An Ancient, Unified Mechanism for Metformin Growth Inhibition in C. elegans and Cancer
Wu et al., Cell, 167:1705-1718

1) Briefly describe the RNAi screen for resistance to metformin treatment (suppression) and RNAi screen for sensitivity to metformin treatment (enhancement) screens. (b) What broad types of genes would you expect to obtain from such screens? (c) What are some reasons that this screen would not identify all the genes involved in metformin effects?

(b) - Genes whose knockdown result in resistance are predicted to be mediators of metformin treatment; could potentially be the binding target of metformin, assuming that binding activates a growth inhibitory gene product.
- Genes whose knockdown result in sensitivity to metformin are predicted to be involved in the same processes (growth) that are being partially disrupted by metformin treatment; could potentially be the binding target of metformin, assuming that binding inactivates a growth promoting gene product.
(c) (i) only a subset of C. elegans genes were tested in the screen (1046 with “metabolism” GO annotation), relevant genes among the ~19000 non-tested genes would not be identified; (ii) because of the high false negative rate of RNAi screens, some genes among the 1046 tested may have been missed.

2) What is the wild type function of CeACAD10? Provide genetic data supporting this conclusion. What and its role in metformin treatment?

- CeACAD10 inhibits worm growth, based on overexpression data showing growth inhibition, and enhancement of the growth inhibitory effects of low-level metformin treatment.
- CeACAD10 is a mediator of metformin treatment, based on loss of CeACAD10 function suppressing metformin treatment, and its expression is induced by metformin treatment.

3) What data is provided by Wu et al. that mitochondria, CeTORC1 and CeACAD10 function in a single metformin response pathway? (b) What about the experimental design leaves open the possibility that they are not in a single pathway?

(a) RNAi of mitochondrial complex I component gas-1, inhibitor rotenone, and RNAi of daf-15 Raptor result in increased CeACAD10 expression and inhibition of body size, analogous to metformin treatment. Wu et al argue that gas-1 and daf-15 act in the same pathway as metformin because the increased CeACAD10 expression and decreased body size of gas-1 RNAi and daf-15 RNAi is not enhanced by metformin treatment.
(b) Failure to see enhancement could be a result of incomplete RNAi knockdown of gas-1 and daf-15.

4) Wu et al. performed an EMS screen to isolate suppressors of metformin induced induction of CeACAD10 expression. (a) What type of genes were the authors attempting to identify? (b) What is the importance of the assays of npp-3 and npp-21 RNAi on body size? (c) Explain the results that Wu et al. use to argue that the nuclear pore complex (NPC) genes are acting downstream, of mitochondria and upstream of CeTORC1; which genes/treatments are epistatic? What type of pathway are the authors assuming? (d) Wu et al. repeated the epistasis analysis using human tissue culture cells; what was the phenotype used to assay pathway activity?
(4a) Genes that act to promote the induction of CeACAD10 following metformin treatment, where loss would result in a failure to obtain induction.
(b) To show that npp genes are acting on both induction of CeACAD10 and on body size, which is effected by mitochondria, CeTORC1 and metformin pathways.
(c) Both rotenone (a mitochondrial inhibitor) and rapamycin (a TORC1 inhibitor) induce CeACAD10 expression. Loss of npp-21 blocks induction by rotenone while it does not block induction by rapamycin; thus npp-21(alk44) is epistatic to rotenone while rapamycin is epistatic to npp-21(alk44). The authors assume that the metformin/mitochondria/NPC/mTORC1 pathway is a regulatory/binary switch pathway, which with the epistasis data, places metformin treated mitochondria upstream of the NPP, which is upstream of CeTORC1.
(d) A direct readout of mTORC1 activity, S6 kinase, as compared to downstream readout of CeTORC1 activity in C. elegans, CeACAD10 expression.

5) Describe the experiment and logic used to argue that it is the nuclear pore complex, rather than a non-nuclear pore complex function of npp-3 and npp-21, that mediate metformin induced growth inhibition.

5) If metformin induced growth inhibition is acting through the NPC, then loss on many different structural and functional NPC gene products should also suppress induction of ACAD10 expression. This is shown in Figure 5A.

6) Describe the data that indicates that it is RagA/RagC and its nuclear entry/export that is altered in metformin/phenformin treatment.

6) Of mTORC1 activators, only RagC shows significant nuclear localization under basal conditions. RagC contains a nuclear export sequence (NES); RagC/NES has increased level in the nucleus, which is fully blocked by phenformin treatment. RagA stimulates increased nuclear localization of RagC, which is blocked by phenformin treatment. Adding 3xGFP to RagC, which decreases passive nuclear entry, was found to block RagA ability to activate mTORC1 in phenformin treated cells.

7) You join the Soukas laboratory as a postdoctoral fellow and you characterize the next set of EMS mutants that strongly suppress induction of CeACAD10 expression following metformin treatment. From whole genome sequencing of 25 alleles you focus on those that affect genes predicted to be involved in transcriptional regulation of CeACAD10. You identify 3 new alleles of skn-1, 4 alleles of the worm ortholog of the conserved hypoxia-inducible factor hif-1, 4 alleles of conserved T-box protein tbx-11; all of these mutations are recessive, with at least one allele likely null, indicated here as (0). You also identify one dominant allele (alk100) in fkh-2, the worm ortholog of human FOXG1. You examine CeACAD10 expression in the null deletion allele of fkh-2 ok683, and find that homozygotes show high levels of CeACAD10 expression, both with and without metformin treatment, while heterozygotes are identical to wild type.

A) Based on the null mutant phenotype, what is the wild type function of hif-1, tbx-11 and fkh-2?

hif-1 and tbx-11 are positive regulators of metformin induced induction of CeACAD10 expression. fkh-2 is a negative regulator of CeACAD10 expression
B) Based on the dominant phenotype of \textit{fkh-2}, provide a hypothesis for the likely affect of the \textit{alx100} mutation. Describe the experiments and the predicted result to test your hypothesis.

The dominant phenotype of \textit{fkh-2(alx100)}, constitutive CeACAD10 expression, is opposite of that of \textit{fkh-2(ok683)}, failure to induce CeACAD10 expression, suggesting that \textit{alx100} is a hypermorphic gain of function mutation.

Perform gene dosage studies. If \textit{alx100} is a hypermorphic gain of function then addition of a wild-type allele should enhance the overexpression phenotype while removal of the wild type allele with a null allele should suppress the overexpression phenotype.

\textit{alx100/alx100/} > \textit{alx100/alx100} > \textit{alx100/ok683}, where > signifies stronger CeACAD10 expression.

C) You perform genetic epistasis analysis to determine how this set of transcription factors controls CeACAD10 expression.

Expression data is of the form that 100\% = full induction of CeACAD10 expression following metformin treatment.

\begin{align*}
\text{skn-1(0)} & \quad 40\% \\
\text{hif-1(0)} & \quad 30\% \\
\text{fkh-2(0)} & \quad 100\% \text{ (with or without metformin)} \\
\text{fkh-2(alx100)} & \quad 0\% \\
\text{tbx-11(0)} & \quad 0\% \\
\end{align*}

\begin{align*}
\text{skn-1(0); hif-1(0)} & \quad 0\% \\
\text{skn-1(0); fkh-2(0)} & \quad 100\% \text{ (with or without metformin)} \\
\text{skn-1(0); fkh-2(alx100)} & \quad 0\% \\
\text{skn-1(0); tbx-11(0)} & \quad 0\% \\
\end{align*}

\begin{align*}
\text{hif-1(0); fkh-2(0)} & \quad 100\% \text{ (with or without metformin)} \\
\text{hif-1(0); fkh-2(alx100)} & \quad 0\% \\
\text{hif-1(0); tbx-11(0)} & \quad 0\% \\
\end{align*}

\begin{align*}
\text{hif-1(0); skn-1(0); fkh-2(0)} & \quad 100\% \text{ (with or without metformin)} \\
\text{fkh-2(0); tbx-11(0)} & \quad 0\% \\
\end{align*}

Draw the genetic pathway that is consistent with the epistasis results above. Explain your reasoning.

\begin{align*}
\text{SKN-1} \quad \text{------- | FKH-2 \ ------} \quad \text{TBX11 \ ------ > CeACAD10} \\
\text{HIF-1} \quad \text{- Assume that these function in a regulatory/binary switch pathway.}
\end{align*}
SKN-1 and HIF-1 have incompletely penetrant phenotypes and likely act redundantly with each other as the double mutant fails to induce CeACAD10 expression. Doubles of skn-1 or hif-1 with other gene null mutants that have 0% induction are uninformative as they show the 0% induction phenotype of the other gene null mutant.

- *fkh-2(0)* is epistatic to the *skn-1(0); hif-1* double mutant, indicating that SKN-1 & HIF-1 act upstream of FKH-2.

- *tbx-11(0)* is epistatic to *fkh-2(0)*, indicating that FKH-2 acts upstream as a negative regulator of TBX-11, which activates CeACAD10 expression.

8) The CeACAD10 likely null mutant, *gk463343* in F37H8.3, only partially suppresses metformin treatment and the *daf-15* Raptor RNAi for relative body area phenotype, suggesting that another gene is acting in parallel to CeACAD10 - F37H8.3 to mediate the relative body area phenotype. Therefore you looked for genes, following RNAi knockdown that resulted in full suppression of metformin + F37H8.3(*gk463343*) and *daf-15* RNAi + F37H8.3(*gk463343*) using the 1046 set of genes with a metabolism GO annotation. This screen identified K09H11.1 (which was apparently missed in the earlier screen). Based on the molecular identity of K09H11.1, provide an explanation for the full suppression of metformin and *daf-15* Raptor phenotypes following simultaneous removal of F37H8.3 and K09H11.1.

Both F37H8.3 and K09H11.1 encode ACAD10 homologs, which likely act redundantly with each other to mediate the growth arrest phenotypes caused by metformin treatment and loss of *daf-15* Raptor.

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Localized *TWIST1* basic domain mutations cause a novel frontonasal dysplasia that can be modeled in *C. elegans*

Kim et al., under review at *American Journal of Human Genetics.*

9) What was the goal of this study?

10) In the Introduction, the authors indicate that *hlh-8*(Glu29Lys) homozygotes have a more severe phenotype than *hlh-8* homozygotes? Is *hlh-8*(Gly29Lys) acting as a dominant negative or an antimorph? Explain your reasoning.

Dominant negative mutants of homo-multimeric proteins, as defined by Ira Herskowitz, *Nature*, 329:219-222, poison wild type subunits in the homo-multimer. Thus a homozygous dominant negative mutant cannot have a more severe phenotype than the homozygous null mutant. In contrast, dominant poisoning mutants that have a more severe mutant phenotype than the homozygous null mutant must be poisoning some other gene product, which results in further exacerbating the phenotype. Thus, *hlh-8*(Gly29Lys) can not be a dominant negative, as originally defined by Herskowitz. The term antimorph, for a poisoning gene product, was defined prior to molecular biology, and includes all types of poisoning activities.

11) Explain the reasoning that the 100 previously describe Saethre-Chotzen syndrome TWIST1 mutations, which are heterozygous in the affected individuals, show dominance due to haploinsufficiency.
These patients show a range of lesions, deletions, nonsense, frame shift and missense mutations distributed throughout the protein – which in aggregate indicate loss of functions. Since the affected individuals are heterozygous for the loss of function mutations, then it is a haploinsufficiency.

12) What information supports the hypothesis that the TWIST1 Glu117Val in Subject 1 is pathogenic for the observed facial dysmorphism affecting the eyes, nose, mouth, ears and hairline?

That Subject 2, with the same constellation of phenotypes, also has a variant at Glu117, Glu117Gly.

13) Figure 3 shows the analysis of hlh-8 alleles, corresponding to knock-in of patient variants, for various phenotypes that provide a readout for gene activity of the orthologous TWIST1 & TWIST genes – these worm phenotypes are call “phenologs” (McGary et al., 2010, PNAS 107:6544-6549)

A) Which allele do the authors use to represent hlh-8 null?

fs2728

B) Are these alleles showing pleiotropy?

Yes. They show phenotypes in multiple tissues - egg laying muscles and muscles involved defecation.

C) Indicate an allele that is showing a strong tissue specific effect and explain your reasoning?

R28W, for Egl (99% mutant) versus Con (0% mutant). Thus hlh-8(R28W) is defective for function in egg laying muscles but essentially wild type for function in defecation muscles – this represents a separation of function. Similarly for E29Q.

D) What are issues with the data presented in Figures 3 (and 4 – 6) that make it harder to compare phenotypic effects of the different alleles?

Error bars are not shown and statistical analysis (significance) is not included.

E) For knock-in hlh-8 homozygous alleles, qualitatively aggregate the various phenotypes (Figure 3) and present an allelic series, from least to most severe mutant phenotype. (In the absence of statistical parameters, it is reasonable to group together alleles with similar penetrance/effect).


F) Based on the ranking of homozygotes, which alleles are hypomorphic, which are likely null, and how would you describe E29K?

null (behave like fs2728): E29D, E29V
Antimorphic poisoning of some other gene product (more severe than null): E29K
14) What data indicate that the missense mutations, particularly E29K, are acting as an antimorph, possibly dominant negative, rather than a haploinsufficiency?

From Figure 6A, wild type and fs2728/+ have identical phenotype, indicating that hlh-8 does not show haploinsufficiency. However, heterozygotes for the E29x mutants show embryo retention, which is acting in the same phenotypic direction as loss of function (Egl, in Figure 3). Thus, since hlh-8 does not show a haploinsufficiency, then the dominant loss of function must be from antimorphic action.

15) How does the analysis of hlh-8 mutants support the proposal that the TWIST1 Glu117Val & Glu117Gly variants are causal for the Sweeney-Cox syndrome?

Essentially the same spectrum of phenotypes (although not necessarily penetrance) in C. elegans (Egl, Con, reduced fertility) are found for knock-ins of Sweeney-Cox syndrome variants as are observed for knock-ins of known pathogenic variants for other syndromes, SCS, BSS and AMS.

16) Qualitatively rank the phenotypes and present an allelic series for heterozygous mutant phenotype (Figure 6A & 6B).

WT = fs2728 (null)/+ < E29D/+ < E29V/+ < E29G/+ < E29A/+ < or = E29Q/+ < E29K/+  

17) What can you conclude about the basis of the observed dominantly acting alleles? Why is it likely an antimorphic/dominant negative mode of action rather than neomorphic?

- hlh-8 is not an obvious haploinsufficient locus in C. elegans  
- E29K, which is the most phenotypically severe of the group, since it is poisoning some other gene product in the homozygous state is very likely also poisoning some other gene product as a heterozygote (is antimorph), explaining its strong phenotypic effect. 
- E29D, E29V, E29G, E29A & E29Q are showing dominant phenotypes and thus must be poisoning (as hlh-8 is not a haploinsufficient locus). They are likely poisoning the wild type gene product (dominant negative, as define by Herskowitz) but cannot rule out that they are poisoning another gene product.

The dominant phenotypes are all acting in the same phenotypic direction as loss of function – indicative of poisoning activity, rather than novel (neomorphic) activity.

18A) C. elegans and Drosophila each contain a single Twist gene while in mammals there are two Twist genes, where their high sequence identity suggests redundant functions while divergence in sequence and expression indicates unique functions, which is supported by the fact that TWIST1 null homozygous mice (embryonic lethal, including abnormal neural tube closure) and TWIST2 null (lethal after birth, from cachexia) are phenotypically distinct.

In Figure 7, data from the hlh-8 knock-in mutants has been used to place the various alleles on a gene activity continuum. Explain the positioning of Glu75Ala & Glu75Gln BSS mutations and the positioning of Glu75Lys AMS mutation on the TWIST2 gene activity continuum.
TWIST2 is not a haploinsufficient locus (Setleis syndrome carriers are normal) and knock-in of Glu75Ala & Glu75Gln corresponding residues into *hlh-8* show a dominant poisoning phenotype, thus they must have less than 50% gene activity. Knock-in of Glu75Lys corresponding residue into *hlh-8* shows the strongest dominant phenotype, and thus is predicted to have less activity than the corresponding Glu75Ala & Glu75Gln. However, there is a complication, in that E29K (Glu75Lys) is poisoning some other gene product and it is unclear how to represent such poisoning on the TWIST2 gene activity continuum.

b) Explain the position of Glu1117Val & Glu117Gly SwCoS mutations on the TWIST1 continuum.

Knocking in Glu1117Val & Glu117Gly corresponding residues into *hlh-8* results in a dominant phenotype that is more severe than *hlh-8/+,* indicating that there is less than 50% gene product activity.

C) What is the reasoning that TWIST1 Glu1117Lys (corresponding to Glu75Lys in TWIST2) heterozygotes may be lethal?

E29K/+ (corresponding to TWIST1 Glu1117Lys) have a more severe *C. elegans* phenotype that the corresponding SwCoS knock-in mutants (E29A/+ & E29Q) and thus must have even less activity. Subject 1 & subject 2 already have severe birth defects, so even less activity is likely to be lethal.