c', 'XMP\_c', 'Glu\_c', 'KG\_c', 'OAA\_c', 'Mal\_c', 'Fum\_c']

Reaction added: AMP\_h + NAD\_c <=> AMP\_c + NAD\_h

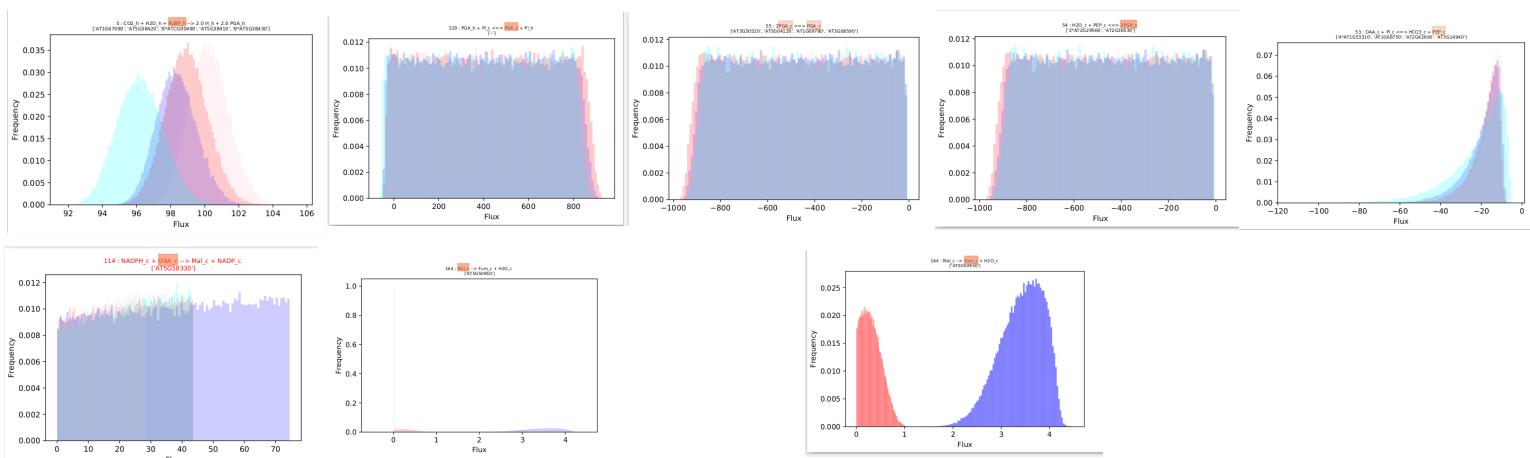
Flux sampling concludes: This pathway is unlikely, see Pathway 2.

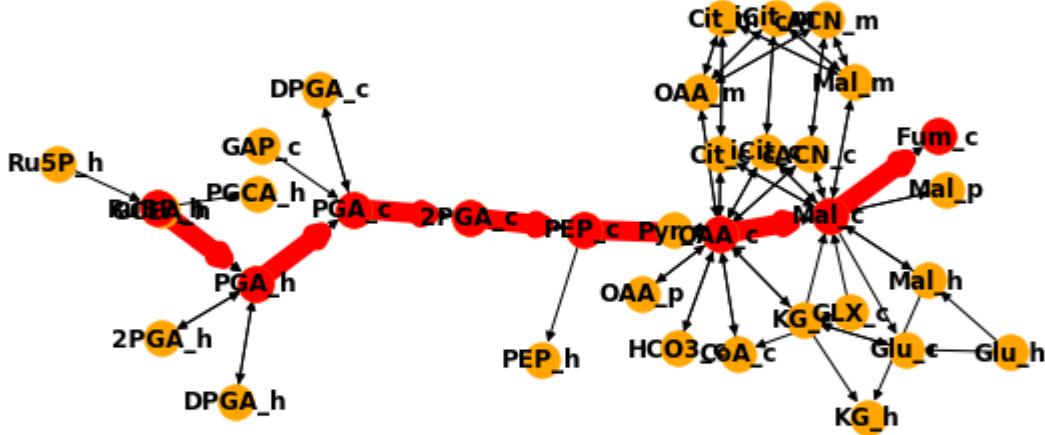
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**Pathway 5:** ['RuBP\_h', 'PGA\_h', 'PGA\_c', '2PGA\_c', 'PEP\_c', 'OAA\_c', 'Mal\_c', 'Fum\_c']

Reaction added: PGA\_h + Pi\_c <=> PGA\_c + Pi\_h

Flux sampling concludes: This pathways is feasible!

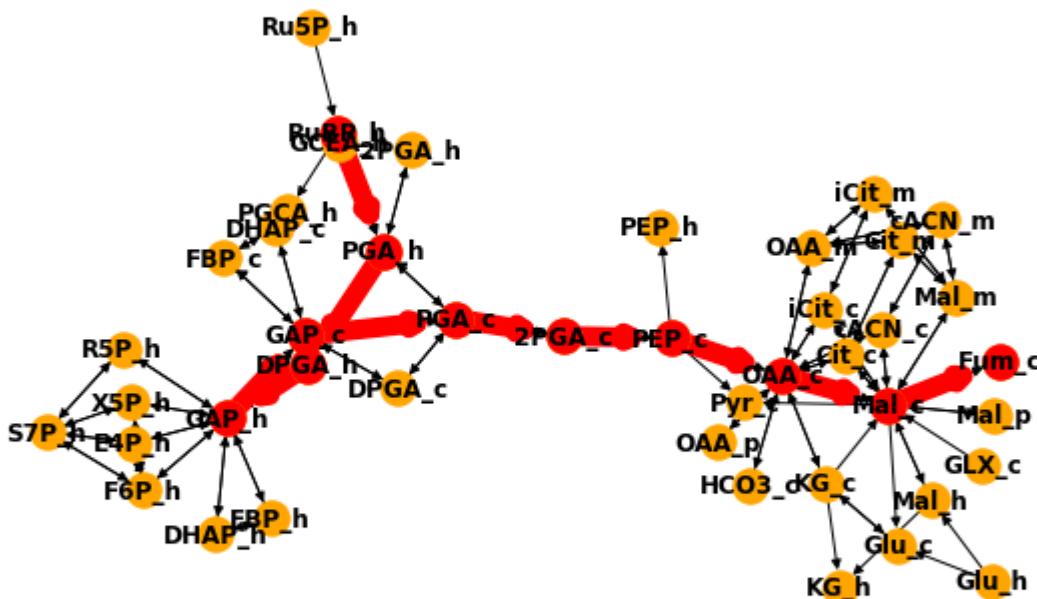
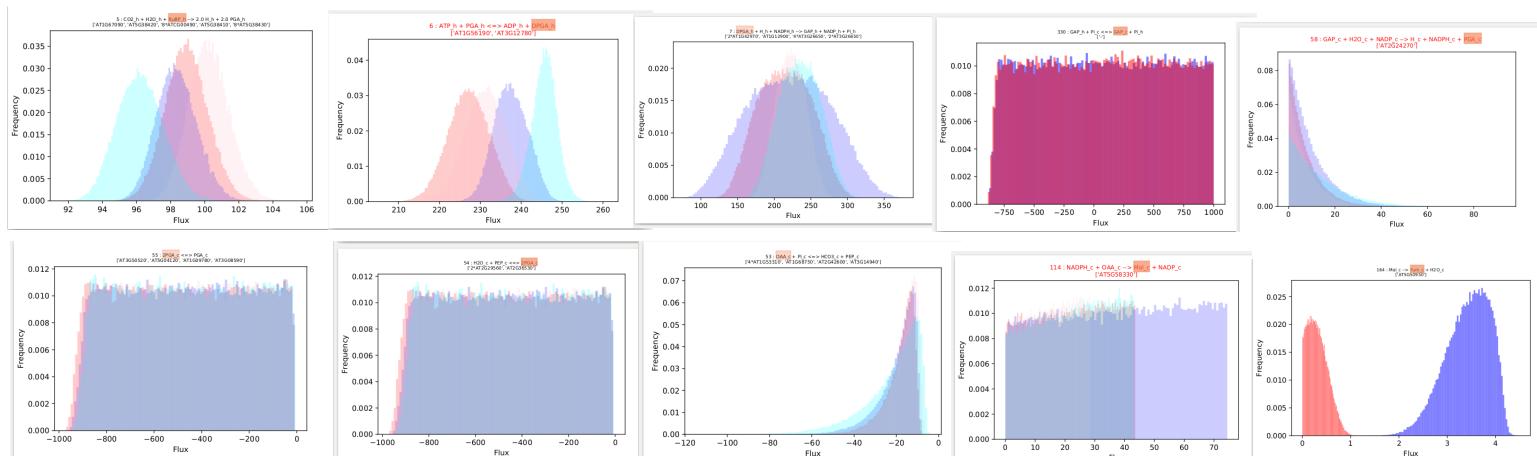




**Pathway 6: ['RuBP\_h', 'PGA\_h', 'DPGA\_h', 'GAP\_h', 'GAP\_c', 'PGA\_c', '2PGA\_c', 'PEP\_c', 'OAA\_c', 'Mal\_c', 'Fum\_c']**

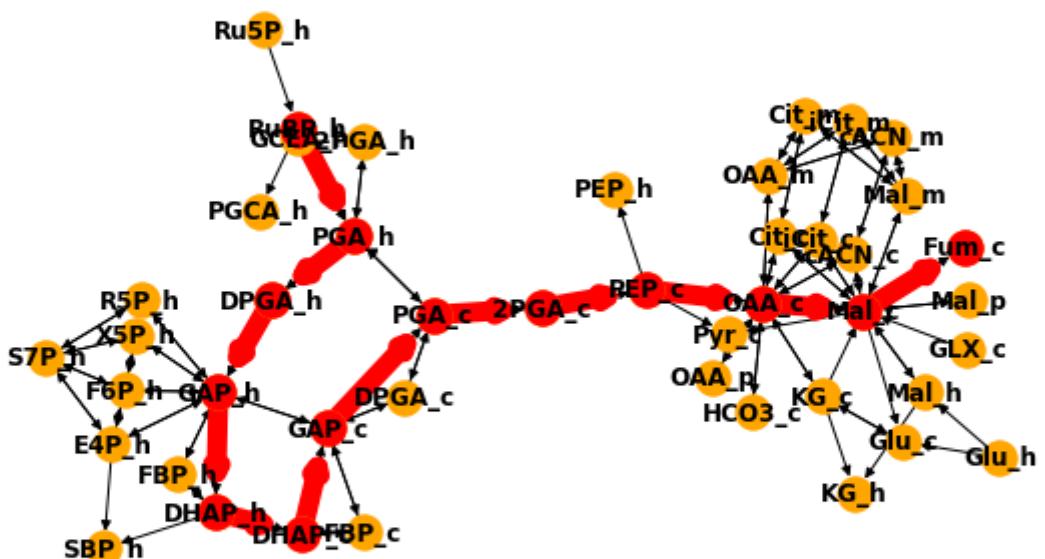
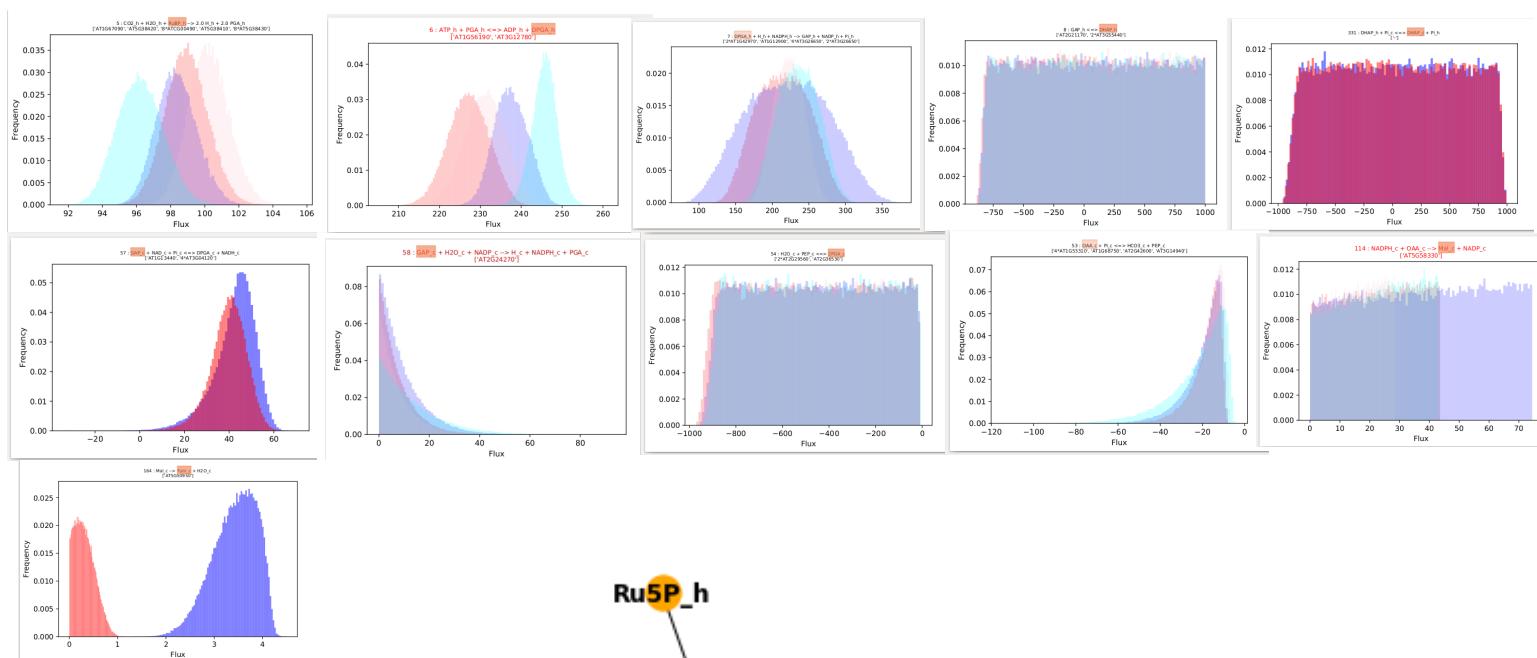
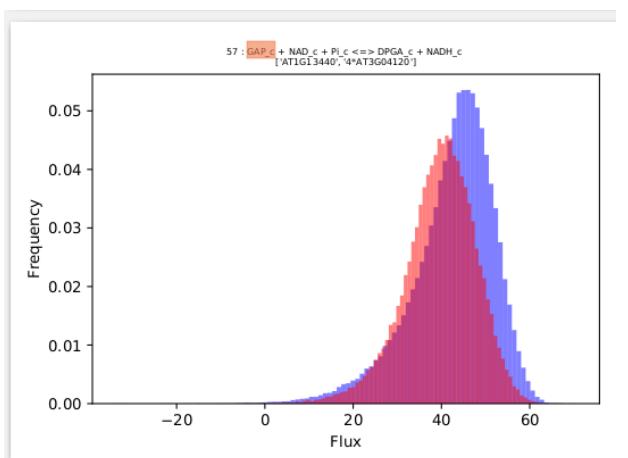
Reaction added:  $\text{GAP}_h + \text{Pi}_c \rightleftharpoons \text{GAP}_c + \text{Pi}_h$

Flux sampling concludes: This pathway is feasible!



**Pathway 7:** ['RuBP\_h', 'PGA\_h', 'DPGA\_h', 'GAP\_h', 'DHAP\_h', 'DHAP\_c', 'GAP\_c', 'PGA\_c', '2PGA\_c', 'PEP\_c', 'OAA\_c', 'Mal\_c', 'Fum\_c']  
Reaction added: DHAP\_h + Pi\_c <=> DHAP\_c + Pi\_h

Flux sampling concludes: This pathway is feasible but arguably less likely because the model flux from DHAP\_c to GAP\_c is a very infrequent solution....



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**Pathways 8 - 12:**

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['RuBP_h', 'PGA_h', 'DPGA_h', 'GAP_h', 'F6P_h', 'G6P_h', 'G1P_h', 'ADPG_h',
'starch1_h', 'Glc_h', 'Glc_c', 'G6P_c', 'UDP_c', 'UTP_c', 'Glu_c', 'KG_c', 'OAA_c', 'Mal_c',
'Fum_c']
['RuBP_h', 'PGA_h', 'DPGA_h', 'GAP_h', 'F6P_h', 'G6P_h', 'G1P_h', 'ADPG_h',
'starch3_h', 'Glc_h', 'Glc_c', 'G6P_c', 'UDP_c', 'UTP_c', 'Glu_c', 'KG_c', 'OAA_c', 'Mal_c',
'Fum_c']
['RuBP_h', 'PGA_h', 'DPGA_h', 'GAP_h', 'F6P_h', 'G6P_h', 'G1P_h', 'ADPG_h',
'starch2_h', 'Glc_h', 'Glc_c', 'G6P_c', 'UDP_c', 'UTP_c', 'Glu_c', 'KG_c', 'OAA_c', 'Mal_c',
'Fum_c']
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Reaction added: Glc\_h --> Glc\_c

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['RuBP_h', 'PGA_h', 'DPGA_h', 'GAP_h', 'F6P_h', 'G6P_h', 'G1P_h', 'ADPG_h',
'starch3_h', 'Mas_h', 'Mas_c', 'Glc_c', 'G6P_c', 'UDP_c', 'UTP_c', 'Glu_c', 'KG_c', 'OAA_c',
'Mal_c', 'Fum_c']
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['RuBP_h', 'PGA_h', 'DPGA_h', 'GAP_h', 'F6P_h', 'G6P_h', 'G1P_h', 'ADPG_h',
'starch3_h', 'Mas_h', 'Mas_c', 'starch2_c', 'G1P_c', 'UDPG_c', 'UTP_c', 'Glu_c', 'KG_c',
'OAA_c', 'Mal_c', 'Fum_c']
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Reaction added: Mas\_h --> Mas\_c

Pathways too unlikely. Did not bother checking against flux sampling....

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**Pathway 13: ['RuBP\_h', 'PGA\_h', '2PGA\_h', 'PEP\_h', 'Pyr\_h', 'KG\_h', 'OAA\_h', 'Mal\_h', 'Mal\_c', 'Fum\_c']**

Reaction added: KG\_c + Mal\_h --> KG\_h + Mal\_c

Flux sampling concludes: This pathways is not feasible, see Pathway 1.

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**Pathways 14-16:**

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['RuBP_h', 'PGA_h', '2PGA_h', 'PEP_h', 'EPSP_h', 'CHR_h', 'Glu_h', 'Glu_c', 'KG_c',
'OAA_c', 'Mal_c', 'Fum_c']
['RuBP_h', 'PGA_h', '2PGA_h', 'PEP_h', 'Pyr_h', 'KG_h', 'Glu_h', 'Glu_c', 'KG_c', 'OAA_c',
'Mal_c', 'Fum_c']
['RuBP_h', 'PGA_h', 'DPGA_h', 'GAP_h', 'R5P_h', 'PRPP_h', 'Glu_h', 'Glu_c', 'KG_c',
'OAA_c', 'Mal_c', 'Fum_c']
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Reaction added: Glu\_h + Mal\_c --> Glu\_c + Mal\_h

Flux sampling concludes: The first two options are not feasible, see Pathway 1. The 3<sup>rd</sup> pathway is feasible although this pathway is unlikely to contribute substantially to Fum\_c production because the flux from PRPP\_h to GLU\_c is too low (<0.4).

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**Pathway 17: ['RuBP\_h', 'PGA\_h', 'DPGA\_h', 'GAP\_h', 'R5P\_h', 'PRPP\_h', 'PRPP\_c', 'AMP\_c', 'IMP\_c', 'XMP\_c', 'Glu\_c', 'KG\_c', 'OAA\_c', 'Mal\_c', 'Fum\_c']**

Reaction added: H\_h + PRPP\_h --> PRPP\_c

Flux sampling concludes: This pathway is unlikely, see Pathway 2.

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## Conclusion

From the above, Pathways 5,6 & 7 are the only feasible shortest pathway which rely on only one reaction with no AT code and is confirmed by flux sampling.

While these represents the shortest possible pathway we cannot confirm whether which one is the most likely pathway.

However, it must be noted that while pathway 7 is feasible, the probability of this being a feasible flux solution upon further model constraints is unlikely given that a flux from DHAP\_c to GAP\_c is a very infrequent solution....

The flux sampling confirms that cytosolic malate is required for cytosolic fumarate accumulation. It further suggests that cytosolic malate is produced from cytosolic oxaloacetate; this flux is significantly increased in response to cold in the wild-type and not in the mutant.

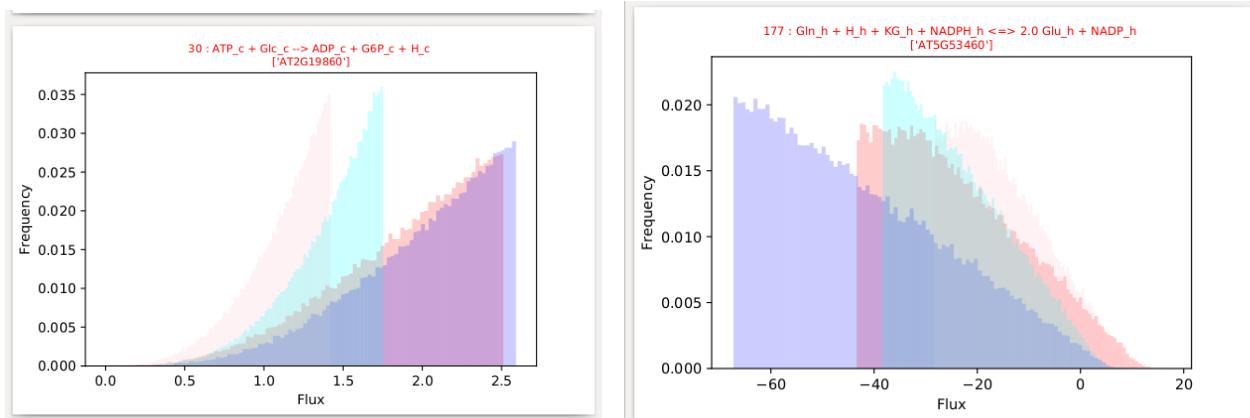
None of the analysed pathways suggest cytosolic malate to be exported from the mitochondrion. Some of the analysed pathways export malate from the chloroplast, by exchanging it for KG or Glu. The flux through these reactions however is low and neither of the two reactions have a confirmed AT code.

While the shortest possible pathways suggests cytosolic oxaloacetate to be produced from PEP\_c, it could also be produced via KG\_c ( $\text{Glu}_c + \text{OAA}_c \leftrightarrow \text{Asp}_c + \text{KG}_c$ ) another reaction which carries a higher flux in the wild-type in response to cold. This flux, however is non-essential (can carry a flux of zero) whereas the conversion of PEP\_c to OAA\_c is essential. The latter has a higher flux potential in response to cold in both the wild-type and the mutant, confirming the fact that the total malate + fumarate storage is increased in both genotypes in response to cold although less so in the mutant.

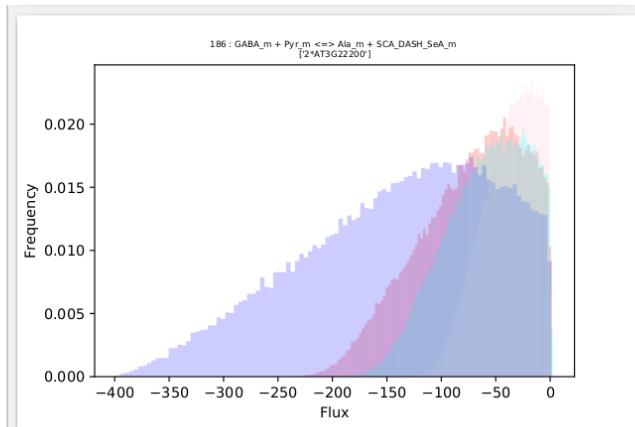
The production of PEP\_c from 2PGA\_c and the production of 2PGA\_c from PGA\_c are also essential (carry a non-zero flux) across all conditions. Our results therefore confirm that under all conditions these reactions must take place and that the flux potential of PEP\_c production is increased in response to cold. The conversion of PGA\_h to PGA\_c however is non-essential and we can therefore draw no conclusion as to whether this is indeed required for obtaining PGA\_c from RuBP\_h or whether alternative pathways in the chloroplast are used.

## Other Interesting Reactions

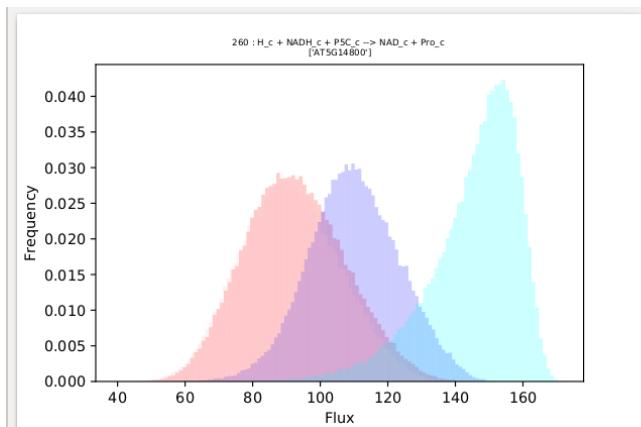
These are interesting results from the flux sampling but don't understand them yet in a pathway context....



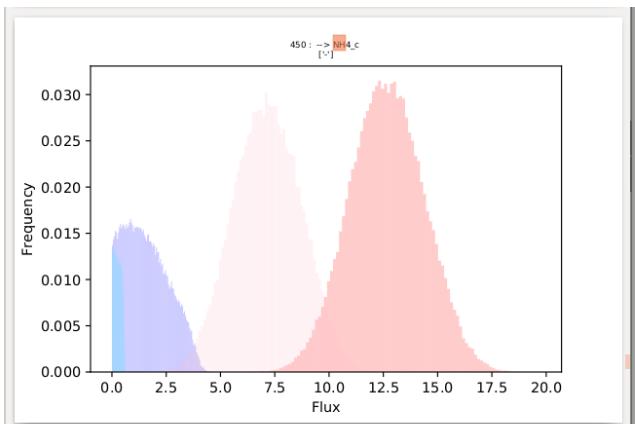
- Lots of differences in the glucose pathways between wild-type and mutant... but because these reactions could technically carry a flux of zero it is hard to comment, would have to phrase it in terms of flux potential....



- With protein constraints incorporated, GABA again stands out to have a greater flux potential in the wild-type in response to cold



- There are quite a few metabolomics studies which mention Proline accumulation in response to stress....



- Increased nitrogen uptake in control conditions but cold wild-type is higher than cold mutant