Co-substrate induced nonlinear flux switching at branch-points can explain overflow metabolism

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1 Abstract

Metabolic behaviours of cells, such as metabolic overflow, involve regulation of metabolic fluxes across different pathways. Two well-established concepts in flux regulation are the transcriptional regulation, involving changes in enzyme levels through gene regulation, and allosteric regulation, involving changes in enzyme catalytic activities through metabolite binding. Here, we describe an additional mechanism arising through co-substrate usage around metabolic branch points. We find that asymmetric usage of a given co-substrate by reactions at and around a branch point introduces nonlinear flux switching at that branch point with increasing influx. This provides cells with an 'inherent self-regulation' embedded in the architecture of metabolic pathways. Using models of different branch point motifs, we explain this regulation mechanism and provide parameter ranges under which it can operate. We then develop a specific model of the yeast pyruvate branch point and NADH usage around it to show that co-substrate based flux switching can explain experimental data obtained during yeast metabolic overflow. Furthermore, the model captures observed effects of altering NADH dynamics on the metabolic overflow initiation point. We conclude that co-substrate dynamics around branch points provides an inherent regulatory mechanism that could explain observed relations between co-substrate dynamics and metabolic behaviors. Additionally, the presented theory offers a quantitative approach to manipulation of co-substrate dynamics for metabolic engineering.

2 Introduction

Overflow metabolism, also known as aerobic glycolysis, 'Warburg', or 'Crabtree' effect, is a broadly observed cellular phenomenon, seen in mammalian cells, yeast, and bacteria [4, 12, 47]. It involves an increased flux into fermentation products, such as lactate, ethanol, and acetate, with increasing glucose consumption rates, despite aerobic conditions and continued respiration [12, 43]. Since respiration produces more ATP per glucose compared to fermentation, and since cells achieve a higher growth rate under respiro-fermentation [4, 6, 42], metabolic overflow is seen as cells trading-off efficiency in energy harvesting for growth rate [33, 43].

Despite this fundamental relevance and seemingly universal presence across species, the underpinning causes of overflow metabolism are still unclear. At an abstract level, overflow metabolism is explained as arising from cellular constraints [26]. At a mechanistic level, however, the exact cause of such a constraint is unclear. Several modelling studies proposed that the protein synthesis can become a constraint under fast grow conditions, and that the switching to fermentation would result due to the lower protein cost of fermentation compared to respiration [4, 29]. This suggestion leads to the hypothesis that metabolic overflow results from cells increasing the levels of fermentation pathway enzymes over those in the respiration

pathways. Proteomics-based measurements, however, are inconclusive on the extent of changes in pathway-specific enzyme levels [16, 28, 32]. In addition, recent experimental studies report contrasting results on protein efficiency of fermentation vs. respiration [22, 36]. It is also found that metabolic flux responses to perturbations, in general, cannot be explained solely by changes in enzyme expression levels [11, 16, 46]. Taken together, these findings show that protein-based constraints, on their own, cannot explain overflow metabolism fully [11, 36].

Within central metabolism, metabolic overflow specifically relates to a branch point involving pyruvate (Fig. 1A). Pyruvate is converted into either organic acids, forming the fermentation pathways, or into acetylCoA, forming the starting point for the tricarboxylic acid (TCA) cycle and the respiratory pathway. This raises the question of how the flux at this branch point is regulated during overflow metabolism and how this might relate to the constraint-based view described above. A seminal modelling study showed that branch points could provide a point of regulation, as the distribution of fluxes among the two branches can be readily altered by regulating the levels or catalytic properties of the enzymes catalysing the reactions on each branch [24]. For the pyruvate branch point specifically, enzymes immediately downstream of the branch point, including pyruvate decarboxylase (PDC) on the fermentation branch and pyruvate dehydrogenase (PDH) on the respiration branch, all have identified allosteric, post-transcriptional, or transcriptional regulators [21, 35, 39]. In the case of PDH, for example, altering post-translational regulation of this enzyme is shown to influence metabolic overflow in mammalian cells [25].

Another interesting feature of the pyruvate branch point - and branch points across the central metabolism in general - is the involvement of co-substrates in reactions at or around the branch point (Fig. 1). Potential functional significance of the placement of these co-substrate-linked reactions around a branch point, and the impact of their dynamics on flux regulation, however, are not explored. The concentrations of co-substrates are commonly assumed as constants in metabolic models, but when this assumption is relaxed, co-substrate dynamics can become flux limiting [17, 19, 23, 40, 48]. Experimentally in yeast and bacteria, modulating NADH dynamics (via introducing enzymes that can consume or produce NADH) can change the level of glucose consumption and the growth rate at which metabolic overflow is initiated [44, 45]. These findings lead to the hypothesis that co-substrate dynamics are interlinked with alteration of fluxes at the pyruvate branch during metabolic overflow.

Here, we provide supporting evidence for this hypothesis using mathematical models and experimental measurements from yeast. We first develop generalized models of metabolic branch points featuring co-substrate involving reactions. Analysis of these models shows that co-substrate dynamics can introduce a type of 'self-regulation', where the flux distribution at a branch point can change in a nonlinear fashion, as influx into the upstream pathway increases. We show that this inherent regulation of flux distribution at the branch point arises from asymmetric co-substrate usage, at and around the branch point. A similar effect can be achieved with differential enzyme affinities at the branch point, but the co-substrate based mechanism is less restricted by parameter choices and achieves higher sensitivity. We then develop a more specific model of the pyruvate branch point in yeast, accounting for NADH dynamics across cytosol and mitochondria. We show that mitochondrial NADH dynamics in this model can drive a nonlinear flux switching into fermentation with increasing glucose influx. This model behaviour qualitatively captures experimentally observed shifts in NADH levels during metabolic overflow[6]. We find that the model can also account for the changes in threshold glucose level, for metabolic overflow initiation, with changes in NADH turnover rates[44, 45]. We conclude that the co-substrate dynamics around metabolic branch points introduce a nonlinear flux switching mechanism that acts as a self-regulatory element and that this can explain the overflow metabolism. As a corollary, and in light of the developed predictive theory, we hypothesize that modulating co-substrate dynamics at branch points can allow explanation and engineering of different dynamic behaviours in cell metabolism.

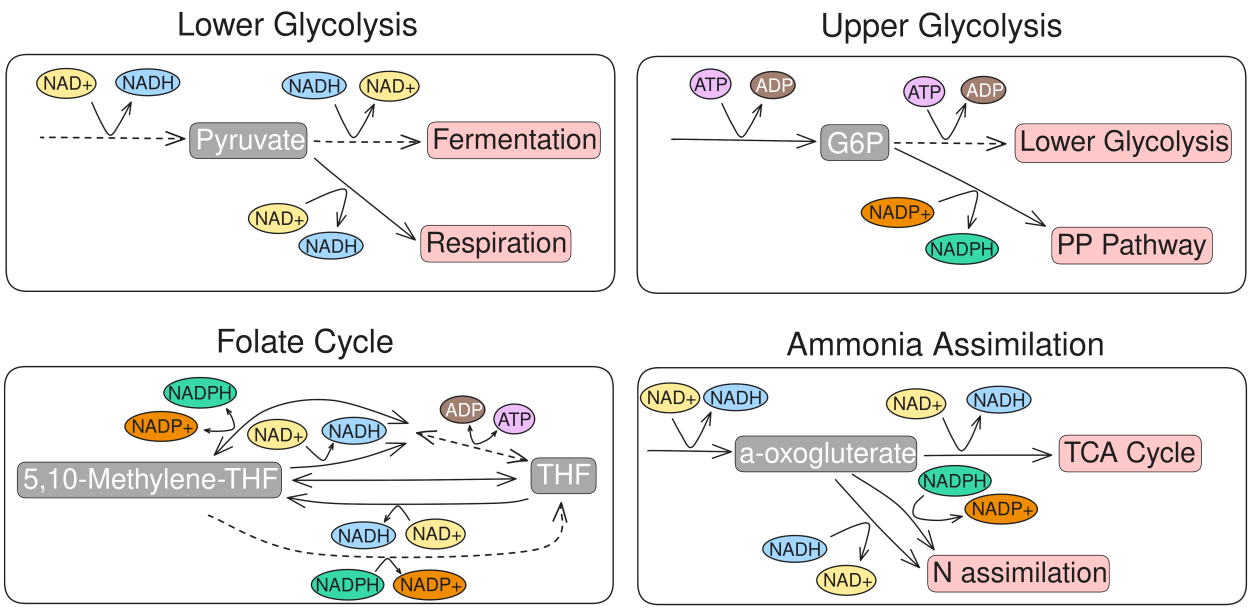

Figure 1: Branch points in central metabolism Cartoon representation of different examples of branch points, involving co-substrate linked reactions. Each cartoon is based on corresponding reaction maps included in the Yeast Pathways database [13] and are simplified for tractability. In particular, dashed arrows indicate multiple reactions, grey and red boxes indicate metabolites and processes, respectively and other colours indicate different co-substrates. EC Numbers and pathways used to create the cartoons are: Lower Glycolysis - 1.2.12 (glycolysis I), 1.1.1.1 (superpathway of of glucose fermentation), 1.1.1.41/1.1.1.286 (TCA cycle, aerobic respiration). Upper Glycolysis - 2.7.1.1/2.7.1.2 (superpathway of glucose fermentation), 2.7.1.11 (glycolysis I (from glucose 6-phosphate)), 1.1.1.49 (pentose phosphate pathway (oxidative branch) I). Folate Cycle (folate transformations I) - 1.5.1.5, 1.5.1.15, 6.3.4.3, 2.1.2.1, 1.5.1.3, and the tetrahydrofolate (THF) oxidation reaction, which does not have an EC assigned. Ammonia Assimilation - 1.1.1.41/1.1.1.286, 1.2.1.- (both from TCA cycle and aerobic respiration), 1.4.1.4 (glutamate biosynthesis from ammonia), 1.4.1.14 (L-glutamate biosynthesis IV).

3 Results

Co-substrate involving reactions (CRs) are common within cell metabolism and can be located at or around branch points. We postulate a role of co-substrate dynamics in the distribution of metabolic fluxes at a branch point. While NADH dynamics around the pyruvate branch point constitutes a key example, which we will analyse further below, branch points are common throughout cellular metabolism (Fig. 1). Interestingly, the different branch points show different placement of CRs at and around them. For example, at the pyruvate branch point in yeast, we have two NADH-linked CRs either at or downstream of the branch point, and another one upstream. In other examples, we have branch points with one or many CRs that link to the same or several different co-substrates (e.g., ATP, NADH, or NADPH) (Fig. 1). These observations raise a question about how flux dynamics arising from CRs placed at and around a branch-point can influence flux distribution at the branch point.

To explore this question, we focus first on simple models that can provide a didactic insight. We first consider the case of a branch point with or without a CR, placed at the branch point (Fig. 2A and B). In each case, and the subsequent cases analysed, we focus on the steady-state flux fraction into one branch - arbitrarily taken as the upper branch - ($F_f = F_{\text{upper}}/(F_{\text{lower}}+F_{\text{upper}})$) as a key variable describing the pathway dynamics. All enzymatic reactions are considered as reversible or irreversible, and modelled using Haldane or Michealis-Menten equations respectively (see *Methods*). Total co-substrate levels are assumed

to be conserved, i.e. the sum of the two forms of the co-substrates (B_0 and B_1) is a constant, B_{T}. In the supplementary information (SI), we show that this assumption can be relaxed without loss of the general results presented here (see SI section and Figure S1). The effects of any 'off-pathway' reactions consuming the co-substrate are modelled as a single, enzymatic reversible reaction with equilibrium coefficient $K_{\mathrm{eq,B}}$. In the context of NADH, for example, this abstract reaction represents all off-pathway processes consuming or producing NADH, such as the respiratory electron transport chain.

Co-substrate dynamics facilitate switch-like flux regulation at a branch point, in response to increasing pathway flux. A model of a branch point without co-substrates has been considered before [24] (Fig. 2A). Here, we first recover the main result from that study, namely that the steady state F_f at the branch point relates to the ratio of the maximal rate of the enzymes at the branch point. For this model, F_f is unaffected by any changes in the upstream pathway influx (Fig. 2D, dashed lines), when the two branch point enzymes have the same substrate affinity (i.e. $K_{M,23} = K_{M,24}$). This is because, in this case, increasing upstream flux has no mechanism to influence on the flux dynamics at the branch point. When the substrate affinity of one of the branch point enzymes is increased by 100-fold compared to the other (i.e. $K_{M,24} >> K_{M,23}$), and the same enzyme is set to have a 10-fold lower maximum rate (i.e. ($V_{24} >> V_{23}$)) we see a nonlinear response of F_f to changes in the upstream pathway influx (Fig. 2D, solid lines). Thus, under a restrictive parameter condition, the branch point dynamics itself can result in a response of F_f to increasing influx, but this response has low sensitivity (Fig. 2G).

We next consider the case of placing a single CR at the branch point, utilizing one form of a co-substrate, B_0 (e.g. NADH or NAD^+) (Fig. 2B). Interestingly, we find that changes in the pathway influx rate now cause a nonlinear change in the steady state F_f at the branch-point (Fig. 2E), even when both branch point enzymes have the same kinetic parameters (i.e. $V_{24} = V_{23}$ and $K_{M,24} = K_{M,23}$). For the case with $K_{M,24} = K_{M,23}$, F_f at low influx rate is determined by the ratio of the maximal rate of the branch point enzymes, as in the model without CR. As the pathway influx increases, however, F_f increases in a nonlinear fashion towards unity, irrespective of the ratio of branch point enzyme activities (Fig. 2E, dashed lines). The magnitude of this response can be quantified by the difference of flux fraction at low and high influx levels, shown as ΔF_f in (Fig. 2E), while its sensitivity (i.e. extent of response nonlinearity) can be measured by the response gradient. We find that the response gradient shows a sharp peak at a narrow range of influx rates (Fig. 2H, dashed lines), confirming a nonlinear, switch-like dynamics in F_f. Compared to the case without a CR, the response generated through co-substrate dynamics does not require specific kinetics of the branch point enzymes and displays a higher sensitivity for the same parameters (compare Fig. 2G and H). When differences in kinetic parameters of branch point enzymes are introduced in the CR-featuring model, this enhances the switch-like regulation and its sensitivity (Fig. 2E and H, solid lines). We conclude that introduction of a CR at the branch point facilitates a dynamic response, a form of a 'self-regulation', causing a switch-like response in F_f at a threshold level of pathway influx.

In the case of the model with equal kinetics of the branch point enzymes, the observed F_f response must be associated with co-substrate dynamics. Indeed, we find that the observed change in F_f coincides with a depletion of one form of the co-substrate, with increasing influx (Fig. 2E and F, inset). This depletion introduces an asymmetry between the branch point reactions in terms of substrate availability, which explains the observed F_f response. Another way of breaking substrate usage symmetry at the branch point would be through allosteric regulation of one of the branch-point enzymes by their substrate. We find that this can introduce a low level of 'self-regulation' in the case without CRs, and increases the extent of response nonlinearity in the case with CRs (see FigureS2).

We find that the above-outlined intuition on the role of asymmetric co-substrate usage at the branch point introducing a dynamical F_f response aligns well with the expected effect of changes in the model parameters and structure, on such response. For example, increasing B_{T} or the off-pathway co-substrate conversion rate V_M^B is expected to reduce the strength of co-substrate induced asymmetry, and either completely abolish the response of F_f to changes in influx, or increase the threshold influx point where this happens. We find that

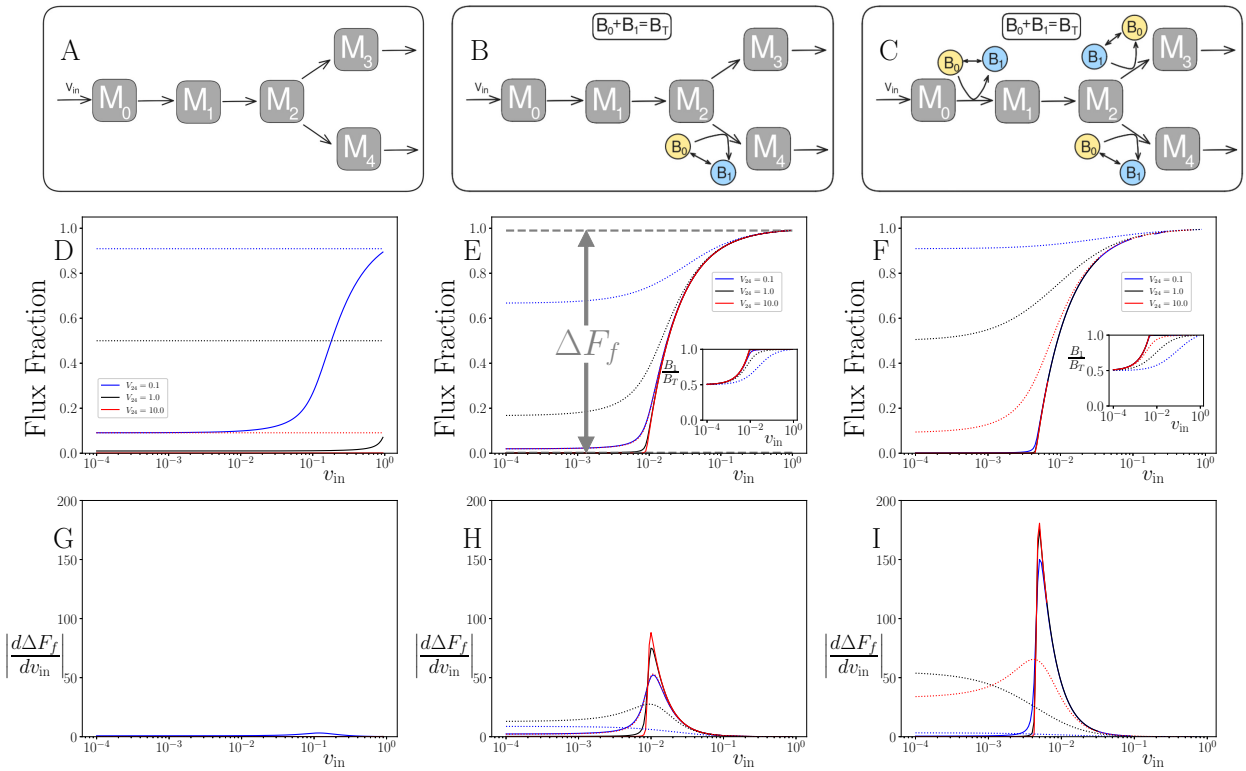

Figure 2: Co-substrates introduce regulation at branch points. (A-C): Simple branch-point motifs without (A), with co-substrates on only one branch (B) and on both the branches and upstream (C). All reactions apart from the background conversion of B_0 and B_1 are modelled as irreversible. (D-F): Simulation results from models corresponding to the motifs shown in (A-C). Each panel shows the steady state flux fraction, F_f, as the ratio of steady state flux from M_2 to M_3 over total flux at the branch point, against increasing influx into the pathway v_{in}. Red/black/blue lines indicate when the v_{24} is larger, equal, or smaller than v_{23} (see legend). For panel D, dashed lines show the case where $K_{M,24} = K_{M,23}$, while the solid lines show the case for $K_{M,24} = 100 \cdot K_{M,23}$, while all other parameters set arbitrarily to 1. For panels E and F, dashed lines show the case for $(B_{\text{T}}, V_M^B) = (10, 0.01)$ with all other parameters set arbitrarily to 1, while solid lines show simulation results with the same parameters, except $K_{M,24} = 100$. Insets in (E,F) show the B_1/B_{T} fraction, with the different colored lines corresponding to simulation results with matching parameters as in the main panel. (G-I): Absolute value of the gradient of the flux fraction with respect to v_{in}.

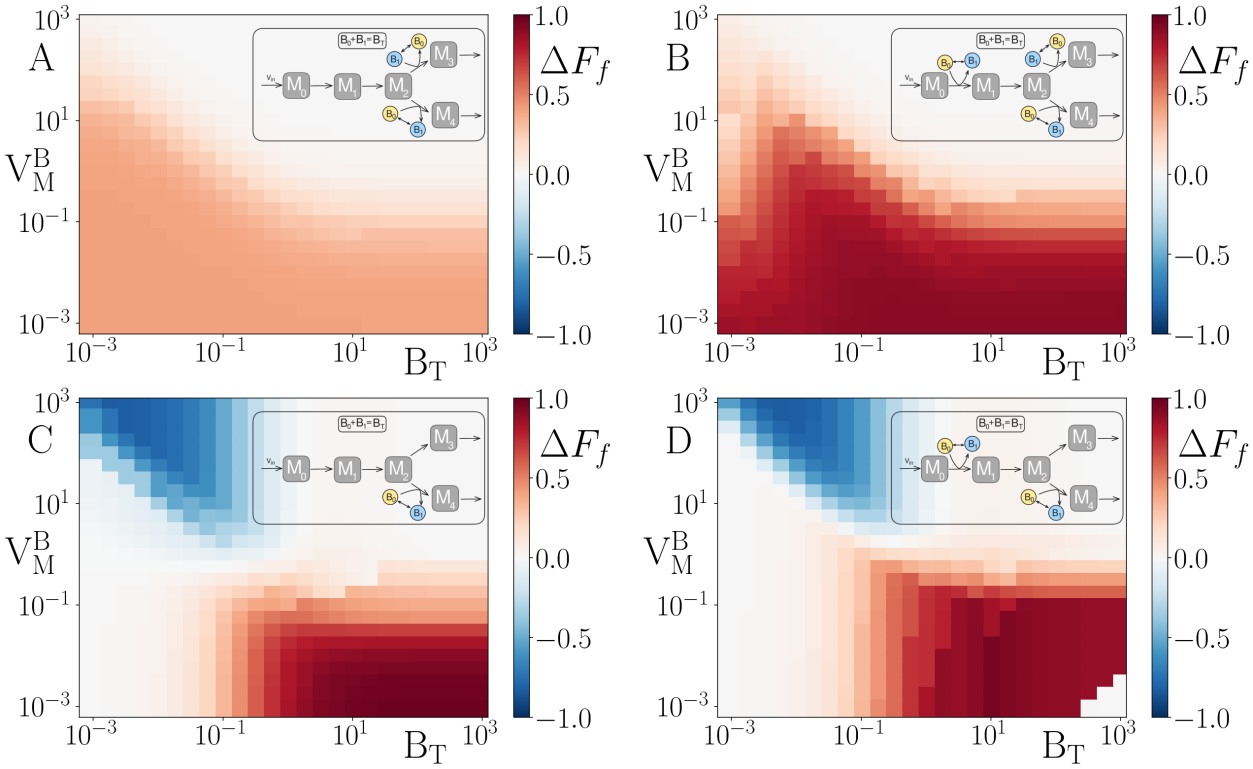

Figure 3: Motif structure influences capacity for regulation. Heatmaps showing $\Delta F_f = F_f(\min(v_{\text{in}})) - F_f(\max(v_{\text{in}}))$ (as shown in Fig 2E) as a function of B_T and V_M^B for metabolic motifs with (A): co-substrates on the branch points, (B): co-substrates on the branch points and upstream, (C): co-substrate on only one branch point, (D): co-substrates on one branch point and upstream. Simulations are performed for each combination of B_T and V_M^B and using $V_{23} = 0.1$ and all other parameters set to 1.

both of these expectations are realized (see Fig. S3). Similarly, a branch point with two CRs that utilise the same co-substrate form, or a branch point with both of the CRs moved downstream of the branch point, should result in symmetry of co-substrate usage at the branch point, and hence abolish the F_f response. Again, these expectations are realised (Fig. S4). Taken together, these results support the notion that the nonlinear F_f response to an increase in influx is facilitated by asymmetric co-substrate usage at a branch point.

'Self-regulation' dynamics is affected by the additional CRs and their locations with respect to the branch-point. In the simple, didactic motif shown in Fig. 2B, we only considered a single CR placed onto the branch point. It is, however, possible to have any combination of CRs across the two different branches, as well as upstream of the branch point. We explored a range of additional pathway 'motifs' involving the locations of CRs (see Fig. 2C, Fig. 3 and Fig. S5). We find that additional CRs can alter response dynamics in ways that are expected from the intuition developed above based on co-substrate usage. For example, in a motif with asymmetric co-substrate usage by the two branch-point reactions, combined with an upstream CR, the response magnitude can be reduced compared to the single-CR motif, for the same branch point enzyme kinetics (Fig. 2F, dashed lines). The same motif, when combined with unequal K_M values for branch point enzymes enhances the nonlinearity of the F_f response, compared to the single-CR motif (Fig. 2F and I, solid lines).

To further explore these effects systematically, we have quantified ΔF_f in different motif structures and under different parameter regimes pertaining to the key parameters B_{T} and V_M^B. This analysis shows that, different motifs can generate a nonlinear F_f response to influx, either resulting in decrease or increase in upper branch flux, and with a range of ΔF_f values (Fig. 3, see also Fig. S5). In general, for the case when branch point enzyme K_M's are equal, creating a F_f response to influx requires at least one CR to be at the branch-point, while an upstream CR generally results in enhancing of the F_f response (Fig. 3 and Fig. S5). Overall, these results indicate that the introduction of CRs across a branch-point provides a flexible mechanism for the introduction of nonlinear flux switching with increasing influx, and that the emergence of this response can be understood from co-substrates becoming limiting and the breaking of co-substrate use symmetry at the branch point.

A simplified model of co-substrate dynamics at the pyruvate branch point in yeast captures the experimentally observed features of metabolic overflow. The motifs analysed above are similar to those seen in the central carbon metabolism (see Fig. 1). In the case of glycolysis, ATP, ADP, NADH, or NAD^+ utilising reactions participate upstream, downstream and at the pyruvate branch point. In particular, NAD^+-linked pyruvate oxidation at the pyruvate branch point initiates the respiratory pathway, while NADH is oxidised in the fermentation pathway (Fig. 1). Thus, the above theory would suggest that both, or either, ATP and NADH dynamics can cause symmetry breaking and nonlinear dynamics, and impact F_f at the pyruvate branch point. The measured total NADH levels, however, are about two fold lower than total ATP levels in eukaryotic and prokaryotic cells [10, 15], and therefore NADH dynamics are more likely to act as a constraint. In addition, chemostat experiments with yeast have shown that metabolic overflow is initiated abruptly at a certain glucose influx level, and that NADH levels are altered at this switching point [6, 42]. Modulation of NADH dynamics is also shown to alter the threshold glucose level for metabolic overflow initiation in bacteria and yeast [19, 44, 45].

Based on these points, we created here a simplified model of NADH-utilising reactions in the lower part of the yeast glycolysis pathways, around the pyruvate branch point (Fig. 4A). This model takes into account the fact that the pyruvate branch point is split across the cytosol and mitochondria, where pyruvate is transferred into mitochondria before entering the TCA cycle, and crucially, the NADH pools for the cytosol and the mitochondria are separated and possibly connected by mitochondrial shuttles [2]. Given the additional features in this more complex model, and specific placement of NADH-involving reactions, we wondered if co-substrate dynamics can still underpin a flux switching (i.e. high ΔF_f) with increasing influx, as seen in the metabolic overflow phenotype observed experimentally (Fig. 4B, see also *Methods*). To parameterise the model, we used *in vitro* determined parameters utilised in previous models of yeast glycolysis [41] where possible, and then performed a sampling approach for other, unknown parameters to cover physiologically relevant ranges [3, 18](see *Methods* and Table S1). For each set of sampled parameters, we simulated the model to obtain F_f response against increasing influx and quantified ΔF_f. We have also quantified any switch-like dynamics in the NADH pool by measuring the extent of change in the fraction of NADH in the cytosol or mitochondria (ΔN_H^m and ΔN_H^c).

We found many parameter sets resulting in high ΔF_f, or both high ΔF_f and ΔN_H^m (Fig. 4C). The former cases of flux switching do not involve any changes in the co-substrates (Fig. 4D), indicating that the switching mechanism in these cases does not involve NADH dynamics. Indeed, we found that cases of high ΔF_f and zero ΔN_H^m are characterized by high, and opposing, differences in the K_M and V_M of the branch point enzymes (Fig. S6C), conforming the enzyme-based mechanism noted with the didactic models presented above. The latter cases of high ΔF_f and ΔN_H^m show changes in NADH dynamics (Fig. 4E). In particular, the flux switching is preceded by a large switch in the mitochondrial NADH fraction (ΔN_H^m) (Fig. 4E, green and blue lines), while cytosolic NADH fraction does not switch and the total NADH fraction change can be explained by the change in mitochondrial NADH fraction (Fig. 4E, red and yellow lines). This indicates that for these cases, the mitochondrial NADH dynamics is the driving factor behind the switching behavior. Supporting this view, we find that for these cases the key parameters underpinning switching relate to co-substrate cycling in the mitochondria (Fig. S6B).

The above findings show that models implementing the specific pathway structure of the yeast pyruvate branch point, and parameterised in a physiologically relevant regime, can display switch-like F_f responses as seen experimentally in metabolic overflow through two distinct mechanisms involving either differential kinetics of pyruvate consuming enzymes or co-substrate dynamics. Altering the model parameters and structure such that either one of these mechanisms is excluded, high ΔF_f is still possible (Fig. S7). This shows that the two mechanisms can operate independently. We note, however, that only the mechanism involving co-substrate dynamics produces a nonlinear alteration of NADH levels during metabolic overflow, as observed experimentally. In particular, whole cell measurements show an abrupt increase in NADH and a decrease in NAD^+ levels after the onset of metabolic overflow [6] (Fig. 4B), as seen in the model (Fig. 4E, red line). In addition, we find that for the models where high ΔF_f is achieved through co-substrate dynamics, altering the recycling dynamics of mitochondrial NADH pools, by shifting the equilibrium dynamics towards NAD^+, results in the shifting of the flux switching point to higher influx levels (Fig. 4E, dashed lines). These observations, as well, match with experimental findings in yeast, showing introduction of foreign NADH consuming reactions shifting the metabolic overflow initiation point to higher glucose levels [44] (Fig. 4E and S8). Additionally, the deletion of NADH-specific mitochondrial dehydrogenases is found to result in shifting of the metabolic overflow initiation point to lower glucose levels in *S. cerevisiae*[30], as also observed in the model by shifting the equilibrium dynamics towards NADH (Fig. S8(D)). Overall, these qualitative agreements between the model behaviour and experimental data indicates that asymmetric dynamics arising from NADH-NAD^+ usage at and around the pyruvate branch point is an integral driver of the metabolic overflow dynamics observed in yeast.

4 Discussion

Here, we analysed the flux dynamics at a metabolic branch point, with and without co-substrate utilising reactions at and around the branch point. Using mathematical models of simple branch point architectures as didactic cases, we shown that (i) differential kinetic paramaters for the branch-point enzymes, or (ii) a specific distribution of co-substrate utilising reactions as two independent mechanisms enabling nonlinear, flux switching at the branch point with increasing influx. This response constitutes a type of self-regulation, as it does not require active control of enzyme levels or their allosteric regulation. To see how the presented mechanisms could apply to overflow metabolism seen in yeast, we developed a specific model of the pyruvate branch point and the NADH usage around it in yeast. Using measured or physiologically relevant kinetic parameters, we found flux switching response to increasing influx through both mechanisms is possible in this model. However, only co-substrate based mechanisms is capable of explaining existing experimental data from yeast on NADH and metabolic overflow dynamics, and their influence on each other. Taken together, these findings demonstrate that branch points and co-substrate use around them act as key, 'built-in' regulatory elements within cellular metabolism.

The qualitative agreement between the presented model and yeast metabolic overflow dynamics provides strong support for involvement of co-substrate dynamics in metabolic overflow behaviour. The observed reduction in NAD^+ levels in yeast during metabolic overflow is captured by the co-substrate model, however, it must be noted that the experimental measurements here are rather noisy. They also pertain to the whole cell, rather than mitochondria and cytosol separately, while the model indicates a larger change in mitochondrial NADH dynamics with metabolic overflow initiation. In the case of effects of NADH dynamics on the threshold point for metabolic overflow, the experiments employed both mitochondrial and cytosolic NADH oxidases, and observed only the former to shift the metabolic overflow threshold, as in the presented model (Fig. 4E and S8). These differences in affecting the cytosolic and mitochondrial NADH pools can be understood, in light of the model, due to the lower NADH pool size in the mitochondria, which is shown in mammalian cells [10]. The model also highlights a key role for NADH shuttling reactions between cytosol and mitochondria for the metabolic overflow initiation, which can be explored by direct inhibition in future experiments.

The explanatory power of the presented theory, based on NADH-dynamics, can extend to other observations on metabolic overflow in yeast and other species. In this theory, metabolic overflow initiation point is predicted to tightly depend on NADH-NAD^+ turnover rates and/or pool sizes. Thus, these key physiological parameters in different species or strains, shaped by adaptations to past environmental conditions such as stable or changing oxygen levels [36], can determine their metabolic overflow phenotypes. In this context, we note that recent proteomics studies showed that Crabtree negative yeasts, which have a higher threshold for metabolic overflow, display significantly higher expression levels of electron transport chain enzymes [27, 36]. This suggests a higher mitochondrial NADH recycling in Crabtree negative yeasts, as supported by the finding of higher NAD^+ and lower NADH levels in Crabtree negative vs. positive yeast [36]. The presented theoretical findings also align with the findings from bacteria, where altering of NADH dynamics also resulted in shifting of metabolic overflow initiation point in *Escherichia coli* [44, 45]. In mammalian cells as well, alterations in NADH dynamics are found to affect flux distributions both at the pyruvate branch point, influencing overflow metabolism [25], and downstream at the alpha-ketoglutarate branch point, influencing the induction of reductive glutamine consumption [14]. These diverse finding from different species, all linking to NADH dynamics support the broader notion that co-substrate dynamics act as flux regulators across branch points.

The presented theory provides a clear mechanistic basis on how co-substrate dynamics can affect metabolic flux distributions. As such, it is linked to a series of historic studies proposing a regulatory role for co-substrates [34], including the phenomenological notion of ATP acting an indicator of cellular 'energy charge' [1, 20]. More recent studies have shown that co-substrate dynamics can act as flux limiting [17, 23, 48] and might underlie the nonlinear dynamics beyond certain pathway structures [19, 40]. The presented work extends these studies by showing a direct nonlinear regulatory effect of co-substrate dynamics around branch points. While we considered here specifically the pyruvate branch point, and NADH dynamics, it is noticeable that key junctions in central metabolism such as the folate cycle, underpinning single carbon transfers, and the TCA cycle, underpinning nitrogen assimilation, involve branch points and extensive co-substrate usage around them. Interestingly, some of these cases feature multiple co-substrates involved in reactions at and around these branch points. Thus, future studies can expand the modelling of co-substrate dynamics to specific branch points and also to capturing the interlinked nature of multiple co-substrates at branch points, in general. There is already experimental findings suggesting, for example, that aerobic glycolysis in mammalian cells involves an interplay between NAD^+-NADH and ATP-ADP dynamics [25].

We presented here a co-substrate based, dynamical explanation for metabolic overflow with increasing glucose influx, which happens in conjunction with increasing growth rate. This explanation is distinct from those explaining metabolic overflow arising from cells altering their protein investment into fermentative and respiratory pathways, according to the proteome efficiency of these two pathways [4, 22, 29]. Those theories operate at a higher, phenomenological level, and do not provide any mechanistic explanations for how cells sense and initiate such a proteome allocation adjustment and how different species or strains can have different metabolic overflow thresholds [4]. Thus, the presented theory could be complementary to the ideas surrounding protein allocation, as co-substrate based flux regulation at branch points can act as a fast-acting, self-regulatory response to achieve flux changes, which can then be facilitated further with slow-acting, and longer-term, changes in enzyme allocation. It would, therefore, be interesting to expand ongoing efforts of generating 'coarse-grained' cellular models, based on resource allocation, with the inclusion of co-substrate dynamics and key branch points from central metabolism.

In recent years, attempts are emerging to modulate co-substrate dynamics for directly engineering cellular metabolism for biotechnological applications [9, 37, 38]. We note that the presented co-substrate based theory - and extensions of it - can provide a predictive basis for these attempts. In particular, specific modulation of NADH turnover rates can be used to engineer metabolic overflow through the pyruvate branch point. Future application of the presented theory to other branch points, or several connected branch-points, can allow engineering of co-substrate dynamics and metabolic fluxes associated with other phenotypes.

5 Methods

Modeling of branch-point motifs. We used ordinary differential equations to model the dynamics of branch point containing pathways, with and without CRs. In the case of CRs, specific co-substrate forms - generically represented as B_0 and B_1 - are assumed to take part in a given reaction and the total amount of co-substrate is assumed to be fixed (i.e. $B_0 + B_1 = B_{\mathrm{T}}$), an assumption relaxed in the supplementary material (see Fig. S1). All reactions are modelled using single or two-substrate versions of the Michealis-Menten or reversible Haldane reaction rates, assuming simultaneous binding of all substrates to the enzyme. The generic forms of these reactions result in rate equations of the following form;

In addition to the CRs directly modelled, a 'background', reversible $B_0 \leftrightarrow B_1$ conversion reaction is implemented to account for reactions not related to the branch-point motif, with an equilibrium coefficient of $K_{\mathrm{eq,B}}$. The resulting reactions and rate equations are incorporated in a series of ODEs for each model and depending on the model architecture. All ODE systems are implemented as a Python script and solved using numerical methods implemented in scipy library. A set of ODEs for an example system (that shown in Fig. 2B) are given in the SI, while the main analysis script is made available at our group's Github repository: .

Simulation of flux behavior. A key metabolic behavior, we have focused here, is the dynamics of flux distribution at a branch point. To quantify this behaviour, we considered the steady-state flux fraction into the upper branch as a key measure describing the pathway dynamics, ($F_f = F_{\mathrm{upper}}/(F_{\mathrm{lower}}+F_{\mathrm{upper}})$). To get F_f for a given pathway architecture, we simulate the ODEs to steady state for different values of influx rate and record system variables, resulting in a response curve as shown in Fig. 2). For the models used in motif analyses, reaction kinetic parameters are set to arbitrary values for simplicity, with the key parameters, such as $K_{\mathrm{eq,B}}$, B_{T}, and maximal rate of the 'background' $B_0 \leftrightarrow B_1$ conversion reaction varied to assess their effects in system dynamics (as shown in Fig. 2 and Fig.3). These phase plane analyses involve running time-course simulations as described above for different parameters sets, to steady state, and obtaining F_f. Simulated systems are assumed to have reached steady state, when all metabolite gradients are smaller than 10^{-5} after a simulation time of 10^5 steps.

Yeast pyruvate branch-point model. The simplified model of the lower glycolysis and the pyruvate-branch point shown in yeast are modelled based on a previous model of the lower glycolysis in yeast [41]. The model is simplified by only focusing on the pyruvate branch point, lumping some of the linear parts of the lower glycolysis into a single reaction, and expanding with reactions around the branch point. In particular, we include the compartmentalisation of NADH across cytosol and mitochondria, and transport of pyruvate into mitochondria. The latter reaction, together with the pyruvate decarboxylase mediated reaction in the cytosol, thus forms the pyruvate branch point in this model. We also include a postulated NADH shuttling mechanism between mitochondria and cytosol, involving the acethaldeyde-ethanol conversion [2], as well as background NADH turnover (accounting for other NADH utilising mechanisms not directly modelled). Kinetic parameters for most reactions are obtained from the previous yeast model [40]. The kinetics of the pyruvate transport dynamics are obtained from measurements with isolated mitochondria [31], while remaining kinetic parameters are obtained from databases [5, 8]. To account for unknown parameters, and allow for variances in measurements, many of the kinetic parameters are further sampled from a wide, physiologically relevant, range, and centered around measured values where available (see Table S1). For the background reaction for NADH turnover, the $K_{\mathrm{eq,B}}$ is set to achieve a NAD^+ to NADH ratio of 100, as observed for the yeast cytosol, but also varied as low as 7, as observed for whole cell metabolite measurements [7].

Re-analysis of experimental data. Experimental data plotted in Fig 4B was obtained from Boer et al. 2010 [6]. Data points for ethanol production rate were extracted from the graph in Supplementary Figure S6 of [6] using plotdigitizer.com/app. Normalized ion counts of NADH and NAD^+ from LC-MS/MS analysis were extracted from Supplementary Dataset 2 in [6]. Each bar indicates normalized ion counts averaged over 4 experiments conducted at any given dilution rate of a glucose-limited chemostat. As cell growth is limited by the dilution rate in a chemostat reactor, we use dilution rate under glucose limitation as a proxy for metabolite influx rate into glycolysis. While model simulations tracked cofactors as fractions, the experimental data was plotted individually for NADH and NAD^+ because ion counts for different molecular species are not directly comparable due to species-specific ionization efficiencies.

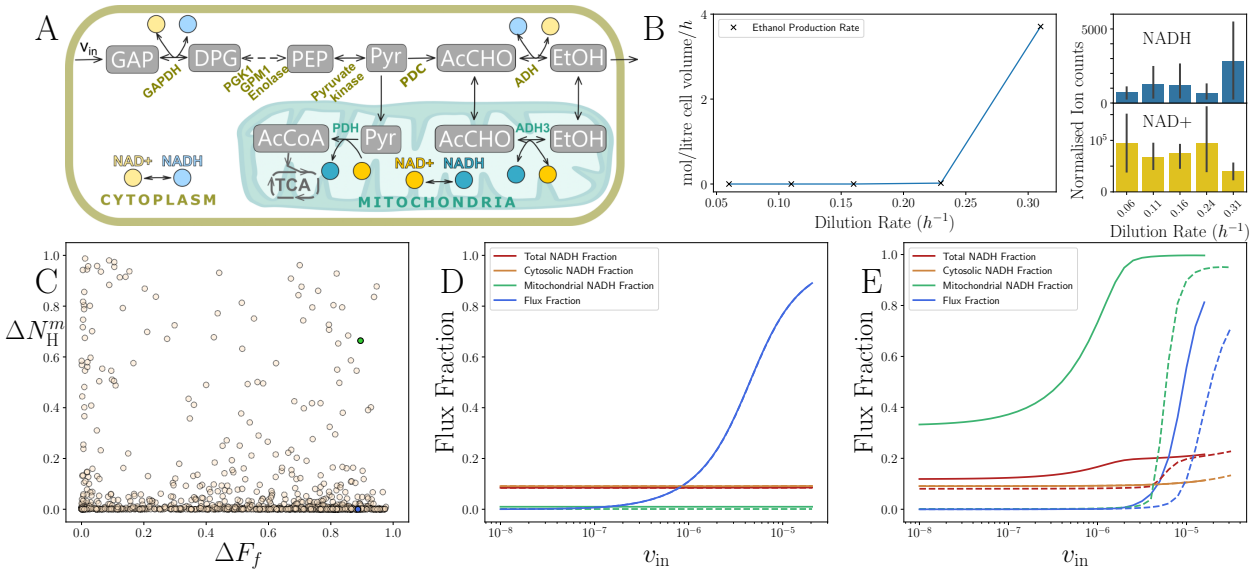

Figure 4: Yeast compartmental model. (A) A cartoon of the pyruvate branch point in yeast, where specific reactions are labelled with the relevant enzyme. Dashed reaction line indicates that multiple reactions have been condensed into one. (B) Experimental data from [6], showing an increase in ethanol production rate and NADH ion counts with dilution rate, as well as a reduction in NAD+ ion counts. (C) Scatter plot of ΔF_f vs. ΔN_{H}^m (change in mitochondrial [NADH]/([NAD+]+[NADH]) fraction). Each point shows simulation results for the model simulated using parameters drawn from the ranges shown in Table S1. Blue/green dots indicate the points plotted in (D/E). (D,E) Plot of v_{in} vs flux fraction and co-substrate fraction for the model simulated with specific parameter values, picked from those sampled in (B). The corresponding points are also indicated in (B). Solid lines are simulation results for the original parameter set, while the dashed lines show results from a simulation with the NADH background reaction equilibrium coefficient $K_{\mathrm{eq,B}}$ altered to favor NAD^+, mimicking the experimental manipulation of NADH dynamics with a NADH-consuming oxidase.

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Supplemental Materials: Co-substrate induced nonlinear flux switching at branch-points can explain overflow metabolism

S1 Supplementary Figures and Table

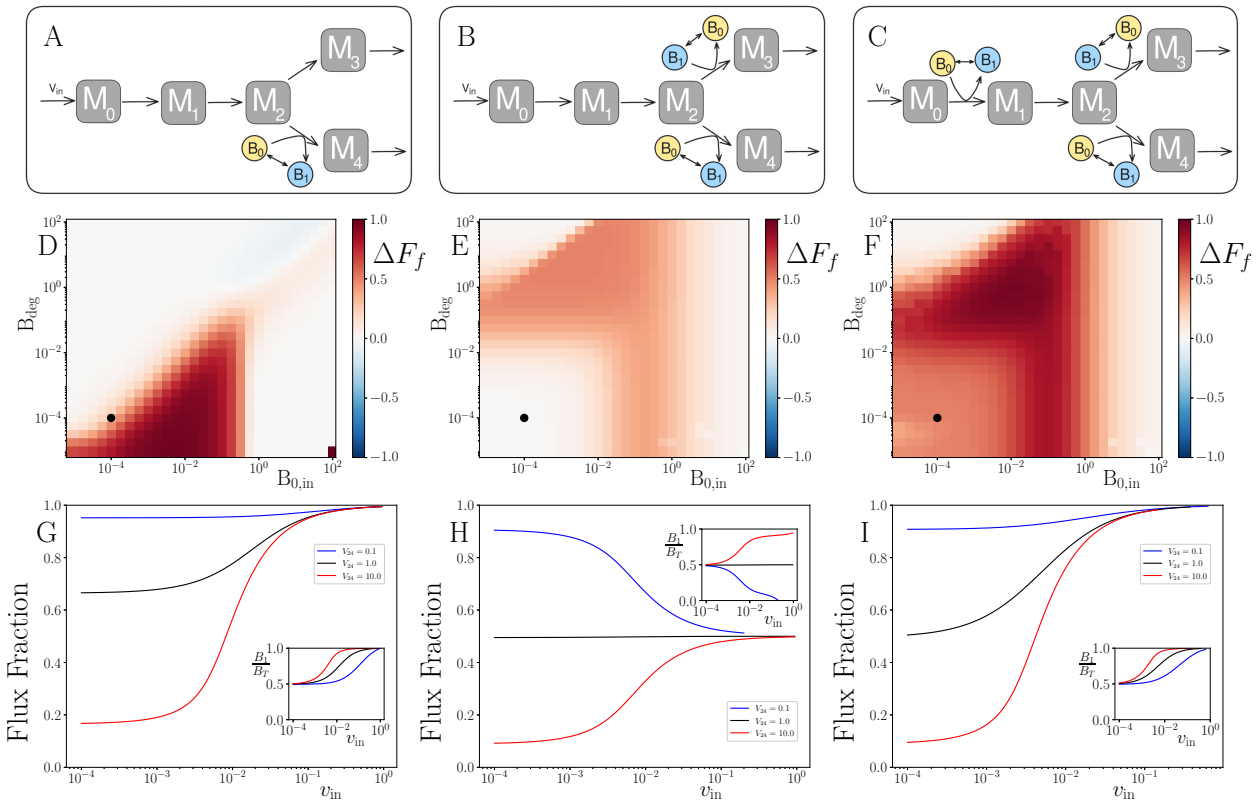

Figure S1: Regulation persists in the presence of synthesis and degredation of co-substrates. (A-C): Simple branch-point motifs with co-substrates on only one branch (B), on both branches (C), and on both the branches and upstream (D). All reactions apart from the background conversion of B_0 and B_1 are modelled as irreversible. (D-F): Heatmaps showing $\Delta F_f = F_f(\min(v_{\text{in}})) - F_f(\max(v_{\text{in}}))$ as functions of $B_{0,\text{in}}$ and B_{deg} for the metabolic systems in the panels above. $(B_{\text{T}}, V_M^B) = (10, 0.01)$, and all other parameters are set arbitrarily to 1 (G-I): Simulation results from models corresponding to the motifs shown in A-D. Each panel shows the steady state flux fraction from M_2 to M_4 vs. the influx into the pathway v_{in}. Red/black/blue lines indicate when the v_{24}/neither/v_{23} reaction is favoured (see legend). Parameters are the same as in the panels above, with ($B_{0,\text{in}}$, B_{deg}) corresponding to the black dot, V_{24} shown in the legend, and all other parameters are set arbitrarily to 1. Insets in (G-I) show the B_0/B_T fraction, with the different colored lines showing simulation results with parameters as in the main panel.

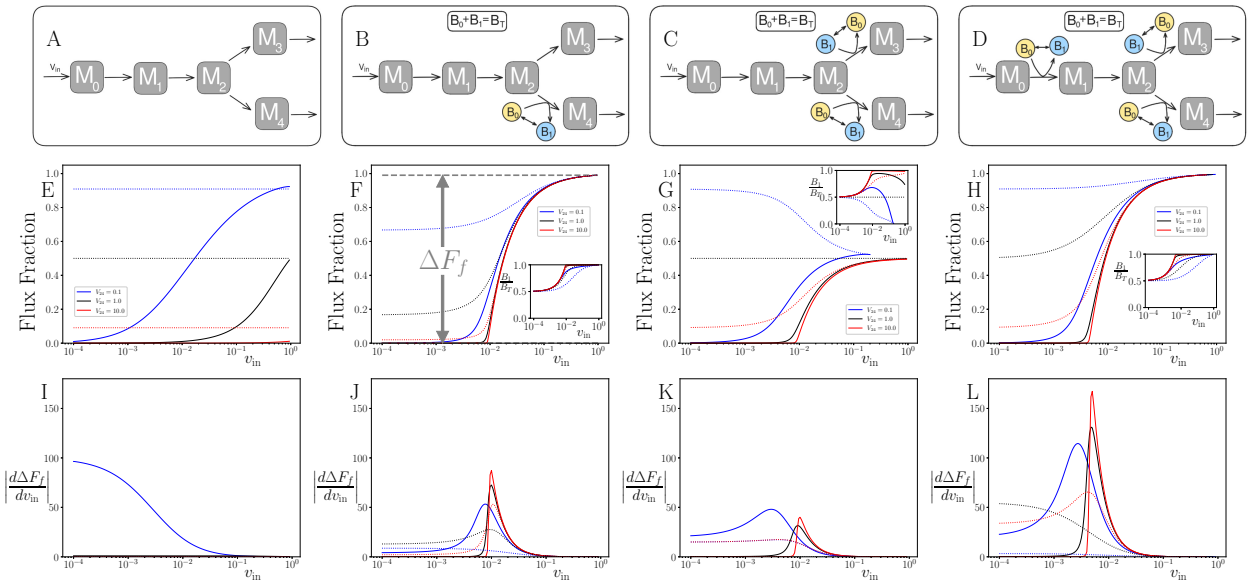

Figure S2: Allosteric regulation enhances nonlinear flux switching at branch points. (A-D): Simple branch-point motifs without (A) and with co-substrates on only one branch (B), on both branches (C), and on both the branches and upstream (D). All reactions apart from the background conversion of B_0 and B_1 are modelled as irreversible. (E-G): Simulation results from models corresponding to the motifs shown in A-D. Each panel shows the steady state flux fraction from M_2 to M_4 vs. the influx into the pathway v_{in}. Red/black/blue lines indicate when the v_{24}/neither/v_{23} reaction is favoured (see legend). Dashed lines show the case for $(B_{\text{T}}, V_M^B) = (10, 0.01)$ with all other parameters are set arbitrarily to 1, solid lines are the same except the $M_2 \rightarrow M_4$ reaction is allosteric in M_2. This is modelled by using m_2^2 and $K_{M,24}^2$ instead of m_2 and $K_{M,24}$ in the Michaelis-Menten equation for this reaction. Insets in (F-H) show the B_0/B_T fraction, with the different colored lines showing simulation results with parameters as in the main panel. (I-L): Absolute value of the gradient of the flux fraction with respect to v_{in}.

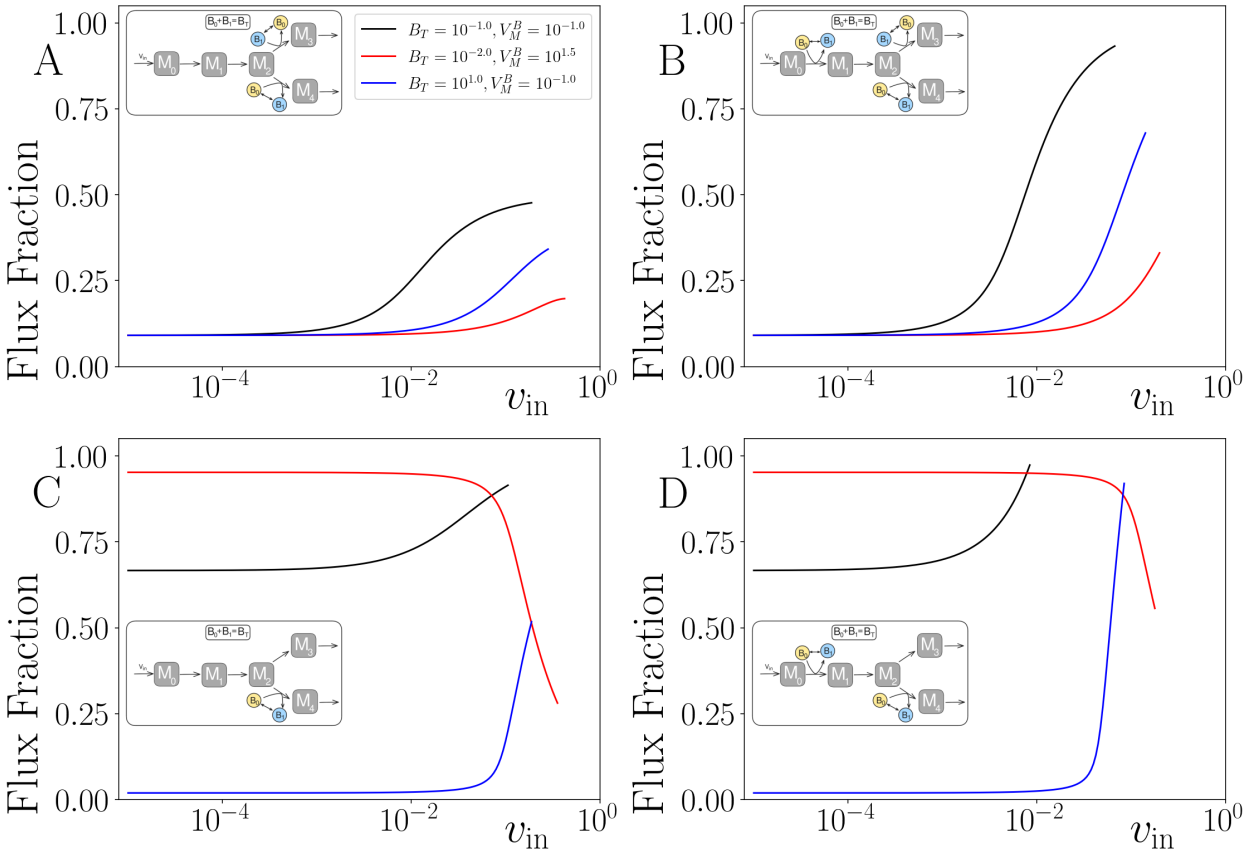

Figure S3: Impact of co-substrate pool size on switching dynamics. Flux fraction vs v_{in} for the motifs shown in the insets, which are the same as in Fig 3. Legend in panel A applies to all other panels.

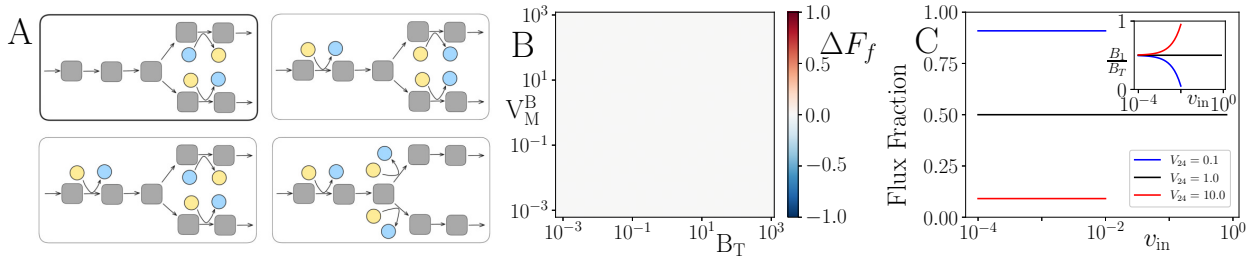

Figure S4: Off pathway or symmetric co-substrate usage does not result in regulation. (A): Motifs showing a metabolic branch points that do result in regulation as v_{in} varies (B) Heatmap of ΔF_f as a function of B_T and V_M^B for the motif with the bold outline in A. $(K_{\text{eq}}^B, V_{24}) = (0.1, 0.1)$, with all other parameters set to 1. (C) Flux fraction vs v_{in} for $(B_T, V_M^B, K_{\text{eq}}^B) = (10, 0.01)$, V_{24} indicated in the legend and all other parameters set to 1.

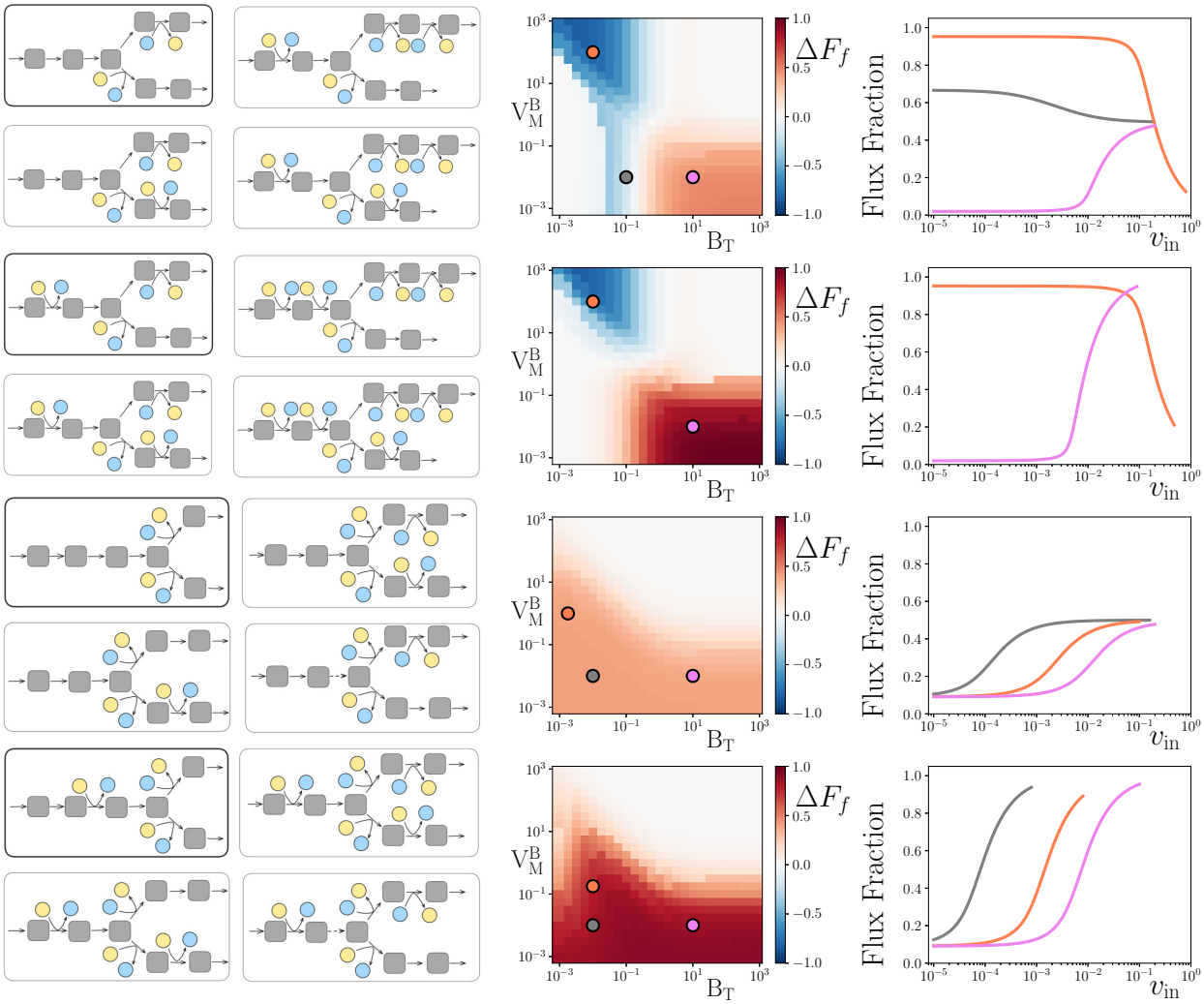

Figure S5: Categorisation of regulation types and associated motifs. (A, D, G, J): Motifs exhibiting similar regulation, showing the adjacent heatmap. (B, E, H, K): Heatmaps showing $\Delta F_f = F_f(\min(v_{\text{in}})) - F_f(\max(v_{\text{in}}))$ for bold motif in adjacent panel. All other motifs in this panel have similar heatmaps. $v_{23} = 0.1$ (top branch $V_{\max}$) and all other parameters set to 1. (C, F, I, L): Flux fraction vs v_{in} for the bold motif in the same row, with parameters corresponding to the dots in the adjacent heatmaps.

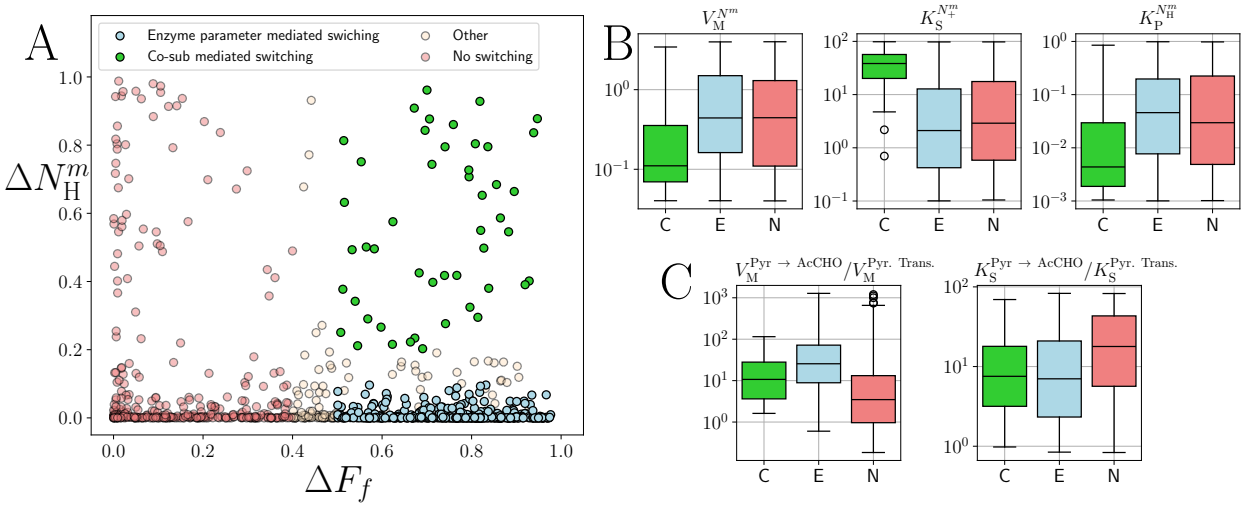

Figure S6: Comparison of parameter regimes in the compartment model (A) Scatter plot of ΔF_f vs. $\Delta N_{\mathrm{H}}^{m}$ for the same parameter set as in Fig 4(C), with regions labelled (see legend). (B/C) Box plots showing important parameter regimes for the different switching cases. Color of box plots shows the points from (A) used. (B) Parameters important for co-substrate mediated switching, (C) parameters important for enzyme mediated switching.

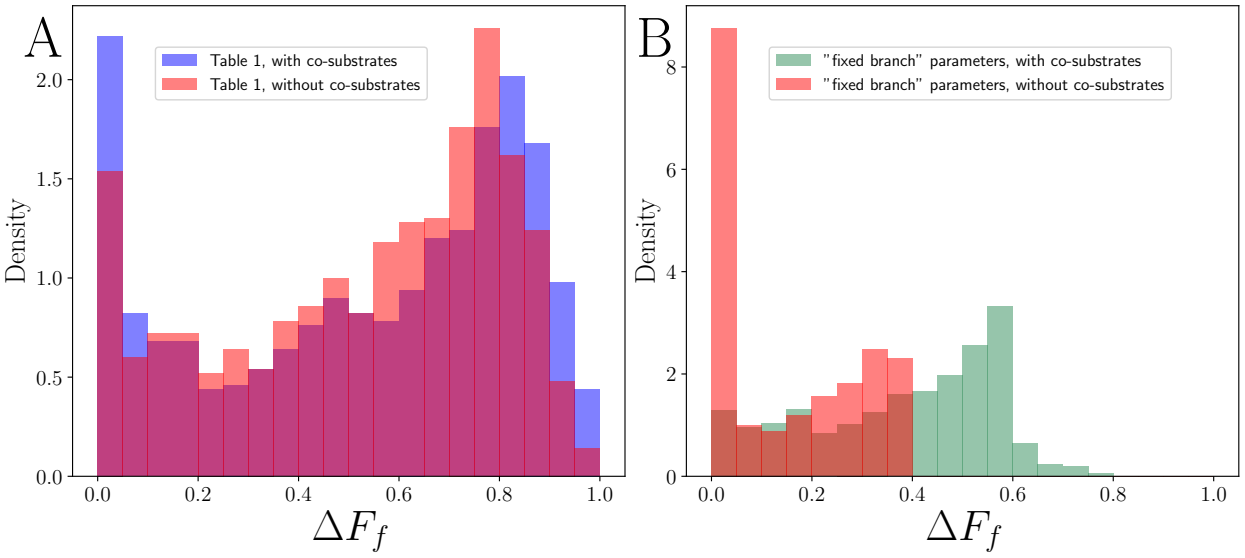

Figure S7: Co-substrate dynamics produces larger switches in the compartment model (A) Histograms for the distribution of ΔF_f in the case with/without co-substrates (blue/red). Parameters as in Table S1. (B) Histograms for the distribution of ΔF_f in the case with/without co-substrates (green/red) for the parameter regime shown in Table S1. but with all the reactions that have Pyruvate as a substrate set to have the same parameters.

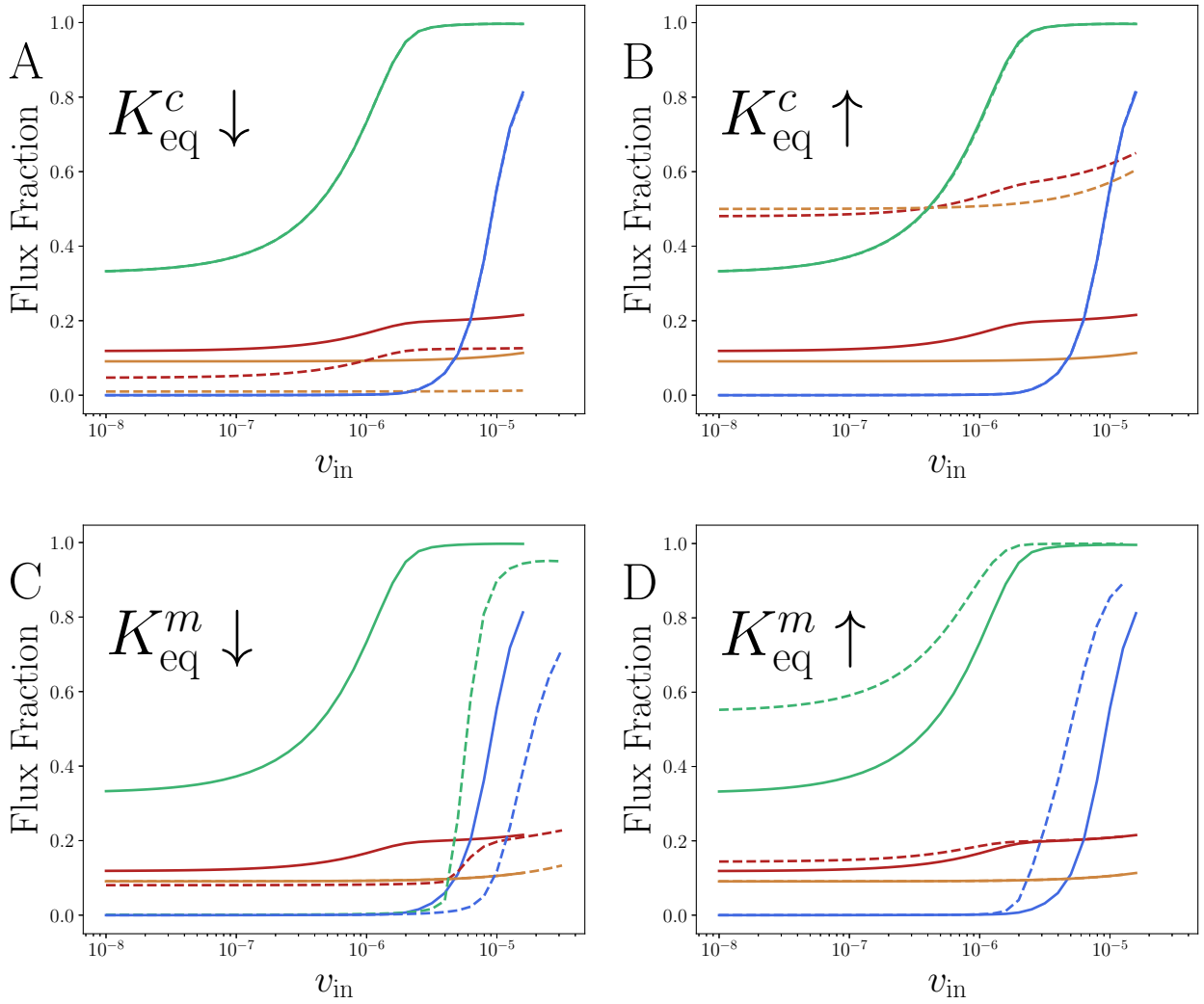

Figure S8: Mitochondrial co-substrates drive switching Effects of changing the NADH bias in the cytosol and mitochondria, by changing the K_{eq} for the background conversion (indicated in each panel). Solid lines are the same in all panels, and are the results from Fig. 4(E). Dashed lines in (A,B) show the effect of changing the cytosolic NADH levels while (C,D) show the same for mitochondrial. Left column shows biasing towards NAD+, while the right column shows biasing towards NADH.

Michaelis-Menten (Reversible): $\nu = \frac{V_M}{K_S}\frac{S-P/Keq}{1+S/K_S+P/K_P}$						
Reaction (enzyme)	V_M (μmol/min/mg)	K_S (mM)	K_P (mM)	K_{eq}	Pool size (mM)	Citation(s)
GAP $\xleftrightarrow{\text{gapdh}}$ DPG	$1-10$	$0.001-1$	$0.001-1$	0.0056		[41]
DPG $\xleftrightarrow{\text{pgk1, gpm1, enolase}}$ PEP	$1-10$	$0.001-1$	$0.001-1$	$0.1-10$		[41]
PEP $\xleftrightarrow{\text{pyruvate-kinase}}$ PYR	$0.4-40$	0.014	0.53	$6.5\cdot10^3$		[41]
AcCHO $\xleftrightarrow{\text{adh}}$ EtOH	$0.3-30$	17	0.17	$1.45\cdot10^4$		[41]
AcCHO $\xleftrightarrow{\text{adh3}}$ EtOH	$0.3-30$	17	0.17	$1.45\cdot10^4$		[41]
NAD+ $\longleftrightarrow$ NADH	$0.06-6$	$0.01-10$	$0.01-10$	0.1	$0.3-3$	
NAD+ $\longleftrightarrow$ NADH	$0.04-4$	$0.1-100$	$0.001-1$	0.01	$0.03-0.3$	
Michaelis-Menten (Irreversible): $\nu = \frac{V_M}{K_S}\frac{S}{1+S/K_S}$						
Reaction (enzyme)	V_M (μmol/min/mg)	K_S (mM)	Citation(s)			
PYR $\xrightarrow{\text{pdc}}$ AcCHO	$0.03-3$	5	[41]			
PYR $\xrightarrow{\text{mcp}}$ PYR	$0.002-0.2$	$0.06-6$	[31]			
PYR $\xrightarrow{\text{pdh}}$ AcCoA	$0.01-1$	$0.06-6$	[5, 8]			
Inactive Transport (Linear): $\nu = v_{in}S_{cyto} - v_{out}S_{mito}$						
Reaction (enzyme)	v_{out} (min^{-1})	v_{in} (min^{-1})	Citation(s)			
AcCHO $\leftrightarrow$ AcCHO	$10^{-4}-1$	$2\cdot10^{-4}-1$				
EtOH $\leftrightarrow$ EtOH	$10^{-4}-1$	$2\cdot10^{-4}-1$				

Table S1: Parameters and parameter ranges used in the yeast model. All ranges are taken over a log-normal distribution. Colour indicates where the reaction happens: yellow, blue and yellow to blue for cytosol, mitochondria and cytosol into mitochondria respectively. Citations to studies used for obtaining parameters are given on the last column of the table. Note that the Pyr $\rightarrow$ Pyr reaction is an active transport reaction, which has the same functional form as an irreversible Michaelis-Menten reaction. In the case of simulations using "fixed branch" parameters, the parameters for the mcp and pdh reactions are set to be equal to the parameters of the pdc reaction.