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# ANALYSING THE ROLE OF REGULATORS IN GLUTAMINE SENSING IN *DROSOPHILA MELANOGASTER*

Master's thesis
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## Abbreviations

| | |
|---|---|
| dAEL | Days after egg laying |
| FoxO | Forkhead box class O |
| GDH | Glutamate dehydrogenase |
| Gln-food | Fly food containing 2.5 % glutamine |
| GLS | Glutaminase |
| GS | Glutamine synthetase |
| GSH | Glutathione |
| HBP | Hexosamine biosynthetic pathway |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| No-Gln-food | Fly food without glutamine |
| OGA | O-GlcNAcase |
| O-GlcNAc | O-linked N-acetylglucosamine |
| OGT | O-linked N-acetylglucosamine transferase |
| TOR | Target of rapamycin |
| TORC1 | Target of rapamycin complex 1 |
| TORC2 | Target of rapamycin complex 2 |
| Uro | Urate oxidase |
| SALL | Spalt-like |
| Salm | Spalt major |
| Salr | Spalt-related |
| Sxc | Super sex combs |

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Tiivistelmä – Referat – Abstract

Glutamine, the conditionally essential amino acid, is a major carbon and nitrogen carrier required for a range of cell functions, such as protein synthesis and maintaining redox balance. While healthy cells adjust their activities in response to glutamine availability, tumor cells display deregulated glutamine uptake and metabolism allowing quick proliferation and survival in cellular stress conditions. Hence, further knowledge of the glutamine sensing network is of interest.

Utilizing *Drosophila melanogaster*, the roles of formerly identified glutamine sensing regulator candidates, Forkhead box O (FoxO), Super sex combs (Sxc), Spalt major (Salm) and Spalt-related (Salr), were explored. *Drosophila* is an efficient model organism for analyzing gene regulatory mechanisms, with its simple genome but conserved genes and metabolic pathways.

Loss-of function and gain-of-function mutants of the candidates were cultured with/without glutamine, and their physiological response and gene expression changes were analyzed. The results show the glutamine intolerant phenotype of FoxO and Sxc deficiency, not dependent on altered food intake levels of larvae. However, glutamine intolerance of Salr and Salm deficiency was not observed.

Moreover, we aimed to gain further insight to the roles of FoxO and Sxc in glutamine metabolism. Since amino acid catabolism produces ammonia, and glutamine metabolism plays a vital role in ammonia detoxification, we performed a pH-based measurement of *foxo* and *sxc* mutant larvae hemolymph on food with/without glutamine. However, we could not associate FoxO or Sxc with regulation of glutamine-derived ammonia clearance. In addition, we explored FoxO downstream regulator candidates. Putative promoter areas of *Paics, Uro, Sesn, salr, Prat2 and Gdh* were cloned into reporter vectors and the luciferase activity was analyzed under the expression of *foxo*. The results indicate that FoxO is a regulator of all of the 6 genes. Next we could utilize the here constructed plasmids to see whether the FoxO-mediated regulation is affected by altered glutamine levels in cell culture.



Avainsanat – Nyckelord – Keywords
Glutamine, nutrient sensing, *Drosophila melanogaster*, metabolism, gene regulation


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Tiivistelmä – Referat – Abstract

Glutamiini on yksi ihmiskehon yleisimmistä aminohapoista; sillä on tärkeä rooli niin hiilen kuin typenkin aineenvaihdunnassa. Monet soluille välttämättömät reaktiot, kuten proteiinisynteesi ja solujen hapetus-pelkistystasapainon ylläpitäminen, ovat siitä riippuvaisia. Solujen aineenvaihduntaa säädellään ravintoaineiden saatavuuden mukaisesti. Syöpäsolut kuitenkin poikkeavat tästä säätelystä, ja esimerkiksi kiihtynyt glutamiinin aineenvaihdunta tarjoaa niille rakennuspalikoita nopeaan lisääntymiseen sekä työkaluja solulle epäsuotuisissa stressiolosuhteissa selviytymiseen. Tästä johtuen olemme kiinnostuneita selvittämään glutamiinin aistinnan ja aineenvaihdunnan säätelymekanismeja syvällisemmin.

Tutkimuksessa käytimme *Drosophila melanogaster* -malliorganismia. Banaanikärpänen on ideaali valinta, sillä vaikka sen genomi on huomattavasti ihmisgenomia yksinkertaisempi, ovat useimmat geenit ja niiden säätelyreitit hyvinkin konservoituneita lajien välillä. Työssä keskityimme aiemmin löydettyjen säätelytekijäkandidaattien, Forkhead box O (FoxO), Super sex combs (Sxc), Spalt major (Salm) ja Spalt-related (Salr), tutkimiseen.

Hyödynsimme kärpäslinjoja, jotka ali- tai yliekspressoivat kandidaattigeenejä ja analysoimme, millaisia fysiologisia tai geneettisiä vasteita havaitsemme, kun kärpäset saavat joko ruokaa glutamiinilla tai ilman glutamiinia. Tuloksista näimme, että *foxo* ja *sxc* mutantit ilmensivät glutamiini-intoleranttia fenotyyppiä. Varmistimme myös, ettei fenotyyppi ollut riippuvainen siitä, että mutantit toukat olisivat syöneet eri määrän ruokaa sen glutamiinipitoisuudesta riippuen. Emme huomanneet selkeää glutamiini-intoleranssia kärpäsillä, joilla Salr tai Salm oli aliekspressoitu.

Pyrimme ymmärtämään syvällisemmin FoxO:n ja Sxc:n rooleja glutamiinin aineenvaihdunnassa. Aminohappojen hajottaminen tuottaa ammoniakkia, ja toisaalta glutamiinin metabolialla on tärkeä rooli ammoniakin detoksifikaatiossa. Siksi olimme kiinnostuneita, voisiko *foxo* tai *sxc* mutanttien hemolymfan pH-arvoissa olla eroja glutamiinia sisältävällä ruoalla vs. glutamiinittomalla ruoalla. Saamiimme tuloksiin nojaten emme kuitenkaan voineet yhdistää FoxO:a tai Sxc:tä glutamiiniperäisen ammoniakin poistamisen säätelyyn.
Lisäksi selvitimme FoxO:n roolia kuuden eri geenin, *Paics*, *Uro*, *Sesn*, *salr*, *Prat2*, ja *Gdh*, säätelyssä lusiferaasi-reportterigeeni analyysilla. Tuloksista näimme, että FoxO säätelee niistä jokaisen ekspressiota. Seuraavaksi voisimme hyödyntää tässä työssä rakennettuja plasmideja selvittääksemme, onko soluviljelmän glutamiinipitoisuudella vaikutusta näiden kuuden geenin FoxO:sta riippuvaiseen säätelyyn.



Avainsanat – Nyckelord – Keywords
*Drosophila melanogaster*, glutamiini, ravintoaineiden aistiminen, aineenvaihdunta, säätelytekijä


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# Introduction

Nutrients activate specific nutrient sensors and their downstream pathways, maintaining the metabolic homeostasis in changing nutritional conditions. Sensing of nutrients is essential for the cell, and, deregulation of nutrient sensing pathways is a hallmark for metabolic diseases, such as cancer (Coller 2014). In 1925, Otto Warburg found that cancer cells use glucose in excess, and even when sufficient oxygen is available, they tend to ferment glucose with aerobic glycolysis into lactate. Moreover, the pressure of the tumor microenvironment can create hypoxic regions within the tumor and initiate the shifting from preferably oxidative metabolism to glycolysis (Al Tameemi et al. 2019). However, converting glucose into secretory lactate disables its use as a carbon source. Glutamine supports the mitochondrial integrity providing carbon for TCA cycle, for generation of essential building blocks, aiding cancer cell proliferation (Boroughs and Deberardinis 2015; Wise et al. 2008).

Many tumors use excess glutamine, and in fact, the survival of certain cancer cells depends on glutamine availability (Choi and Park 2018). Moreover, efficient glutamine metabolism is essential for normal cell survival, as for instance, immune cells utilize glutamine as their main energy and carbon source (Coller 2014; Altman and Dang 2012). Thus, targeting glutamine metabolism as a cancer treatment has the issue of impairing the normal cell function. To address this issue, further understanding of glutamine metabolism is needed. Hence, the goal of the thesis is to explore components of the glutamine sensing network as well as their role in glutamine metabolism utilizing *Drosophila melanogaster* as a model organism.

## Glutamine

Amino acids are nutrients containing nitrogen, carbon, hydrogen, oxygen and a side chain specific to the amino acid. They are further divided into essential, nonessential and conditionally essential amino acids. L-glutamine (from here on referred to as glutamine), the most abundant free amino acid in the human body, was formerly considered nonessential, as it can be synthetized in sufficient amounts by the body and does not necessarily need to be supplied through dietary protein (Watford 2015). However, the nonessential nature has been questioned as in some cases of physiological malfunction, circulatory and tissue glutamine concentrations significantly decrease (Watford 2015). This evidence suggests glutamine to be merely conditionally essential. Furthermore, the ability for the body to synthetize glutamine has likely been maintained during evolution because of the critically important nature of glutamine for cell homeostasis (Kumada et al. 1993). Interestingly, glutamine synthetase (GS), the enzyme responsible for glutamine synthesis, is likely one of the oldest functioning enzymes in evolutionary history (Kumada et al. 1993).

## Glutamine metabolism

The expression and activity of GS and glutaminase (GLS), regulate the glutamine concentration in different tissues of the body. GS catalyzes the condensation reaction of glutamate and ammonia to form glutamine (Fig. 1) requiring one ATP (Newsholme et al. 2003). Glutamine serves as an important nitrogen donor for nucleotide and cytoplasmic protein synthesis, hence, GS is abundant in cytosol, where it ensures glutamine availability (Cruzat et al. 2018). In addition, the skeletal muscle is a key tissue for glutamine synthesis and supplies the amino acid also through muscle protein catabolism (Hakvoort et al. 2017). Around 80 % of glutamine in the body is found in this tissue (Cruzat et al. 2018). However, the GS activity is relatively low in skeletal muscle, as the fairly high intercellular glutamine concentration regulates the enzyme activity (Cruzat et al. 2018).

GLS is responsible for the glutamine hydrolysis reaction forming glutamate and an ammonium ion (Fig. 1) (Cruzat et al. 2018). For example, in the gut glutamine is mainly used for amino acid synthesis and energy production, and it is an important tissue for glutamine consumption with a high GLS activity (Cruzat et al. 2018). Moreover, glutamate can be further deaminated into α-ketoglutarate, an intermediate of the TCA cycle, through a reversible reaction (Fig. 1) catalyzed by glutamate dehydrogenase (GDH) requiring reduction of NAD(P)+ to NAD(P)H (Plaitakis et al. 2017). Thus, GLS activity in the mitochondria has a central role in amino acid and lipid synthesis.

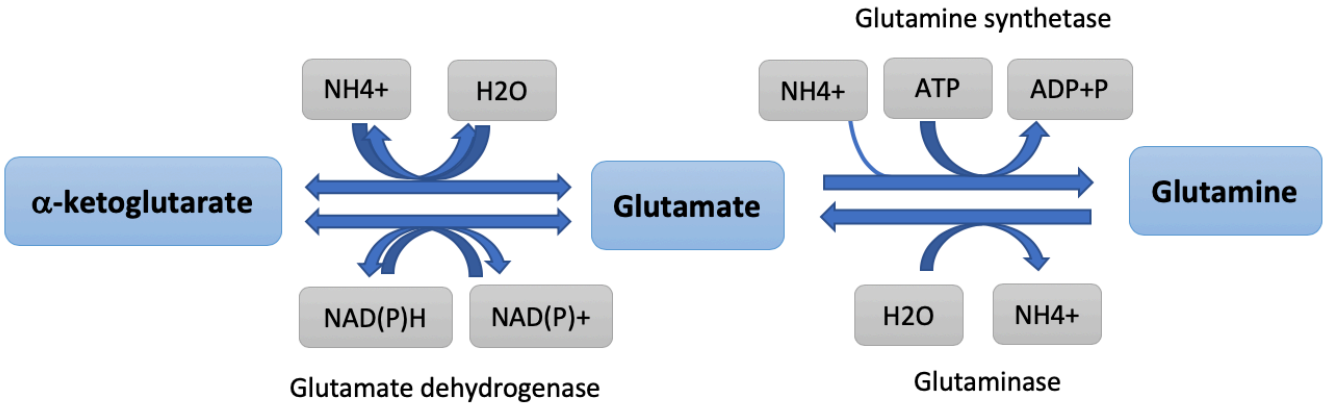


**Figure 1. Enzyme-catalyzed reactions between glutamine, glutamate and α-ketoglutarate.** Glutamine synthetase catalyzes the reaction where glutamate is converted into glutamine requiring one ATP and NH4+. Glutamine can be dehydrogenated to glutamate with glutaminase activity. The reversible reaction between glutamate and α-ketoglutarate is catalyzed by glutamate dehydrogenase and it requires NAD(P)H/NAD(P)+ as a coenzyme. Also, a route for ammonia detoxification is visible in α-ketoglutarate to glutamine.

## Glutamine as a pH and redox buffer

Glutamine metabolism in the liver is important for pH homeostasis. Ammonia derived from amino acid catabolism is toxic in excessive concentrations in the blood (Liu et al. 2018). The detoxification of ammonia is possible through incorporation into α-ketoacids to generate amino acids, such as glutamine (Fig. 1), or through transport or diffusion out of the cell followed by urea cycle in the liver (Hakvoort at al. 2017; Liu et al. 2018). Thus, glutamine is an interorgan ammonia transporter, and therefore, serves as a buffer for pH homeostasis.

In addition, glutamine plays an important role in production of glutathione (GSH), the antioxidant consisting of glutamate, cysteine and glycine. Not only does glutamine provide a source for glutamate, but in turn, glutamate concentration is responsible for regulation of cysteine uptake (Sappington et al. 2016; Cruzat et al. 2018). GSH serves as a redox buffer in oxidative stress conditions and is essential for cell survival (Cruzat et al. 2018). Glutamine metabolism produces also Nicotinamide adenine dinucleotide phosphate (NADPH), which is a major electron donor important for GSH function and lipid synthesis (Nguyen and Durán 2018; Choi and Park 2018).

### Glutamine as a signaling molecule

Target of rapamycin (TOR) plays an essential role in sensing the amino acid status (Nicklin et al. 2009). It is not an actual amino acid sensor, but a master regulator, receiving growth factor, cellular energy and nutritional signals from several pathways, and responding to them by controlling the cellular response (Zhang et al. 2000). Particularly, cell growth and proliferation are affected by TOR signaling (Nicklin et al. 2009). In mammalian species, TOR functions in two different multiprotein complexes TORC1 and TORC2 (Nicklin et al. 2009). Glutamine is involved in TORC1 localization to lysosomes as well as in uptake of leucine into cells which is important for TORC1 activation (Csibi et al. 2013). TORC1 activity initiates phosphorylation of eIF4E binding proteins and ribosomal S6 kinase, which affects protein synthesis through increase in translation (Havula 2017). Thus, glutamine levels can alter gene expression and influence cell growth through TOR activity (Choi and Park 2018).

### Glutamine in cancer

Amino acid transporters and enzymes involved in amino acid metabolism, are expressed in tissue- and developmental specific manner in normal cells (Lukey et al. 2017; Kandasamy et al. 2018). Similarly, tumor cells can overexpress certain enzymes and transporters such as those associated with glutamine. For example, the MYC oncogene overexpression is characteristic in various cancers (Choi and Park 2018). It enables high rates of glutamine transport in the cell through upregulating the expression of glutamine transporters (DeBerardinis and Cheng 2010; Wise et al. 2008). In addition, MYC is involved in upregulating the mitochondrial GLS activity (Choi and Park 2018). Thus, resulting in increased glutamine metabolism and even glutamine dependency in cancer cells (Choi and Park 2018; Wise et al. 2008).

The plethora roles of glutamine for the cell make it an ideal nutrient for cancer cells (Figure 2). These include (1) providing a carbon source for TCA cycle, (2) being involved in oncogenic signaling regulating cell growth through TOR activity, (3) serving as an important nitrogen donor for nucleotide and amino acid synthesis, and, (4) supporting the generation of reducing equivalents, GSH and NADPH, which maintain redox homeostasis enabling cancer cell survival in stress conditions (DeBerardinis and Cheng 2010).

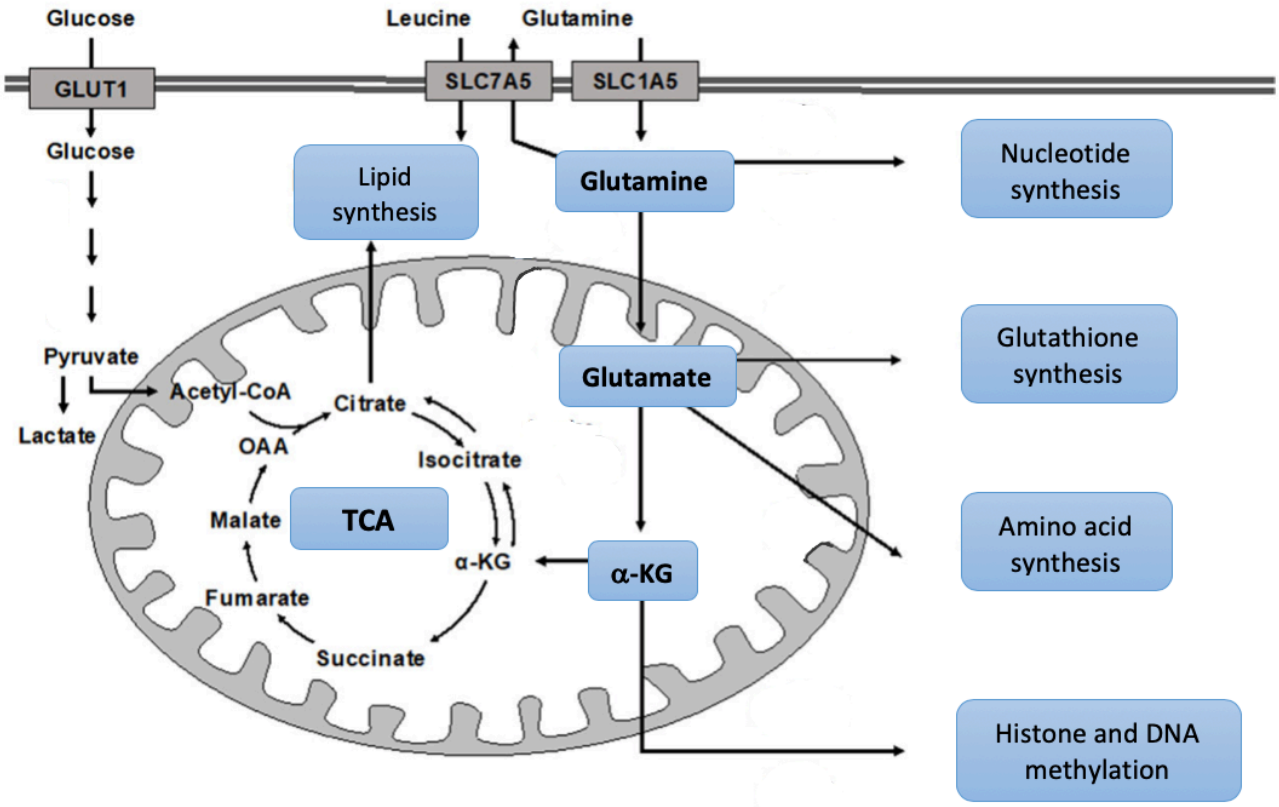


**Figure 2. Glutamine and glucose are important nutrients for cancer cell survival.** Glutamine and glucose provide the necessary precursors for tumor energy production and biomolecule synthesis for proliferation. Glutamine enters the cell via plasma membrane transporters, for example SLC1A5. Glutamine is involved in regulating leucine uptake into the cell. Moreover, glutamine is necessary for nucleotide, glutathione (GSH) and amino acid synthesis. Also, glutamine-derived α-ketoglutarate (α-KG) has been found to play a role in histone and DNA methylation (Nguyen and Durán 2018). (figure modified from: Nguyen and Durán 2018)

## *Drosophila melanogaster* as a model organism

*Drosophila melanogaster* is a powerful model organism for studying nutrient sensing and its effects on metabolism (Droujinine and Perrimon 2016; Havula 2017). Holometabolous insects, like *Drosophila*, are characterized by four distinct developmental stages (Fig. 3A). In addition to morphology, they differ in feeding behavior (Staats et al. 2018). During the larval stages, *Drosophila* feed constantly to increase the body mass and energy storage for growth and metamorphosis (Havula 2017). Following encapsulation, the pupa stage is a non-feeding stage devoted to developing the adult structures such as head, legs and wings from the imaginal discs (Droujinine and Perrimon 2016). After eclosion, the adult flies feed less in comparison to larva, as they do not grow but focus merely on somatic maintenance and reproductive fitness (Droujinine and Perrimon 2016; Havula 2017). An advantage of utilizing *Drosophila* to study nutrition-dependent signaling pathways, is that the diet of the constantly feeding larva can be strictly controlled. Thus, together with the short lifecycle (Fig. 3A), the above-mentioned features allow rapid monitoring in differences during development from larva to pupa stage when studying gene function in response to altered nutritional status.

Although the *Drosophila* genome is composed of only four chromosomes, about 60 % of the genes are functionally similar to mammalian ones (Staats et al. 2018). As in human, sensing of specific nutrients activate their downstream signaling pathways, to maintain homeostasis in cellular as well as organismal level in the fly (Staats et al. 2018). In fact, metabolic pathways, such as the TOR and insulin-like signaling pathway (Fig. 3B), are highly conserved between *Drosophila* and human (Havula 2017). Thus, making it appealing to study the role of regulators in nutrient sensing first in the fly.

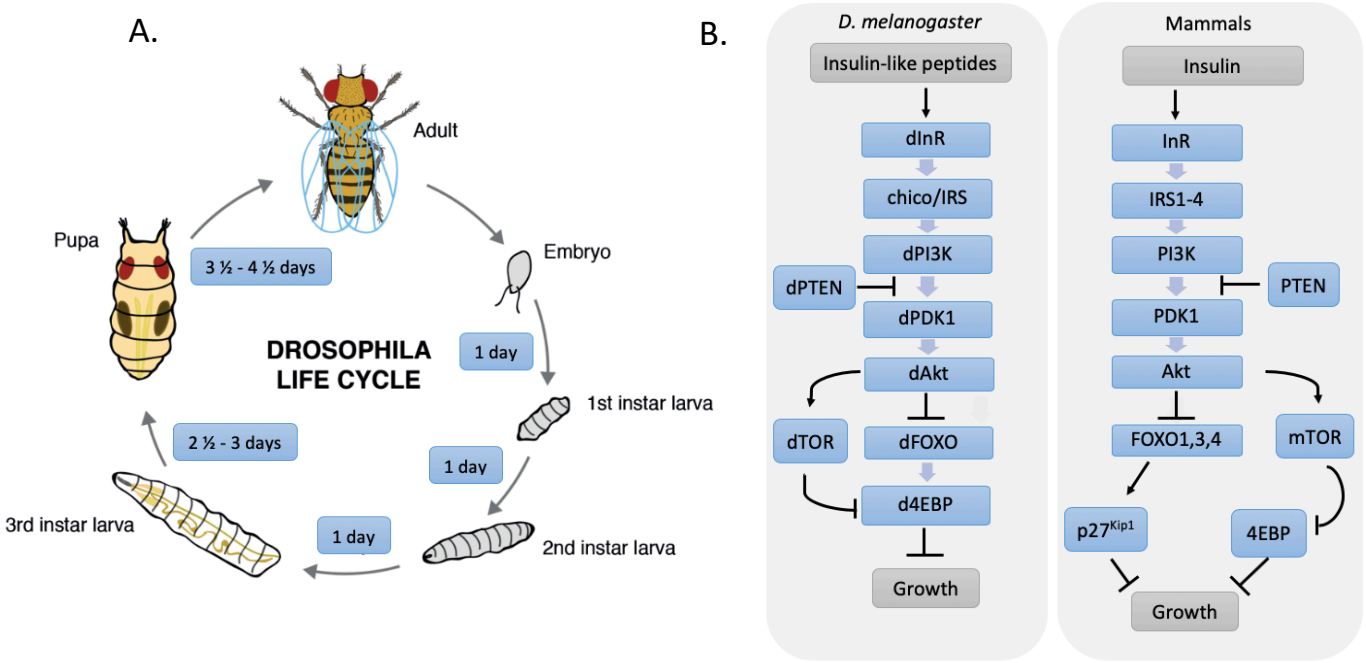


**Figure 3. Benefits of utilizing *Drosophila* as a model organism to study metabolism.** A. The quick generation of flies; in 25 °C it takes about 10 days for an egg to develop into an adult fly. *Drosophila* is a holometabolous insects with four life stages: embryo, larva, pupa and adult stage. B. Insulin-like signaling pathway is highly conserved between mammals and the fly. (figure 3A modified from: Havula 2017)

In addition, flies are easy and cost-effective to keep, and, balancer chromosomes enable maintaining even lethal mutations in balanced fly stocks (Kaufman 2017). There is a variety of genetic tools, for example, for tissue specific expression with the GAL4/UAS system as well as RNAi lines for silencing gene expression (Pandey and Nichols 2011; Kaufman,2017). Stock centers such as Bloomington *Drosophila* Stock Center and Vienna *Drosophila* Resource Center, offer genetically modified flies for research purposes, and the Flybase database enables convenient way for sharing the research data.

## Forkhead box 'Other' transcription factors

Forkhead transcription factors are further classified into subfamilies according to differences in the DNA binding domain found in their 'winged-helix' structural motif (Barthel et al. 2005). The subfamily Forkhead box 'Other' (FoxO) consists of proteins essential for regulating cell metabolism through insulin-like signaling in fluctuating nutritional conditions (Fu and Tindall 2008). Though, not much is known about the regulation of the expression of FoxO factors, the post-translational

regulation through phosphorylation, acetylation, ubiquitylation as well as protein-protein interaction, is well established (Fu and Tindall 2008).

Oxidative stress and nutrient deprivation promote FoxO activites, aiding its nuclear localization and binding to the FoxO recognition element to activate or repress target gene transcription (Fig. 4) (Fu and Tindall 2008). On the contrary, in the presence of insulin and growth factor signaling, FoxO activity is inhibited through PI3K/PDK1/Akt-mediated phosphorylation resulting in relocation to the cytosol (Barthel et al. 2005). FoxO factors have been associated with cancer development as, for example, the Akt signaling pathway regulating FoxO activities (Fig. 4) is known to be dysregulated in cancer (Fu and Tindall 2008).

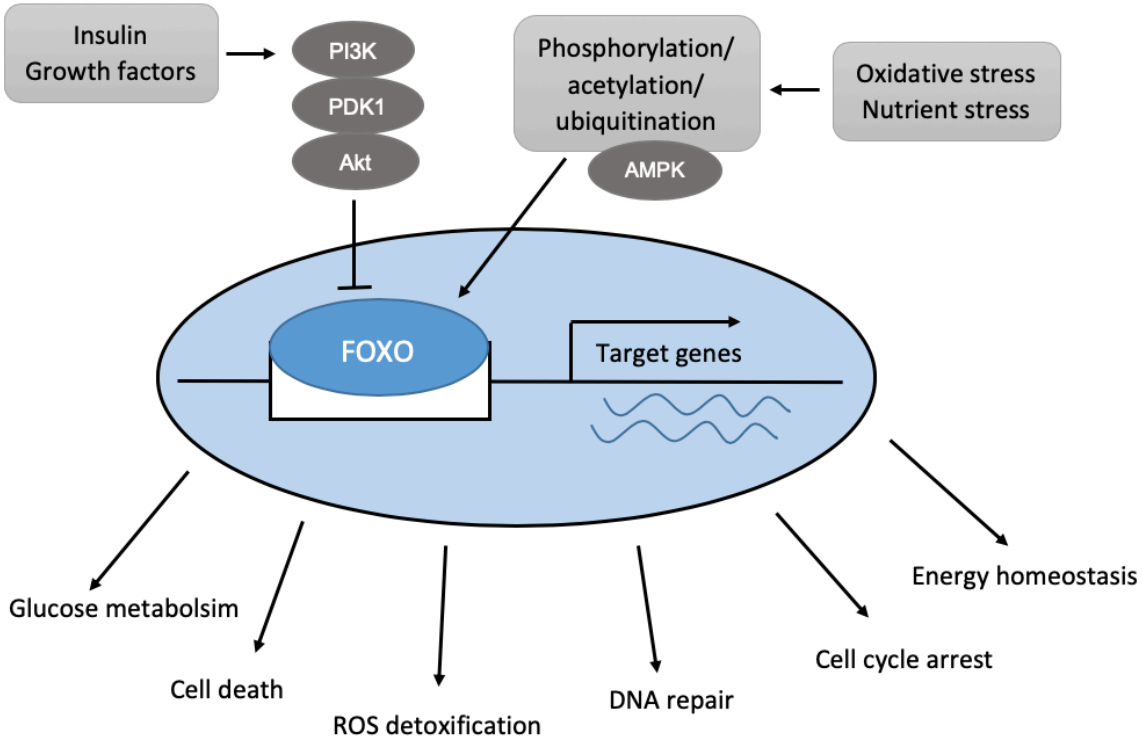


**Figure 4. The diverse roles of FoxO transcription factors.** FoxO transcription factors are regulated in response to environmental conditions. Akt-mediated phosphorylation inhibits FoxO activities as FoxO is translocated from nucleus to cytoplasm, whereas oxidative stress and nutrient deprivation aid its location into nucleus and promote FoxO activities. For example, the AMP-activated protein kinase (AMPK) phosphorylates FoxO. FoxO-dependent regulation is responsible for a variety of functions, regulating genes involved with e.g. apoptosis, cell cycle arrest, oxidative stress resistance, damaged DNA repair, glucose metabolism and energy homeostasis.

The mammalian genome encodes four FoxO factors; FOXO1, FOXO3, FOXO4 and FOXO6; which are differentially expressed in different tissues, whereas the *Drosophila* genome encodes only one FoxO homolog, dFOXO (from now on referred to as Foxo) (Fu and Tindall 2008). In 2008, Kramer et al. showed that Foxo activity is dependent on amino acid levels in *Drosophila*. Also, FOXO1, FOXO3 and FOXO4 have been found to upregulate GS expression in mice (Kamei et al. 2014; van der Vos et al. 2012). Thus, FoxO is an important regulator in amino acid metabolism and has a role in controlling glutamine levels through GS activity.

## Super sex combs *O*-GlcNAc transferase

*O*-linked N-acetylglucosamine (*O*-GlcNAc) transferase (OGT) catalyzes the addition of an *O*-GlcNAc sugar onto a serine or threonine residue in proteins found inside the cell (Fig. 5) (Sinclair et al. 2009). The proteins can then bind to the genome and potentially alter gene regulation. The substrate for *O*-GlcNAcylation is the end product of hexosamine biosynthetic pathway (HBP) requiring inputs of glucose, glutamine, glucosamine and nucleotide metabolism (Fig. 5) (Hardivillé and Hart 2014). In HBP, glutamine fructose-6-phosphate amidotransferase transfers an amino group from the input glutamine amide to fructose-6-phophate, producing glucosamine-6-phosphate and glutamate.

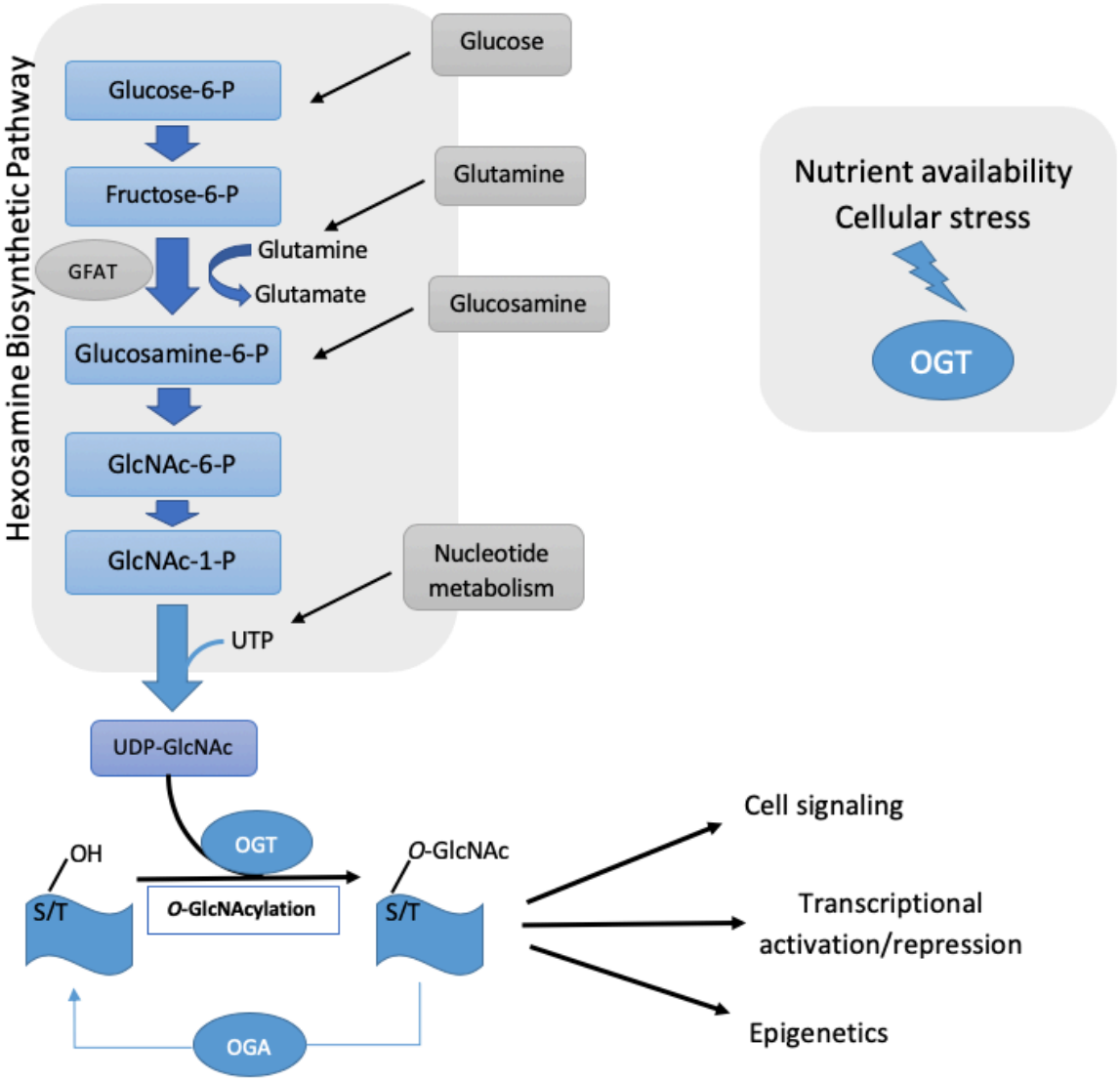


**Figure 5. Hexosamine biosynthetic pathway and O-GlcNAcylation.** Hexosamine biosynthetic pathway (HBP) synthetizes the UDP-GlcNAc which is the substrate for OGT (Sxc in *Drosophila*) mediated *O*-GlsNAcylation. The synthesis requires inputs from glucose, glutamine, glucosamine and nucleotide metabolism (uridine-5'-triphosphate, UTP). Glutamine fructose-6-phosphate amidotransferase (GFAT) transfers an amino group from glutamine to fructose-6-phophate producing glucosamine-6-phosphate and glutamate. The *O*-GlcNAcylation is a reversible reaction; *O*-GlcNAc can be deconjugated by *O*-GlcNAcase (OGA). *O*-GlcNAcylation takes place in serine or threonine residues (S/T) of a target protein. Nutrient availability and cellular stress have been shown to regulated OGT activities (Wu et al. 2017). OGT-mediated O-GlcNAcylation can affect for example cell signaling, such as insulin signaling dynamics; transcriptional activation or repression and epigenetics such as gene silencing (Wu et al. 2017).

OGT-mediated post-translational regulation has been shown to control FOXO1 activities among many other target proteins (Hardivillé and Hart 2014). The *Drosophila* homolog for human OGT belongs to the Polycomb group and is also known as super sex combs (Sxc) (Sinclair et al., 2009). Mariappa et al. (2015) have shown that in *Drosophila* the Sxc-mediated regulation is especially important during the embryonic and larval development. Also, Sinclair et al. (2009) have found that the *sxc* mutant lethal phenotype in *Drosophila* is rescued with the human OGT transgene, thus, *Drosophila* is a powerful model organism for studying the role of OGT in mammals.

### Spalt major and Spalt-related transcription factors

Mammalian Spalt-like (SALL) gene family consists of four zinc finger transcription factors; SALL 1, 2, 3 and 4; of which SALL4 is known to be dysregulated in various cancer (Zhang et al. 2015; Miettinen et al. 2014). The *Drosophila* homologs belonging to SALL family are Splat major (Salm) and Spalt-related (Salr) transcription factors. In wing disc, Salm and Salr are both regulated by the Decapentaplegic signaling pathway to control *Drosophila* wing disc development (Organista et al. 2015). The role of Salm and Salr in metabolic regulation have been poorly understood as the research on these transcription factors has focused mainly on wing patterning (Barrio and de Celis 2004; Sánchez et al. 2010; Organista et al. 2015). However, unpublished data in the lab (Liu 2019) have explored the Salr gene regulatory network and further uncovered several metabolic targets. Moreover, Salr was found to have a role in regulation of starvation-responsive processes (Liu 2019).

## Aim of the thesis

The aim of the thesis work is to analyze regulators in glutamine sensing, focusing on the formerly identified candidates, Foxo and Sxc. In addition, possible candidates, Salr and Salm, are tested for glutamine intolerance. We utilize *Drosophila melanogaster*, as it is an efficient model organism for analyzing gene regulatory mechanisms in nutrient sensing and downstream metabolic pathways. We take a look at the role of Foxo and Sxc in regulation of glutamine-derived ammonia clearance and observe possible Foxo downstream regulation candidates to explore the Foxo regulatory pathway further.

## Materials and methods

The fly lines (Table 1) used for the thesis work were maintained on standard laboratory fly food containing: 0.6 % agar, 1.8 % dry baker's yeast, 6.5 % malt, 2.4 % nipagin, 0.7 % propionic acid and 3.2 % semolina.

Table 1. Fly-lines used in the study

| Fly line | Source |
|---|---|
| $Sxc^{1}$ 3058 | BDSC |
| $Sxc^{6}$ 7182 | BDSC |
| $Foxo^{94}$ 42220 | BDSC |
| $Foxo^{25}$ 80944 | BDSC |
| $W^{1118}$ | BDSC |
| CG-GAL4 | BDSC |
| Tub-GAL4 | BDSC |
| Salm RNAi 3029 GD | VDRC |
| Salm RNAi 3030 GD | VDRC |
| Salm RNAi 101052 KK | VDRC |
| Salr RNAi 29549 | BDSC |
| KK ctrl 60100 | VDRC |
| GD ctrl 60000 | VDRC |
| Trip ctrl 36303 | BDSC |

Bloomington *Drosophila* Stock Center (BDSC)
Vienna *Drosophila* Resource Center (VDRC)

## Experimental setting

Flies were cultured with standard laboratory food, and kept in 25 °C. The flies were transferred to an egging chamber on apple juice plate with dry baker's yeast. The apple juice plate was switched to a new-one in one day and the flies were allowed to lay eggs for 24 hours. The plate was then removed from the egging chamber and kept in 25 °C for another 24 hours, after which, the first instar larvae could be collected from the apple juice plate for further analysis.

Food with glutamine (Gln-food) and food without glutamine (no-Gln-food) contained; 0.5 % agar, 2.5 % dry baker's yeast, 2.4 % nipagin and 0.7 % propionic acid; with or without 2.5 % L-glutamine, respectively.

Each experiment was performed in triplicate, and 30 larvae per tube or per plate were cultured unless otherwise specified.

## Pupation for glutamine intolerance

First instar larvae were transferred to vials with Gln-food and vials with no-Gln-food (see 'Experimental setting'). They were kept in 25 °C and the number of pupas was recorded daily for a total period of 9 to 10 days.

## Measurement of food intake

Coloured food for the measurement of food intake was prepared as the Gln-food and no-Gln-food (see 'Experimental setting') with the exception that food colorant, supplier Dr. Oetker containing 2.2 % E133, was added in 0.05 % final concentration to both of the foods.

First instar larvae were transferred to plates containing no-Gln-food (with no food colour-dye) and kept in 25 °C for 3 days. After the 3 days the third instar larvae were transferred to plates, 10 larvae per plate, containing coloured food. The plates were kept in 25 °C for 3 hours. Empty Eppendorf tubes were weighted. After the 3 hours, the larvae were collected from the coloured food to the Eppendorf tubes, 10 per tube, and frozen in liquid nitrogen to disable further metabolization of the food ingested. The Eppendorf tubes with the larvae were weighted and the larvae body mass was calculated.
The larvae were homogenized in 100 µl of PBS, centrifuged and the supernatant was further diluted 1:1 in PBS. After centrifugation 125 µl of the sample was transferred to a 96 well plate for the measurement. Standard samples for calibration curve were prepared diluting the food colour-dye in PBS in concentrations: 0, 1.1, 2.2, 4.4, 6.6, 8.8 and 11.0 µg/well. The absorbance of the samples and standards was measured in 629 nm wavelength. The results were normalized according to the larvae body mass.

## pH-measurement of larvae haemolymph

First instar larvae were transferred to vials containing the standard laboratory food and kept in 25 °C for 3 days. The third instar larvae were then transferred to plates containing Gln-food and no-Gln-food and the larvae were allowed to feed in 25 °C for 24 hours. After 24 hours the larvae were moved from the food on to a microscopy slide, 15 larvae per replicate, for the collection of larvae haemolymph. 3 µl of the haemolymph was pipetted into a 0.5 ml tube containing 2 µl 3mM pH indicator dye-colour diluted from Solvent Green 7 hydrate (Sigma). The sample was centrifuged to remove protein which could affect the measurement, and the supernatant was collected into a new tube and kept on ice.
pH standards for the calibration curve were prepared in pH: 6.6, 6.8, 6.9, 7.2, 7.4, 7.6, 7.8 and 8.0. For the standard samples 2 µl of the pH indicator dye was mixed with 3 µl of a given standard solution. The samples and standard samples were measured with NanoDrop in wavelengths 405 nm and 450 nm using the pH 6.8 standard solution without indicator dye as a blank for the samples, and each standard with no pH indicator dye as a blank for a given standard.

## Luciferase reporter gene assay

Primers were designed to target an area about 1 kb before the transcription start site of a selection of genes (Table 2). The putative promoter areas were amplified with PCR using genomic DNA of $W^{1118}$ flies as a template. The inserts were cloned into a pGL3-basic luciferase reporter vector (Promega) and transformed into *E. coli* competent cells. The plasmids were isolated, and the inserts were sequenced to confirm the successful cloning. Plasmids containing Foxo were received from previously made stocks. The Drosophila S2 cells were cultured in M3 medium with

1X Insect Medium Supplement and 2 % fetal bovine serum. The Effectene Transfection Regent kit form Qiagen was used for the transfection. The luciferase activity was analyzed under expression of *foxo* in cell culture with Dual-Luciferase Reporter kit (Promega), and the measurements were performed with the EnSpire Multimode Plate Reader. Renilla was used as a transfection efficiency control for normalization.

Table 2. Genes for the luciferase reporter gene assay

| Gene (transcript) | Chromosomal position amplified |
|---|---|
| *Paics* | X: 12,760,566…12,761,619 |
| *Uro* | 2L: 7,779,094…7,780,128 |
| *Sesn* | 2R: 23,712,887…23,713,943 |
| *salr* (RC) | 2L: 11,357,863…11,358,982 |
| *Prat2* (RA) | 3L: 6,913,905…6,914,966 |
| *Prat2* (RB) | 3L: 6,914,839…6,915,876 |
| *Gdh* (RA, RF) | 3R: 23,943,057…23,944,110 |

# Results

## Role of Foxo and Sxc in glutamine sensing

Foxo and Sxc have been formerly identified as regulator candidates in glutamine metabolism with RNA-seq analysis (Liu et al., unpublished data in the lab). The loss-of-function mutants of these candidates can be exploited in order to observe the physiological response to glutamine availability to further analyze their role in glutamine sensing. Thus, we aimed to confirm the glutamine intolerant phenotype of Foxo and Sxc deficiency. To achieve this goal, the loss-of-function larvae were fed food with/without glutamine and the pupariation kinetics was observed.

*Foxo*$^{25}$ encodes a point mutation G to A (3R:14,067,088) leading to a premature stop codon, hence, a loss-of-function allele. Whereas *foxo*$^{94}$ has an over 20 kb deletion leading to a *foxo* null allele. In the results, we can see that the heterozygous control larvae (*foxo*$^{94}$/*w*$^{1118}$) gain a clear advantage from having glutamine available (Fig. 6A). And, the *foxo*$^{94/25}$ larvae pupate more efficiently without glutamine in their diet (Fig. 6A). However, the difference is not as outstanding as in the control since only about 20 % of the Foxo deficient flies pupate during the 9 days after egg laying (dAEL) regardless of the glutamine availability. This is in line with research from Slack et al. (2011), where they show that *foxo*$^{94/25}$ flies develop slower from egg to adult stage in comparison to wild type flies on regular fly food.

*Sxc*$^{1}$ allele encodes a G to A point mutation (2R:5,327,732) which leads to a nonsense mutation, however, still capable of expressing truncated form of Sxc. *Sxc*$^{6}$ has a splice acceptor mutation (G to A, 2R:5,320,418) manifesting a null mutation. In the results, we can see that similarily to Foxo, Sxc deficient larvae pupate more efficiently without glutamine in their diet (Fig. 6B). However, the

difference in pupation kinetics of *sxc*$^{6/1}$ larva is more considerable as over 70 % pupate on food without glutamine, whereas only 17 % of the larvae on glutamine-rich food develop into pupa in 10 dAEL.

## Influence of glutamine on the food intake levels of *foxo* and *sxc* mutant larvae

Alterations in food intake levels could potentially play a role in the observed glutamine intolerance of *foxo* and *sxc* mutants. To exclude the possible effect of altered food intake level on the glutamine intolerant phenotype, we performed food intake analysis. Indeed, there was no significant difference in the food uptake of the Foxo deficient larvae on different diets (Fig. 6C). Also, they were feeding comparably to the *w*$^{1118}$/*foxo*$^{25}$ control larvae. In the *foxo*$^{94}$/*w*$^{1118}$ control, there was a slight statistical difference in the feeding behaviour, as the larvae were feeding more on the no-glutamine diet.

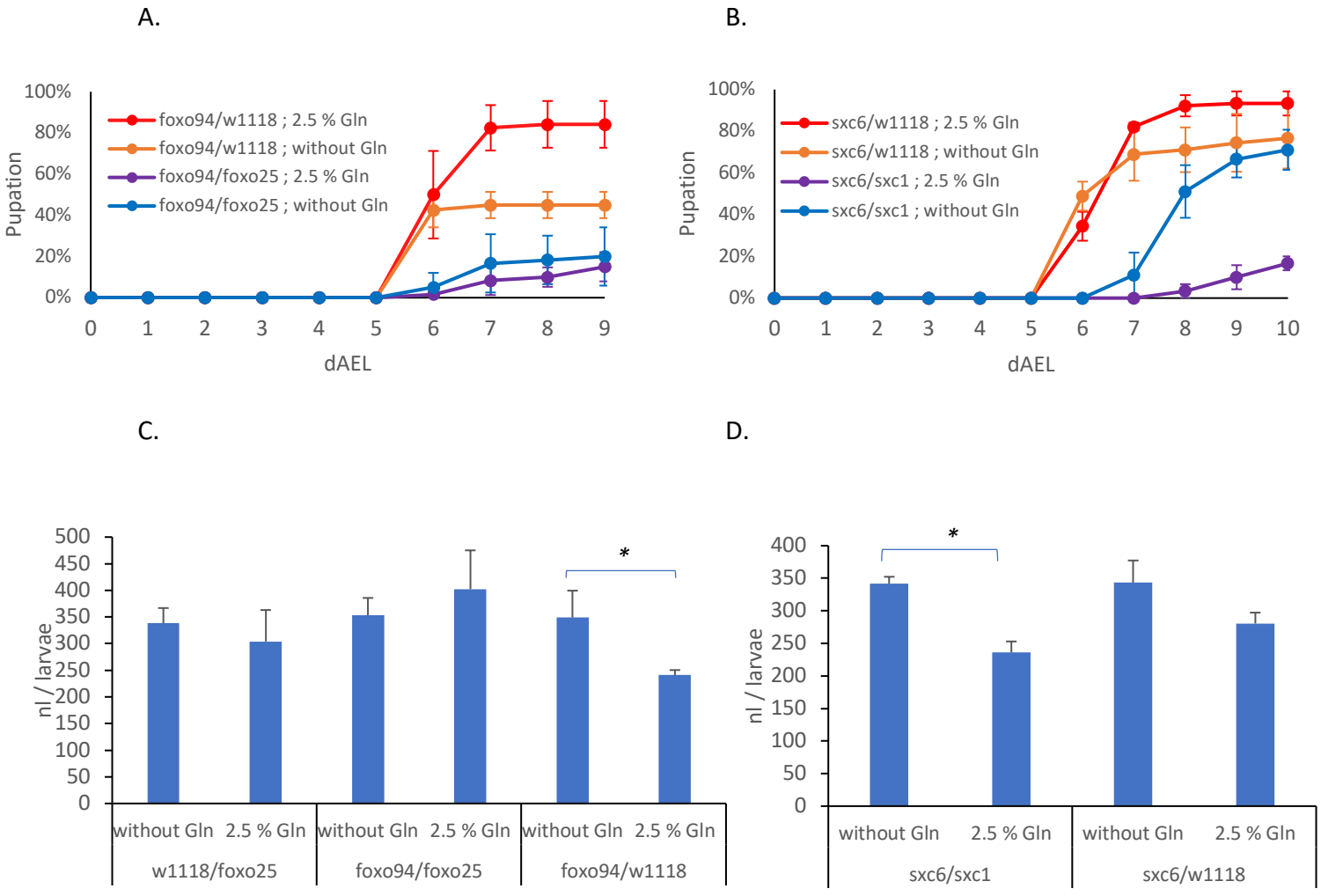


**Figure 6. Foxo and Sxc in glutamine sensing.** A. and B. Pupation kinetics of Foxo (A) and Sxc (B) deficient larvae indicate the glutamine (Gln) intolerant phenotype. The *foxo*$^{94}$/*w*$^{1118}$ and *sxc*$^{6}$/*w*$^{1118}$ controls show the developmental advantage of glutamine-rich diet (red line) in comparison to no-glutamine food (orange line). In contrast, the *foxo*$^{94/24}$ and *sxc* $^{6/1}$ mutant larvae pupate more efficiently on no-glutamine food (blue line vs. purple line). C. and D. Food uptake analysis of *foxo* (C) and *sxc* (D) mutants exclude the effect of altered food uptake levels on the glutamine intolerance. Positive error bars indicate the standard deviations. *$p$< 0,05 for student's t-test.

The Sxc deficient larvae were feeding less on the glutamine-containing diet in comparison to food without glutamine (Fig. 6D). However, when comparing this difference to the one seen in the wildtype larvae food intake levels, there was no statistically significant difference between the wildtype and *sxc* mutant larvae. Still, we can see a major difference in the puparation kinetics between the two of them (Fig. 6B). Thus, we consider that the altered food intake level does not effect on the glutamine intolerant phenotype of Sxc deficient larvae.

## Haemolymph pH of *foxo* and *sxc* mutant larvae on glutamine diet

Glutamine metabolism plays an important role in acid-base balance in the body. Ammonia derived from amino acid catabolism is toxic in excessive concentrations in the blood, and moreover, glutamine is an interorgan ammonia transporter serving as a buffer for pH homeostasis. Therefore, we were interested to see whether Foxo and Sxc play a role in glutamine catabolism derived ammonia detoxification. Thus, we performed pH-measurement of larvae haemolymph for glutamine-derived ammonia assessment. We found no major differences in the pH of *foxo* mutant larvae on the two diets (Fig. 7A). However, in the Sxc deficient larvae we observed a minor trend of an increase in the larval haemolymph pH level on the glutamine food (Fig. 7B). However, there was no statistically significant difference between the results of Sxc deficient larvae on different diets, as the standard deviation of this observation was high.

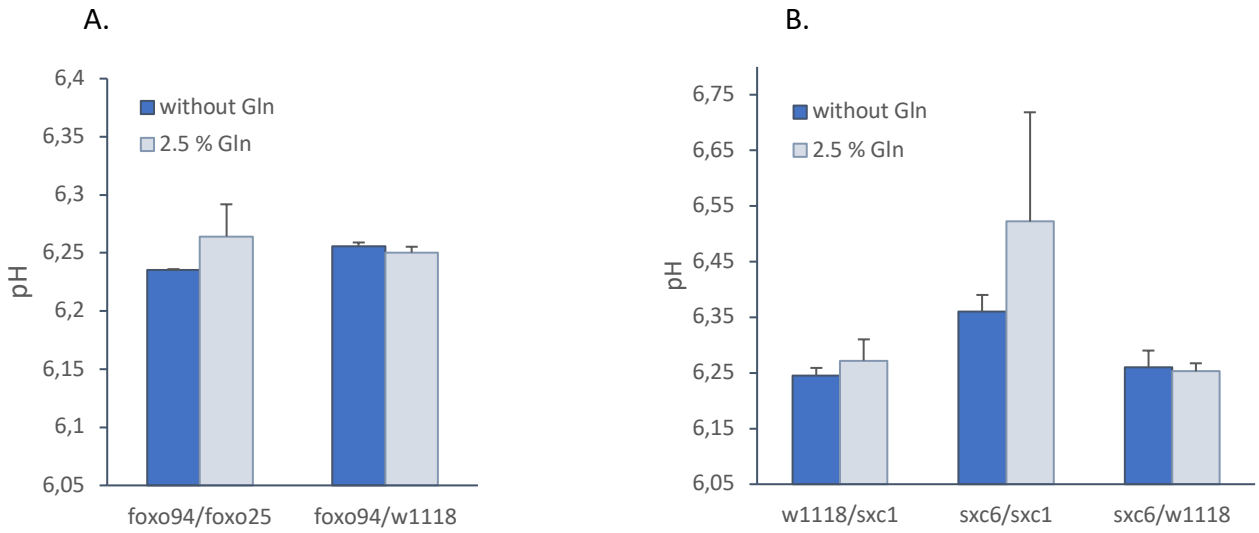


**Figure 7. pH-measurement of Foxo and Sxc deficient larvae.** A and B. pH-measurement of Foxo (A) and Sxc (B) deficient larvae haemolymph show no major difference in pH-values of mutant larvae on Gln-food versus no-Gln-food. Positive error bars indicate the standard deviations.

## Role of Salm and Salr in glutamine sensing

Salm and Salr have been formerly identified as possible candidates in glutamine metabolism with RNA-seq analysis (Liu et al., unpublished data in the lab). Therefore, we were interested to explore the glutamine tolerance of Salm and Salr deficient larvae. Salm RNAi lines; 3029, 3030 and 101052; and Salr RNAi line 29549 were combined with Tub-GAL4 and CG-GAL4 driver lines for universal and fat body specific gene silencing, respectively.

Salm RNAi line 3029 (Fig. 8A and 8B) crossed with the Tub and CG driver lines did not show signs of glutamine intolerance, as the larvae gained a developmental advantage on the glutamine diet. The Salm 3030 RNAi with Tub driver line (Fig. 8C) larvae were pupating more efficiently without glutamine in their diet. However, the GD ctrl used for the experiment was not working properly as the control larvae did not gain an advantage of having glutamine in the food. Also, the Salm 3030 RNAi with CG driver line (Fig. 8D) larvae were pupating better on the glutamine diet. The Salm 101052 RNAi with Tub driver line (Fig. 8E) larvae pupated equally on both diets, whereas Salm 101052 RNAi with CG driver line (Fig. 8F) larvae gained an advantage on glutamine-containing food.

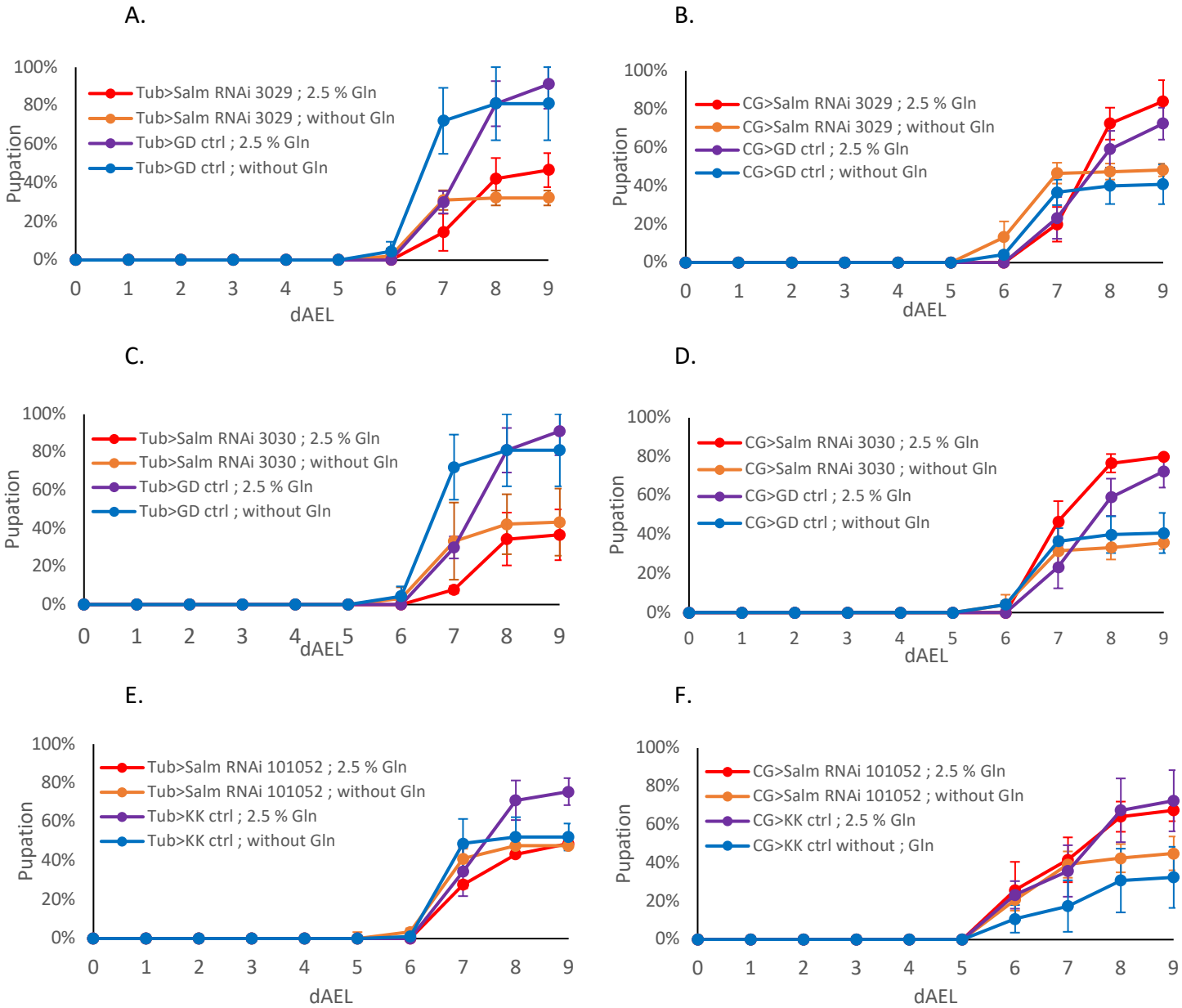


**Figure 8. Pupation kinetics upon Salm and Salr knockdown.** A, B, and C. Tubulin-specific silencing of gene expression with Salm RNAi lines 3029, 3030 and 101052, on 2.5 % glutamine (Gln) vs no-Gln-food. D. Tubulin-specific silencing with Salr 29549 RNAi line. E, F and G. Fat body-specific silencing of gene expression with the three different Salm RNAi lines. D. Fat body-specific silencing with Salm 29549 RNAi line.

Salr 29549 RNAi line with Tub-driver line larvae pupated similarly on both diets (Fig. 9A), and, we could observe a minor advantage on the glutamine diet for the control larvae. With CG driver line, the Salr deficiency gained an advantage on glutamine-containing food (Fig. 9B). However, in both Tub and CG driver line results, we can see that the control larvae are not pupating efficiently enough as only around 50 % are pupating on the glutamine-containing food. Therefore, to confirm the results of Salr 29549 RNAi line for glutamine intolerance, the experiment should be repeated.

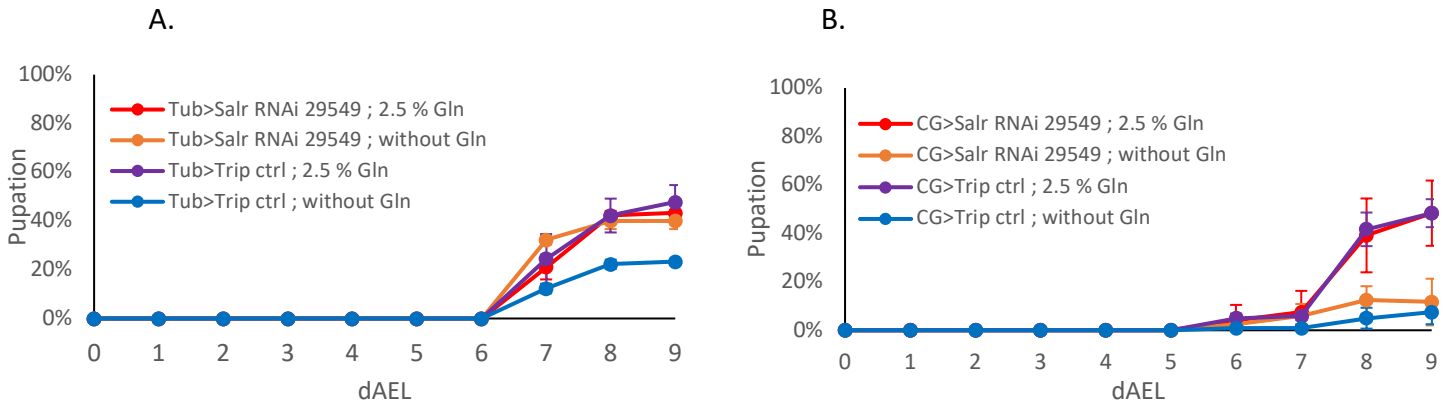


**Figure 9. Pupation kinetics of Salr deficiency.** A. Tubulin-specific silencing with Salr 29549 RNAi line. B. Fat body-specific silencing with Salm 29549 RNAi line.

## Downstream regulatory targets of Foxo

We have confirmed that Foxo is a regulator in glutamine metabolism. Therefore, we aimed to explore the Foxo regulatory network further. In the luciferase reporter gene analysis results (Fig. 10) we can see that Foxo increases the promoter activity of all of the 6 genes: *Paics*, *Uro*, *Sesn*, *salr* (transcript Salr-RC), *Prat2* (transcripts Prat2-RA, Prat2-RB) and *Gdh* (transcripts Gdh-RA, Gdh-RF). The fold change in luciferase signal under the expression of *foxo* is greatest in Prat2 transcript RB, with an over 40-fold change, and in Uro, with a fold change over 20.

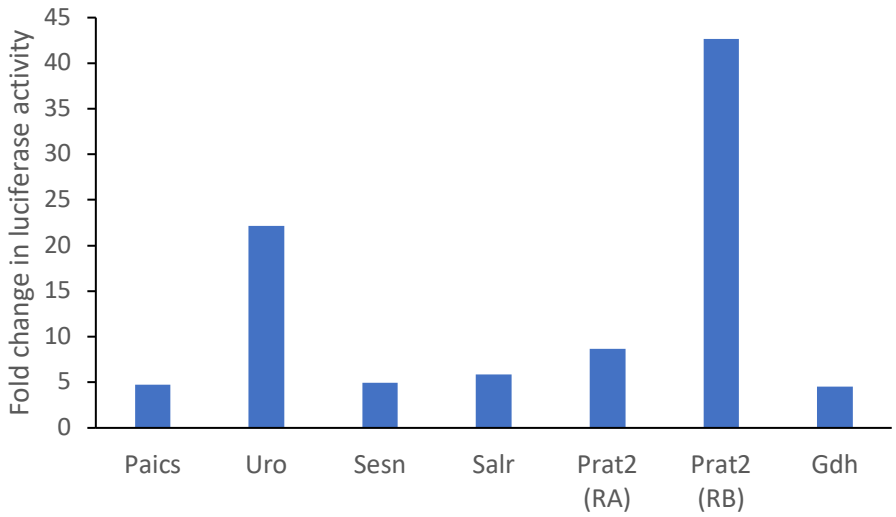


**Figure 10. Luciferase activity under the expression of *foxo*.** The fold change in the activity of the putative promoter areas of 6 genes; *Paics*, *Uro*, *Sesn*, *salr* (transcript Salr-RC), *Prat2* (transcripts Prat2-RA and Prat2-RB) and *Gdh* (transcripts Gdh-RA and Gdh-RF); under the expression of *foxo* in cell culture.

However, the raw data of the luciferase reporter gene experiment (not included) indicated that the here used Foxo concentration, 20 ng/well, had an effect on the cells. Plasmids containing Renilla were co-transfected as a transfection efficiency control, 5 ng/well, for normalization. The wells with Renilla and Foxo had a much lower control signal in comparison to the wells containing Renilla with empty plasmid. Thus, Foxo could have a possibly toxic effect on the cells which could potentially influence the luciferase reporter gene analysis results.

## Discussion

Foxo and Sxc have been formerly identified as regulator candidates in glutamine metabolism. We aimed to show the glutamine intolerant phenotype of mutant larvae. Indeed, in the results we could see that the pupation rate of Foxo and Sxc deficient larvae decreased when they were fed food with glutamine in comparison to having no glutamine in their diet. Thus, the mutant larvae were not able to metabolize glutamine properly, which confirmed that Foxo and Sxc are regulators involved in glutamine metabolism.
In addition, we considered Salr and Salm for glutamine intolerance. In the results, we could not see a clear developmental advantage on food without glutamine in three different Salm RNAi lines and one Salr RNAi line together with Tub or CG driver lines used for the experiment. Thus, glutamine intolerance of Salr and Salm deficiency was not observed.

We were interested to see, whether the glutamine intolerant phenotype of Foxo and Sxc could be dependent on altered food intake levels of the mutant larvae. For example, Zinke et al. (1999) have associated *Drosophila* pumpless deficiency with decrease in food intake levels of larvae in response to specific amino acids. Nevertheless, the results indicated that there was no significant difference on food intake of *foxo* or *sxc* mutant larvae feeding on food with/without glutamine. Thus, the phenotype was not dependent on glutamine-responsive alteration in food intake levels.

We studied the role of Foxo and Sxc in glutamine metabolism further. Ammonia is a potentially toxic metabolite in increased blood concentrations. Therefore, the pH-levels of *foxo* and *sxc* mutant larvae were observed to see whether they could be associated with regulation of glutamine-derived ammonia clearance. Foxo upregulates GS expression, which is involved in mediating the ammonia detoxification through catalyzing the reaction; glutamate + ammonia $\xrightarrow{GS}$ glutamine. This could suggest that loss-of-function of Foxo could affect the hemolymph ammonia concentration. For example, in 2014 Kamei et al. showed that FOXO1 deficiency results in reduced ammonia detoxification after ammonia infusion in mouse blood.
However, we could not see increase in larvae hemolymph pH-levels of Foxo deficiency. Also, there was no notable increase in Sxc deficient larvae hemolymph on glutamine-rich diet, though a minor increase was observed. Therefore, the results did not indicate that Foxo or Sxc would be associated with glutamine-derived ammonia clearance. Though we could neither exclude the possibility merely with the pH-based measurement. For example, in the above-mentioned study, Kamei et al. (2014) utilized a microdiffusion based method to determine the ammonia blood levels in mice.

Furthermore, the observed overall pH-values of Foxo and Sxc deficiency as well as the wildtype larvae were fairly low; between 6.24 and 6.74. For example, Ghosh and O'Connor (2014) find the larval haemolymph pH-values around 6.7-6.8 low in comparison to the normal haemolymph pH range around 7.0-7.5. The low pH-values could be due to the fact that after collecting the larvae from the food, they were not rinsed with $H_2O$ before harvesting of the haemolymph. Therefore, there was still food left on the larvae body and as the food contained e.g. propionic acid, it could possibly affect the results. However, as all the samples were treated in the same way, we believe that this did not effect on the overall results of the measurement.

In addition, we observed the Foxo regulatory pathway further. The luciferase reporter gene analysis results indicate that Foxo has a role in regulation of *Paics*, *Sesn*, *salr* (transcript Salr-RC), *Gdh* (transcripts Gdh-RA and Gdh-RF), *Prat2* transcript Prat2-RA, and in particularly, *Uro* and *Prat2* transcript Prat2-RB. Interestingly both *Uro* and *Prat2* play a role in the purine metabolic pathway involved in regulation of ammonia levels (Lang et. al 2019; Ji and Clark 2006). *Prat2* encodes phosphoribosylamidotransferase, an enzyme important for purine synthesis (Ji and Clark 2006). It has two transcripts, Prat-RA and Prat-RB, of which the Prat2-RB is likely the more common-one (Ji and Clark 2006). Purine degradation produces uric acid, and in *Drosophila*, urate is further converted into allantoin through a reaction catalyzed by urate oxidase (Uro) (Lang et al. 2019). In 2019, Lang et al. found that overexpression of Foxo reduces uric acid levels, suggesting that Foxo is involved in uric acid processing. Our result, that Foxo upregulates *Uro* expression, is in line with this finding, and is possibly one way of the Foxo-dependent regulation in reducing urate levels in *Drosophila*. Thus, according to our results, we could hypothesize that Foxo regulates ammonia-balance through activation of purine-urate metabolism via *Prat2* and *Uro*.
However, the raw data of the luciferase reporter analysis indicated Foxo having a potentially toxic effect on the cells, which could influence the results. Therefore, next we could try to titrate an adequate amount of Foxo per well, which would have a more stable Renilla signal to the wells containing empty plasmid in cell culture. Once the proper concentration was found, we could repeat the luciferase analysis to verify the results.

## Conclusions and future prospects

Here, we have used *Drosophila melanogaster* as a model organism to analyze regulator candidates in glutamine sensing. We have confirmed the glutamine intolerant phenotype of Foxo and Sxc deficiency and shown it to be independent on altered food intake levels of larvae. Furthermore, the Salm and Salr RNAi lines did not show a glutamine intolerant phenotype with Tub-GAL4 or CG-GAL4 expression lines.

We considered the roles of Foxo and Sxc in glutamine metabolism further. With measurement based merely on pH-level differences, we could not associate Foxo or Sxc deficiency with regulation of glutamine-derived ammonia clearance. Furthermore, we explored the downstream Foxo regulatory pathway, revealing that Foxo has a role in regulation of *Paics*, *Uro*, *Sesn*, *salr*,

*Prat2* and *Gdh*. Next of interest would be to see whether the Foxo-mediated regulation of the above-mentioned genes is affected by altered glutamine levels in cell culture. Thus, we could gain further insight to the Foxo-mediated regulation in the glutamine metabolism pathway.

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