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Relationship between Vitamin B6, DNA damage and diabetes

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GLOSSARY

AGEs: Advanced Glycation End-products, a heterogeneous group of compounds produced when reducing sugars, such as glucose, react in a non-enzymatic way with amino groups of proteins, lipids and nucleic acids through a series of reactions, forming bases of Schiff and Amadori rearrangements.

ALA: alpha-lipoic acid, an antioxidant compound that antagonizes the formation of AGEs.

CABs: chromosome aberrations. Rearrangements including chromatid and isochromatid deletions, chromosome and chromatid exchanges

CSK: tyrosine-protein kinase, a negative regulator of Src-family kinases (SFKs).

DM: diabetes mellitus, a metabolic disease characterized by defects in insulin action and/or secretion.

DSBs: double-strand breaks, breakage affecting the two strands of the DNA double helix

FLP: flippase, a site-specific recombinase which mediate mitotic recombination at the FRT sites

FRT: flipase recognition target sites

GDM: gestational diabetes mellitus, pregnancy complication characterized by insulin resistance

γ-H2Av: phosphorylated form of the *Drosophila* H2A histone variant involved in DDR process.

IR: insulin resistance, reduced response of tissues to insulin signalling

LD: lipid droplets, organelles that regulate the storage and hydrolysis of neutral lipids

MARCM: mosaic analysis with a repressible cell marker, a system to generate mitotic labelled clones.

PDXK or PLK: pyridoxal kinase, enzyme involved in vitamin B6 activation.

PL: pyridoxal

PM: pyridoxamine

PN: pyridoxine

PLP: pyridoxal 5'-phosphate, the active form of vitamin B6

PNPO: pyridoxine/pyridoxamine 5'-phosphate oxidase, enzyme which catalyzes the oxidation of PNP and PMP into PLP.

ROS: Reactive oxygen species

sgll*: sugarlethal* the *Drosophila* ortholog of *PNPO* gene

T1D: type 1 diabetes mellitus, hyperglicemia caused by impaired insulin secretion

T2D: type 2 diabetes mellitus, hyperglicemia caused by impaired insulin action.

4-DP: 4-deoxypyridoxine, a vitamin B6 inhibitor

SUMMARY

The active form of vitamin B6, the pyridoxal-5' phosphate (PLP) is a cofactor for more than 150 reactions involved in protein, carbohydrate and lipid metabolism. In addition, it is able to counteract Reactive Oxygen Species (ROS) and Advanced Glycation End products (AGEs). In eucaryotes PLP is produced, in the salvage pathway, by the concerted action of pyridoxal kinase (PDXK) and pyridoxamine/pyridoxine oxidase (PNPO) which recycle PLP precursor from food. PLP has been associated to different pathologies including diabetes and cancer although underlying mechanisms remain in large part still unknown. It has been previously demonstrated that mutations in *Drosophila Pdxk* gene (*dPdxk*^{1}) cause diabetes and chromosome aberrations (CABs) and also that these phenotypes are linked by a cause-effect relationship. The first aim of this thesis has been to verify whether also the inactivation of the other gene of the salvage pathway, *PNPO*, encoded by *sgll* gene, produced the same phenotypes observed in *dPdxk*^{1} mutants. To this purpose we silenced *sgll* gene by RNA interference and characterized the resulting phenotypes. This analysis revealed a significant frequency of CABs and diabetic phenotypes such as hyperglycemia, small body size, impaired lipid storage and accumulation of AGEs associated to Sgll depletion. These results allowed us to confirm the hypothesis that PLP deficiency produces CABs through the genotoxic effect of AGEs in turn triggered by high glucose.

Our second aim has been to investigate whether human PDXK variants present in the population can impact on DNA integrity and can be considered predictive of cancer risk. For this purpose, we expressed four human PDXK variants (carrying missense mutations) into *dPdxk*^{1} mutant flies and tested them for CABs as well as for diabetic phenotypes, finding that none of them was

able to completely rescue the CAB phenotype, hyperglycemia nor AGE accumulation. Biochemical analysis of these variants revealed a compromised catalytic activity and/or a reduced affinity for their substrates, which explained their "loss of function" behaviour. These results suggested that mutations in PDXK human gene can impact on genome integrity *via* AGEs and predispose to cancer.

Our third purpose was to test whether low PLP levels can impact on cancer in *Drosophila*. Thus we tested the effects of the PLP inhibitor 4-deoxypyridoxine (4-DP) on Ras and Ras/Scr cancer models generated by mosaic analysis with repressible marker (MARCM) strategy. This analysis showed that 4-DP caused enlargement of primary tumors as well as appearance of secondary tumors in both cancer models. Taken together all the results collected in this work have contributed to confirm and elucidate the relationship between vitamin B6 diabetes and cancer.

1. INTRODUCTION

1.1 The Pyridoxal 5'-phosphate (PLP)

1.1.1 Discovery of Vitamin B6, food sources and diet recommendations.

Pyridoxal 5'-phosphate (PLP) is the active form of vitamin B6. Its formula was first published by Ohdake in 1932 which found this vitamin as a by-product in experiments aimed at isolating vitamin B1 from rice-polishings. Later, György described the vitamin B6 as the active "rat pellagra prevention factor" in the yeast eluate (György, 1934). In 1938 the crystalline vitamin B6 was isolated from yeast and, after determination of its structure, György named the vitamin pyridoxine due to its structural homology to pyridine (Fig.1) (György et al., 1939). In the same year, Stanton, Harris and Folkers, performed the synthesis of vitamin B6 (Harris et al., 1939).

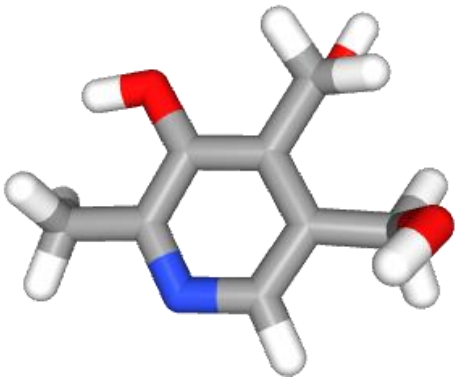


Figure 1. 3D structure of pyridoxal 5'-phosphate (PLP)

Humans are not able to synthesize vitamin B6 but recycle it from B6 precursors as pyridoxal (PL), pyridoxamine (PM), and pyridoxine (PN) contained in food. Plant-based foods are the most

important vitamin source, because they easily meet all the daily nutrient, due to their ability to produce vitamins themselves. The richest sources of vitamin B6 include cereals, fish, beef liver and other meats, vegetables like potatoes, legumes, bananas, nuts, avocados, and other fruits, egg and yolks. Bioavailability of vitamin B6 is estimated to be 75% from a varied diet (Stover and Field, 2015).
The National Institute of Health (NIH) of United States of America has established that the Recommended Dietary Allowance (RDA) goes from a minimum of 2 mg to a maximum of 100 mg per day for adults to obtain a PLP concentration of at least 20 nmol/L (Hellmann and Mooney, 2010).

1.1.2 Vitamin B6 structure and biosynthesis

Vitamin B6 refers to a group of six chemically related compounds, all containing a pyridine ring in the centre: pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL) and the respective 5'-phosphorylated forms (PNP, PMP, PLP). These compounds differ in the chemical group in position 4': PM has an amino methyl group, PL an aldehyde and PN a hydroxyl methyl group. Thanks to the presence of a very reactive aldehydic group, PLP is able to react with all nucleophiles in the cell, with primary or secondary amines forming the aldimmine (Schiff's base) and with the amino group of lysine residues. The aldehydic group can also react with cysteine to form a thiazole ring (Safo et al., 2004, Parra et al., 2018).

Vitamin B6 biosynthesis occurs through two essential ways: de novo biosynthesis and salvage pathway (Mooney et al., 2009). The first way includes two different pathways: the deoxyxylose 5'-phosphate (DXP)-dependent pathway and the DXP-

independent pathway. The first is limited to a small number of bacteria and has been extensively studied in *Escherichia coli* (Hill et al., 1996). The second route is wide-spread among archaea, plants, fungi and most bacteria.
In the DXP-dependent pathway vitamin B6 (Fig. 2) is synthesized from the condensation of deoxyxylulose 5-phosphate and 4-phosphohydroxy-L-threonine catalysed by the concerted action of PdxA and PdxJ enzymes. The DXP-independent pathway relies instead on the activity of the PDX1 and PDX2 proteins which produce PLP from a pentose (ribose 5-phosphate or ribulose 5-phosphate) and a triose (glyceraldehyde 3-phosphate or dihydroxyacetone phosphate) in the presence of glutamine (Fitzpatrick et al., 2007).
In addition to the de novo pathway, bacteria can synthesize PLP in the salvage pathway where PL, PN, and PM are phosphorylated by PdxK or PdxY kinases, and the PMP and PNP are oxidized to PLP by PdxH activity (Fig. 2) (Mukherjee et al 2011).

In humans Vitamin B6 is produced only by salvage pathway where PL, PN and PM are first phosphorylated by a single ATP-dependent PDXK (or PLK) kinase, and then oxidated to PLP by a FMN-dependent pyridoxine pyridoxamine oxidase (PNPO) which are the orthologs of *PdxK* and *PdxH* bacteria genes respectively (Di Salvo et al., 2003).

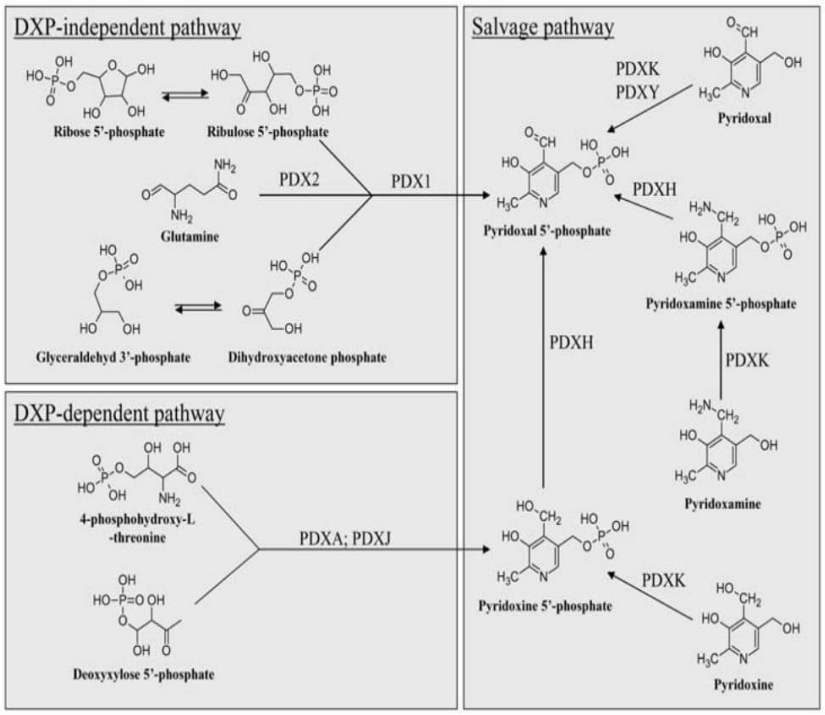


Figure 2. Vitamin B6 synthesis: de novo and salvage pathways. PDXK: pyridoxal kinase; PDXH: pyridoxal oxidase (Mooney et al., 2009)

Vitamin B6 catabolism is also an important aspect for cellular homeostasis of the compound. The first step is the dephosphorylation of PLP/PMP/PNP and it represents a major control for the pool of available active vitamin B6 cofactor, however it is basically unknown how the vitamin is degraded in eukaryotes after pyridoxal phosphatase activity (Jang et al., 2003).

In mammals, once ingested phosphorylated B6 vitamers are hydrolysed to PL, PM and PN by intestinal phosphatase. The absorbed vitamers though blood circulation reach the liver, where

they are phosphorylated by PDXK, with PNP and PMP further oxidized to PLP by PNPO. Then PLP re-enters the circulation bound to albumin. Delivery of active cofactor to the tissues, however, requires hydrolysis of circulating PLP to PL by tissue nonspecific alkaline phosphatases. Once entered the cells, PL is re-phosphorylated by PDXK and then targeted to apo-B6 enzymes (Bohney et al 1992; Clayton 2006). PLP is a very reactive aldehyde that easily combines with amino and thiol groups. Thus at one hand it is important to keep intracellular-free PLP concentration below toxic levels, and on the other hand to maintain enough PLP levels to saturate all PLP-dependent enzymes. How this process is accomplished in cells is so far largely unknown. However, recent evidence suggests that PLP-binding protein (PLP-BP), may have an important regulatory function in PLP homeostasis (Darin et al. 2016).

1.1.3 Vitamin B6 functions

Vitamin B6 performs co-factor role for many pyridoxal phosphate (PLP) dependent enzymes and is involved in more than 150 biochemical reactions including transaminations, decarboxylations, racemizations and deaminations of amino acids (Mooney et al., 2009; Di Salvo et al., 2011).
Vitamin B6 also contributes to biosynthesis of fatty acids, to degradation of stored carbohydrates, such as glycogen, to biosynthesis of plant hormones and neurotransmitters such as epinephrine, dopamine, serotonin and the conversion of glutamate into gamma-aminobutyric acid (GABA) (Fig. 3) (Parra et al., 2018).
PLP is also involved in the folding of PLP enzymes (Cellini et al., 2006; Cellini et al., 2014) and it is important for the biosynthesis of tetrapyrroles such as heme, cobalamin and chlorophyl (Parra et al., 2018). PLP can also modify the expression and action of steroid hormone receptors (Tully et al., 1994) and may have an effect on the immune system (Salhany et al., 1993). Moreover,

vitamin B6 is able to counteract the Advanced Glycation end products (AGEs) which are genotoxic compounds linked to senescence and associated to diabetes complications (Booth et al., 1997).
Vitamin B6 also is able to quench reactive oxygen species (ROS) (Havaux et al, 2009). The antioxidant properties rely on the ability of vitamin B6 vitamers to directly react with the peroxyl radicals with both their hydroxyl (–OH) and amine (–NH2) substituents on the pyridine ring.

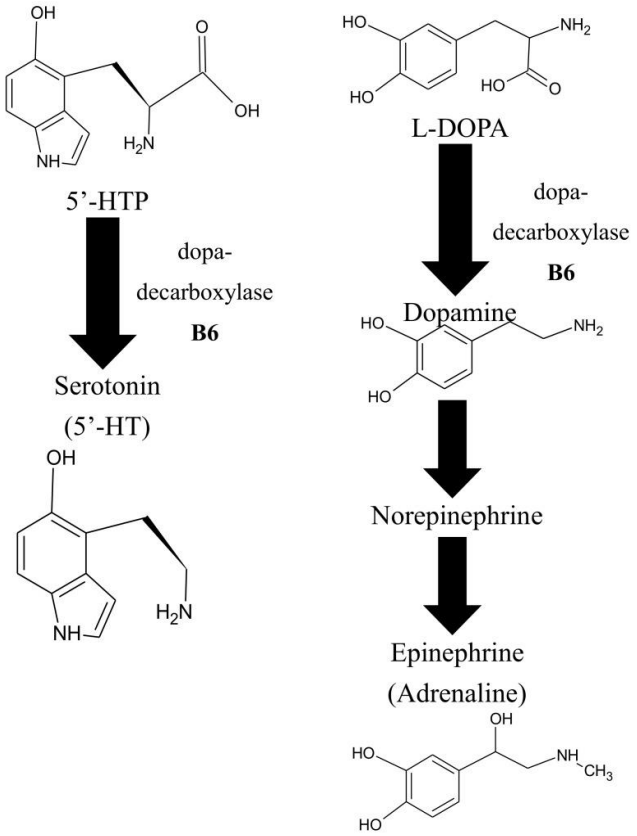


Figure 3. PLP-dependent enzymes involved in neurotransmitter biosynthesis. B6: vitB_6; L-DOPA: L-3'-4'-dihydroxyphenylalanine; 5'-http: 5'-hydroxytryptophan; 5'-HT: 5'-hydroxytryptamine (Parra et al., 2018).

1.1.4 Pyridoxal kinase (PDXK o PLK)

Humans pyridoxal kinase is encoded by *PDXK* gene (also named *PLK*) located on chromosome 21q22.3. Pyridoxal kinase catalyzes the phosphorylation of PN, PM and PL, in the presence of MgATP, leading to the formation of PNP, PMP and PLP. The mechanism of this reaction has been elucidated for the sheep and *E. coli* enzymes, showing that it occurs by a random sequential substrate addition. However it has not clarified yet whether this mechanism occurs also in humans (Di Salvo et al 2011).
The crystal structure of human PDXK has been elucidated showing that the enzyme is an homodimer with an active site that opens as a groove where ATP binds and, stretches deepen into the protein, where vitamin B6 vitamer binds (Fig.4A) (Li et al., 2002; Li et al., 2004; Cao et al., 2006). PDXK is a member of the ribokinase superfamily, thus it shares some features with the other members of the family. In particular it displays the same typical tertiary structure of the central nucleus, composed of β sheets surrounded by α helices, and a well-preserved geometry of the binding sites for ATP and substrates (Di Salvo et al., 2011).
In addition it presents a lid that covers the active site. This structure has been hypothesized to enable the binding of the substrate and for the subsequent catalysis (Cheng et al., 2002). In all the pyridoxal kinases, the lid has become a loop-like structure or a β-loop-β-filament structure, commonly referred to as a flap. In addition PDXK shows another loop structure in the active site, the Thr-Gly dipeptide, which is important to sequester the substrate for the catalysis and also for substrate specificity.
It has been shown that a conserved aspartate residue (Asp235) in the active site plays a crucial role in the PDXK catalytic activity. This residue makes a hydrogen-bond interaction with the C5'-OH group of the substrates, and it has been hypothesized that it deprotonates this group with the consequence that the negatively charged O5' atom makes a direct nucleophilic attack on the ATP γ-phosphate (Gandhi et al 2009).

The driving force to bind the ATP and enable the catalysis, for many kinases, is provided by bivalent and monovalent cations (Di Salvo et al., 2004). The interaction between amino acid residues and metal cations helps to neutralize the negative charge of the phosphate groups and stabilize the transition state during the transfer of the phosphate group from ATP to the substrate. It has been proposed that Zn2+ and K+ are the metals needed for the activity of both human and sheep PDXK in physiological conditions (Li et al. 2002). However, a more recent study on the human enzyme showed that Zn2+ stimulates the enzymatic activity under non-physiological substrate concentrations and/or at pH 6, (McCormick et al. 1961; White and Dempsey 1970). In contrast, under physiological conditions at pH 7.3, Mg2+ is the required divalent metal ion, whereas Zn2+ inhibits the reaction (Di Salvo et al. 2004).

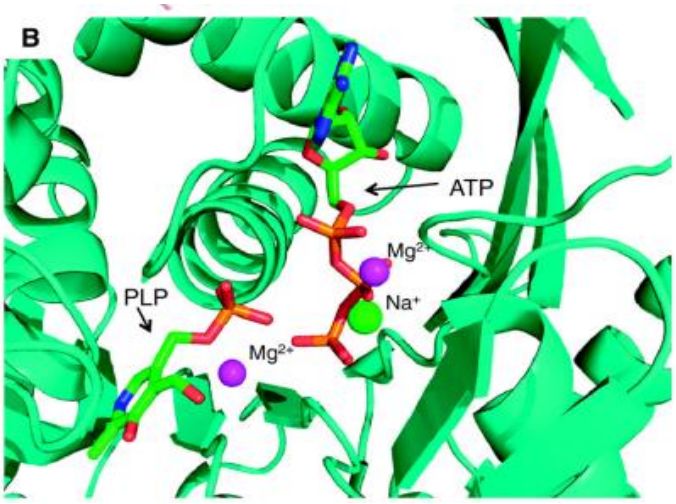


Figure 4. Close-up view of the active site of human PDXK in the form of a non-productive complex with MgATP, PLP and Na + (green sphere) (Di Salvo et al., 2010).

Altered levels of PDXK have been associated to cancer (see below) and to impaired adipogenesis (Moreno-Navarrete, 2016). In addition *PDXK* mutations have been recently associated to

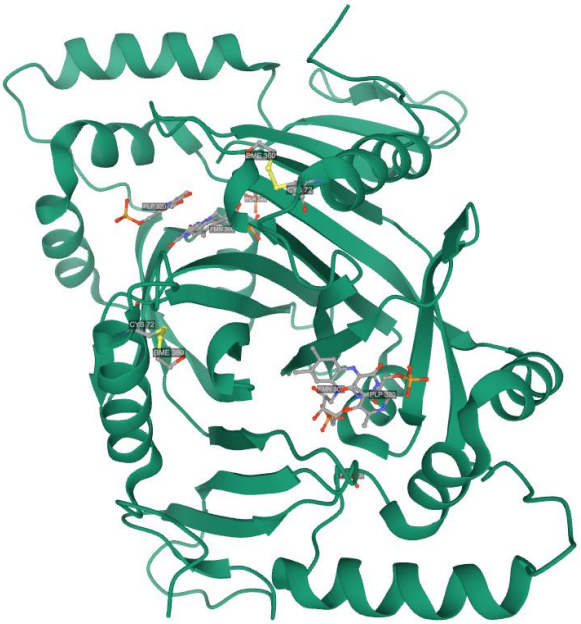
polyneuropathy (Chelban et al., 2019). In *Drosophila* mutations in the corresponding *dPdxk* gene cause chromosome aberrations and diabetes (Marzio et al., 2014). It has been also proposed that *PDXK* could be associated to Parkinson disease (Elstner et al., 2009) although this association is still controverse (Guella et al., 2010). However, it has been shown that the inhibition of this gene activity by the directed expression of an RNAi transgene in the Ddc-Gal4-expressing neurons phenocopies PD-like symptoms in *Drosophila*, and therefore may represent a novel animal model of PD (Githure M'Angale and Staveley 2017).

1.1.5 Pyridoxine/pyridoxamine 5'-phosphate oxidase (PNPO*)*

In humans pyridoxine/pyridoxamine 5'-phosphate oxidase, is encoded by *PNPO* gene located on chromosome 17q21.2. PNPO is a dimeric protein and each monomer uses an FMN molecule as a cofactor (Di Salvo et al., 1998). In the salvage pathway PNPO catalyzes the the FMN-dependent oxidation of the 4′-hydroxyl group of PNP or the 4′-amino group of PMP into the aldehyde group of PLP (Zhao and Winkler 1996).
PNPO has been purified and characterized from various sources, including humans. This enzyme has been highly conserved in the evolution and represents the smallest member of the flavin-containing oxidase family (Safo et al., 2001).
The analysis of crystal structure showed that the human PNPO enzyme is a dimer, with two FMN binding sites (Musayev et al., 2003) (fig 5). Each monomer shows the typical two-domain architecture (domain 1 and domain 2); the two catalytic sites are located at the interface between the two subunits of the dimer and each binds the cofactor FMN and the substrate PNP or PMP. FMN is located in a deep groove at the interface between the two subunits of the enzyme and contacts, by hydrogen bonds, conserved residues of both subunits (Safo et al. 2000). The substrate, PNP or PMP, binds opposite to FMN, with the

phosphate group pointing towards the outside of the catalytic cavity, unlike the phosphate group of the FMN pointing towards the bottom of the cavity. Several functional and structural studies have also shown that PNPO contains on each subunit a non-catalytic site that closely binds a second PLP molecule. This binding site, in addition to be involved in the delivery of PLP to enzymes can also play a role in the regulation of PLP (Yang and Schirch 2000; M.K. Safo et al. 2001; Musayev et al. 2003).

A



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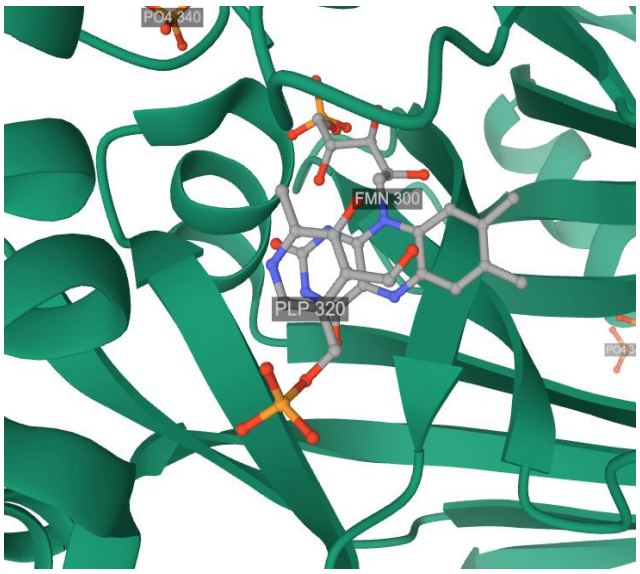


Figure 5. Crystallographic structure of PNPO. (A) ribbon diagram of the dimeric structure of the Human Pyridoxine-5'-Phosphate Oxidase. (B) Close-up view of the active site of human Pyridoxine-5'-Phosphate Oxidase in the form of a non-productive complex with FMN and PLP (Musayev et al., 2003)

Mutations in the *PNPO* gene have been associated with a severe form of neonatal encephalopathy that responds to PLP but not to PN (Mills et al., 2005). Consistently urine and cerebrospinal fluid of patients affected by encephalopathy revealed a reduced concentration of PLP-dependent enzymes involved in the metabolism of the neurotransmitters (Mills et al., 2005). Later, gene sequencing in some patients combined to Genome wide association (GWAS) studies showed that different mutations in the gene can produce a large spectrum of phenotypes including cases able to respond to PN but not to PLP (Steffen et al., 2012; Mills et al., 2014; Di Salvo et al., 2017) The mechanisms through which the *PNPO* mutations cause epilepsy have not been fully elucidated, but it is conceivable that they are linked to the role of PLP as cofactor of enzymes implicated in the synthesis of some

neurotransmitters. To date only few studies have been carried out in animal models. In *Drosophila* a missense mutation in this gene result in a lethal phenotype when flies are grown on a medium containing only 4% sucrose (hence the name of the *sugar lethal* gene, *sgll*) Lethality was recovered in a dose dependent manner by PLP and PN. However when the expression of the *PNPO* gene was drastically reduced through RNA interference, PLP alone and no longer PN was able to recover the phenotype (Chi et al., 2014). Recently it has been shown that *sgll* mutations cause epilepsy also in flies (Chi et al 2019).

1.1.6 Vitamin B6 associated pathologies

In developed countries the risk of developing vitamin B6 deficiency is rather unlikely as this vitamin in contained in a wide variety of foods (Contestabile et al 2020). However a decreased or excessive intake of this nutrient may cause many detrimental effects. Studies carried out in experimental animals and humans showed that an excessive PLP intake can produce signs of toxicity mostly affecting the peripheral nervous system (Di Salvo et al., 2012). PLP deficiency has been associated to several diseases such kidney diseases and malabsorption syndromes including celiac disease and inflammatory bowel diseases (Kowlessar et al., 1964; Merrill and Henderson, 1987). In addition, PLP concentrations tend to be low in people with alcohol dependence (Cravo and Camilo, 2000), pregnant women and obese individuals (Ferro et al., 2017; Merrill et Henderson, 1987). Certain genetic diseases, such as homocystinuria, can also cause vitamin B6 deficiency (Clayton, 2006). Moreover, some drugs such as isoniazid, cycloserine and penicillamide, may reduce PLP availability (Mizuno et al., 1980; Lainé-Cessac, 1997).
Importantly, reduced PLP availability can also be determined by mutations in genes encoding enzymes involved in vitamin B6 metabolism, which cause severe neurological disorders

(Contestabile et al 2020). Vitamin B6 deficiency can also contribute to the onset or progression of serious diseases such autism, schizophrenia, Alzheimer, Parkinson, epilepsy, Down's syndrome, cancer and diabetes. The impact of vitamin B6 on the two last diseases will be discussed in the following paragraphs.

1.2 Involvement of vitamin B6 in diabetes and cancer

Growing evidence indicates that PLP plays a protective role against diabetes and cancer. However molecular and cellular mechanisms underlying the beneficial effect of vitamin B6 in these two pathologies are still debated. Therefore, gaining a robust basic knowledge of how PLP influences diabetes and cancer is the first needed step to ideate personalized therapeutic/preventive treatments.

1.2.1 Relationship between PLP and diabetes

1.2.2 Diabetes mellitus

DM is a group of metabolic disorders characterized by hyperglycemia due to defects in insulin secretion and/or action. People with diabetes have an increased risk of developing a number of serious health problems. Consistently, high blood glucose levels can lead to serious diseases affecting heart and blood vessels, eyes, kidneys, nerves and teeth. Type 1 (T1D), and type 2 (T2D) represent the most common forms of diabetes. T1D is a multifactorial disease which accounts for 5–10% of diabetes; it is caused by autoantibody-mediated destruction of pancreatic beta cells which impairs insulin secretion (Katsarou et al., 2017). T2D accounts for 90–95% of all diabetes and is typical of mature age, though nowadays it affects a growing number of young people. T2D is characterized by a reduced response of tissues to insulin (insulin resistance, IR) (Olokoba et al., 2012) and result from an interaction between environmental factors and a strong

hereditary component. Genetic studies using linkage analysis, candidate gene approaches and Genome-wide association studies (GWAS) have led to the discovery of a large number of T2D associated variants. However these mutations can explain only a small proportion (~10%) of the heritability of T2D (Zhang et al 2020). More common risk factors associated to T2D are obesity, hypertension, high concentration of HDL and triglycerides and reduced physical activity.

Gestational diabetes mellitus (GDM) is a common complication which affects about 7% of pregnancies and disappears after childbirth, although GDM women remain at risk of developing T2D after pregnancy (Plows et al., 2018). The causes of GDM are not so far completely understood. It has been hypothesized that placental hormones combined to other factors may sometimes interfere with the action of insulin, causing IR. Fetal exposure to maternal hyperglycemia leads to fetal hyperglycemia providing excess nutrition that in turn accelerates fetal growth leading to macrosomia and neonatal disturbance in glucose metabolism (Plows et al., 2018).

1.2.3 Vitamin B6 and diabetes

Several studies carried out on both humans and animal models associate vitamin B6 to diabetes. Population studies revealed reduced plasmatic PLP levels in T1D and T2D patients (Satyanarayana et al., 2011; Ahn et al., 2011; Nix et a., 2015). In addition vitamin B6 levels appear to be inversely related to the progression of diabetes (Ellis et al., 1991; Nix et al., 2015). Low PLP levels have also been associated with GDM. By examining 14 GDM patients, Bennink and Schreurs (1975) found that 13 out of 14 women displayed reduced PLP levels successfully restored by pyridoxine administration. Similar results have been obtained by Spellacy and coworkers (1977), which reported the beneficial effect of pyridoxine therapy in GDM women.

It has not clearly established whether PLP deficiency represents a cause or an effect of diabetes. Some studies reported that low PLP levels can contribute to the development of diabetes while others showed that PLP levels are decreased by diabetes itself. However, both mechanisms seem to be plausibly true, allowing to hypothesize the existence of a vicious circle that correlates vitamin B6 and diabetes (Mascolo and Vernì, 2020).

Mechanisms underlying the link between vitamin B6 and diabetes

By considering that PLP works as a coenzyme in a plethora of metabolic reactions and also that possesses antioxidant properties, it seems plausible that reduced vitamin B6 levels can impact on different diabetic contexts with different mechanisms. However, two main routes emerged by searching for molecular and cellular mechanisms able to correlate diabetes and vitamin B6: the tryptophan pathway and the lipid metabolism. It has been indeed shown that tryptophan metabolism is often impaired in diabetes (Bennink and Schreurs, 1975; Connick et al., 1985) and also that in obese people, altered adipogenesis and lipotossicity can promote insulin resistance (Cnop, 2008; Longo et al., 2019). Tryptophan (TRP) is an essential amino acid, needed for serotonin, N-acetylserotonin, and melatonin biosynthesis. About 95% of TRP is metabolized through the kynurenine (KYN) pathway (Fig 6) in which are involved enzymes which depend on PLP for their activity: the aminotransferases (KAT) and the kynureninase (KYNU). As KYNU is more sensitive to deficiency of PLP with respect to KAT (Van de Kamp and Smolen, 1995), decreased PLP availability results in the accumulation of kynurenic acid (KYNA) and xanthurenic acid (XA) (Bender et al., 1990; Rios-Avila et al., 2013; Yess et al., 1964; Takeuchi et al., 1989).

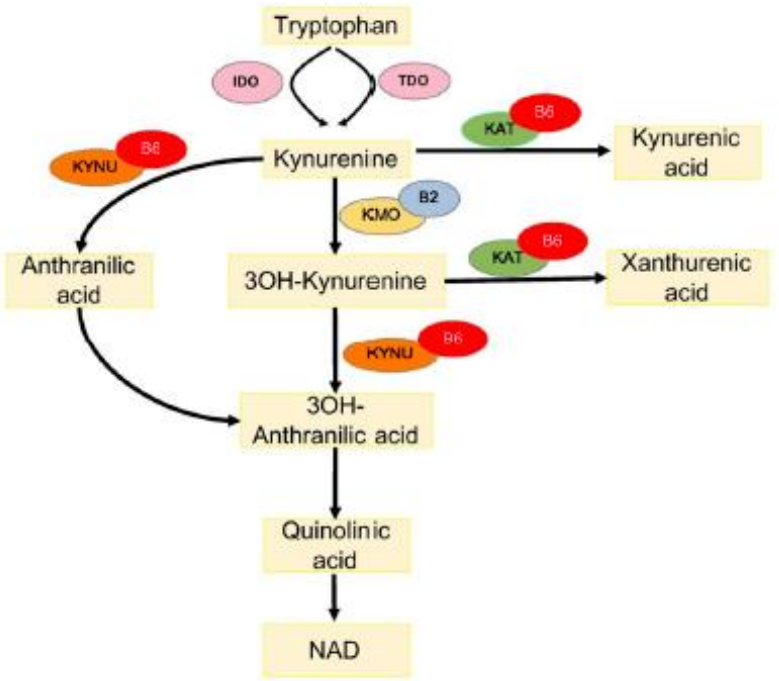


Figure 6. Tryptophan metabolism via the kynurenine pathway. IDO, indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; KAT, kynurenine aminotransferase; KMO, kynurenine 3-monooxygenase; KYNU, kynureninase; 3OH-kynurenine, 3-hydroxy kynurenine; 3OH-anthranilic acid, 3-hydroxyanthranilic acid; B6, vitamin B6 (pyridoxal 50-phosphate); and B2, vitamin B2 (flavin adenine dinucleotide) (Mascolo and Vernì 2020)

It has been proposed that these compounds can interfere in different ways with insulin biological activity (Bennink and Schreurs, 1975; Spellacy et al., 1977; Oxenkrug et al., 2013). TRP metabolites may be responsible for the (1) formation of chelate complexes with insulin (XA–In), which have reduced activity compared to pure insulin (Kotake et al., 1975); (2) formation of Zn++ ion–insulin complexes in cells that cause toxic effects on pancreatic beta cells (Ikeda and Kotake, 1986; Meyramov et al., 1998); (3) inhibition of insulin release from

pancreas (Rogers and Evangelista, 1985), and (4) induction of pathological apoptosis in pancreatic beta cells (Malina et al., 2001).

The other route through which reduced PLP levels can contribute to diabetes and in particular to IR is by impairing lipid metabolism. Evidence indicates that PLP is a regulator of the genes involved in adipogenesis and systemic insulin sensitivity (Moreno-Navarrete, 2016). It has been shown that a vitamin B6-deficient diet results in a significant reduction in adipose tissue and lipogenesis in rat models (Huber et al., 1964; Radhakrishnamurty et al., 1968). In addition, it has been shown that vitamin B6 administration increased intracellular lipid accumulation in 3T3-L1 adipocytes (Yanaka et al., 2011) and decreased macrophage infiltration and adipose tissue inflammation in mice (Sanada et al., 2013; Sanada et al., 2014) Furthermore, it has been shown that obese people have low circulating levels of vitamin B6 (Aasheim et al., 2008).
The exact mechanisms through which PLP influences lipid metabolism leading to diabetes are so far not completely clarified. It has been proposed that PLP might activate peroxisome proliferator-activated receptor- (PPAR), one of the master nuclear receptor involved in the expression of adipogenesis genes (Yanaka et al., 2011). Alternatively, PLP might conjugate with RIP140, a nuclear transcription factor, by enhancing its co-repressive activity and its physiological function in adipocyte differentiation (Huq et al., 2007; Bird, 2018). Moreover, based on the finding that an altered DNA methylation is associated with adipose tissue dysfunction in T2D patients (Nilsson et al., 2014), given that PLP is a coenzyme for serine hydrossymethiltranferase (SHMT), vitamin B6 might contribute to maintain the correct methylation pattern.

1.2.4 PLP, diabetes complications and AGEs

T1D as well as T2D produces long term complications affecting different organs including heart, kidney and eye (Brownlee, 2005). Growing evidence showed that these diseases are associated with the accumulation of genotoxic compounds named Advanced Glycation End products (AGEs) induced by hyperglycemia. It has been shown that PLP is able to counteract AGEs (Booth et al., 1997) and, interestingly, several studies demonstrated that vitamin B6 supplementation decreased the clinical signs of both retinopathy and neuropathy in diabetic individuals; this evidence suggested the hypothesis that PLP may prevent diabetes complications by blocking AGE formation (Mascolo and Vernì 2020). AGEs were first discovered in the early 1900s as yellowish brown colour products originated from a reaction in which amino acids were heated with reducing sugars (the Maillard reaction). Further studies indicated that reducing sugars, i.e., glucose, react non-enzymatically with amino groups of proteins, lipids and nucleic acids to form a Schiff base, an early glycation product, which is converted into more stable Amadori products which progress to covalent adducts and accumulate on proteins. The Maillard reaction generates highly reactive dicarbonyl compounds which include methylglyoxal, glyoxal and 3-deoxyglucosone (Fig 7). These molecules can also be generated from other pathways such as glucose autoxidation, lipid peroxidation, and the polyol pathway (Sergi et al., 2020). AGEs and their dicarbonyl precursors are toxic compounds that compromise the function of the extracellular matrix components by stimulating the production of cytokines and reactive oxygen species through AGE-specific receptors (RAGE). They also modify intracellular proteins by altering their enzymatic capacity, by reducing their degradation capacity and interfering with the ability to recognize receptors (Singh et al., 2001). In addition, AGEs are also considered genotoxic because they attack DNA through oxygen free radicals generated during the various phases of the glycation process (Krymkiewicz, 1973; Stopper et al.,

2003). It has been proposed that vitamin B6 counteracts AGE formation by trapping the 3-DG, one of the AGE pathway metabolites (Nakamura et al., 2007; Nakamura and Niwa, 2005). In vitro experiments showed that incubation with PLP considerably decreased concentration of 3-DG in a dose-dependent way, whereas PL and PM resulted less reactive. Other studies demonstrated that PM can form stable complexes with metal ions, thus catalysing the oxidative reactions associated with the advanced stages of protein glycation cascade (Adrover et al., 2008). Moreover, a density-functional theory (DFT) study indicated that PM might react with reactive carbonyl compounds, generated as byproducts of protein glycation, thereby, counteracting further protein damage (Ortega-Castro et al., 2010). More recently, Ramis and collaborators (2019) emphasized also the importance of PM antioxidant role in neutralizing AGE formation.

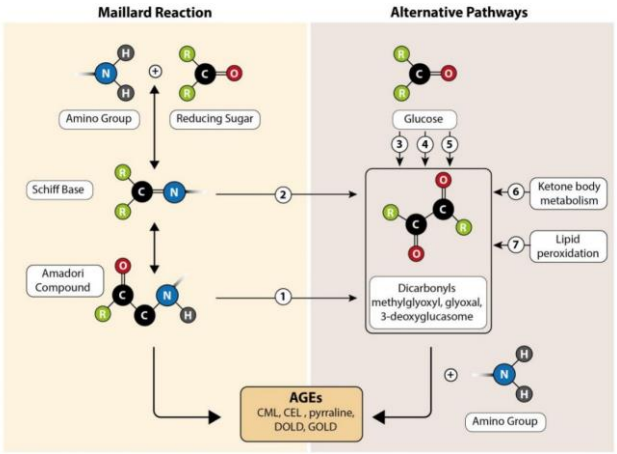


Figure 7. Formation of advanced glycation end products (AGEs). Left panel: Maillard reaction. Right panel: Alternative pathways, Hodge pathway: fructosamine, non-oxidative Amadori product cleavage (1); Namiki pathway: cleavage of dicarbonyl compounds from aldimines (2); Wolff pathway: metal catalyzed glucose autoxidation (3); glycolytic pathway intermediates, for example, glyceraldehyde 3 phosphate (4); polyol (sorbitol

aldose reductase) pathway (5); amino acid derived ketone body metabolism (6); lipid peroxidation (7). These pathways lead to formation of reactive dicarbonyls, which if not detoxified form AGEs, (e.g., carboxyethyl lysine [CEL], carboxymethyl lysine [CML], glyoxal lysine dimer [GOLD], 3-deoxyglucosone lysine dimer [DOLD], and pyrroline) **(Sergi et al., 2020).**

1.2.5 Relationship between PLP and cancer

Vitamin B6 is at one hand an antioxidant molecule, and, on the other hand a cofactor for enzymes involved in cell proliferation. Thus, it is not surprising that this vitamin has been associated to cancer (Fig.8). However how exactly vitamin B6 influences cancer is still debated (Galluzzi et al., 2013). Until the early 1980s, reducing vitamin B6 availability was considered a promising therapeutic approach against cancer (Galluzzi et al., 2013). However, later, an increasing number of observations highlighted a strong inverse association between vitamin B6 dietary intake and cancers in particular affecting the gastrointestinal tract (Kayashima et al., 2011; Gylling et al., 2017) and lungs (Zuo et al., 2019).

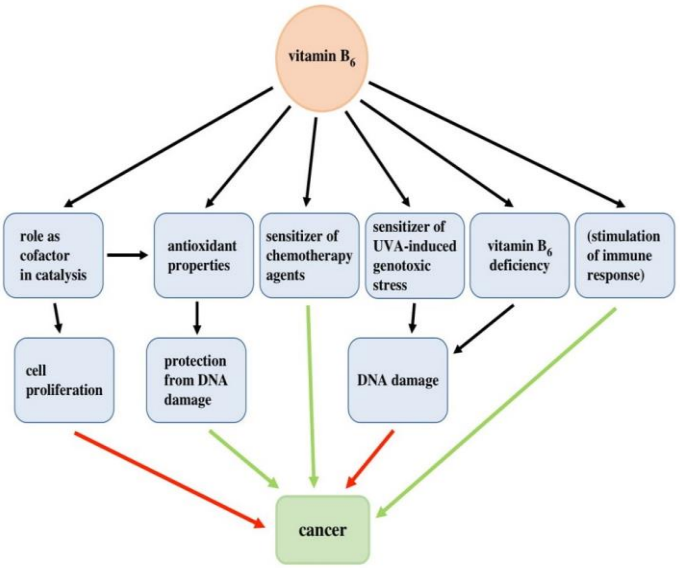


Figure 8. Relationships between vitB6 and cancer. In the scheme, green arrows represent a protective effect against cancer, whereas red arrows indicate a promoting cancer effect (Contestabile et al., 2020).

Interestingly, a marked association has been found between the expression of genes involved in the synthesis of PLP and cancer. The *PNPO* gene has been found overexpressed in colorectal cancer and represents is one out of seven genes, selected among 6487, whose altered expression levels have a prognostic value in this cancer (Chen et al., 2017). *PNPO* is also overexpressed in ovarian cancer, while in contrasts *PNPO* knockdown stimulates cell apoptosis and reduced cell proliferation, migration and invasion in vitro (Zhang et al., 2017). In breast invasive ductal carcinoma *PNPO* resulted to be overexpressed, and its expression levels have been found inversely correlated with the overall survival of patients (Ren et al., 2019).
PDXK gene has been found upregulated in non-small cell lung cancer (NSCLC) (Galluzzi et al., 2012) as well as in myeloid leukaemia cells where its knockdown blocks cell proliferation (Chen, 2020). However, on the other hand, *PDXK* knockdown in human NSCLC cells is able to protect against the cytotoxic activity of different agents, including cisplatin, whereas PN administration improved, in a manner that depends on the presence of *PDXK*, cisplatin anti neoplastic effect by amplifying DNA damage. High intratumoral expression levels of PDXK improve disease outcome among NSCLC patients, irrespective of therapy (Galluzzi et al 2012). These data suggest that the vitamin B6 can behave in different ways in different cancers; the effects produced by increased or decreased levels of the vitamin in different contexts might result from the developmental stage of a given tumor (Galluzzi et al, 2013) or alternatively from a balance between vitamin B6 antiproliferative and growth promoting actions (Contestabile et al., 2020).

1.3 *Drosophila* as a model system to study the relationship between PLP, diabetes and cancer

Model organisms offer suitable contexts to study the physiopathology of many human diseases by overcoming the difficulties associated with human research. Sophisticate genetic approaches have been developed to inactivate genes in a targeted manner to gain insight on specific functions. In the past decade, thanks to the discovery that main metabolic molecular pathways are well conserved, *Drosophila melanogaster* has emerged as an ideal model organism also for studies related to metabolic diseases (Owusu-Ansah and Perrimon, 2014; Padmanabha and Baker, 2014; Teleman et al., 2012). Most common forms of diabetes have been modeled in flies. In particular, T1D fly models have been obtained by ablation of a cluster of brain cells involved in insulin secretion and by mutations in insulin encoding genes (Rulifson et al., 2002; Broughton et al., 2005; Zhang et al., 2009). T2D models have been produced by mutations in insulin signalling pathway genes as well as by feeding flies with a sugar rich diet (Musselmann et al., 2011; Merigliano et al., 2018). *Drosophila* possesses a simple karyotype made of a small number of chromosomes. This feature allows to easily detects chromosome aberrations in preparations from brains which represent one of few larval tissues in active division. In addition *Drosophila* has be proven to be also an excellent model to study cancer biology (see paragraph 3).

Like mammals, *Drosophila* produces PLP through the salvage pathway, by recycling precursors taken up with food thanks to the activity of dPdxk and Sgll (the counterpart of PNPO) enzymes. During a screening aimed at identifying genes involved in chromosome integrity it has been discovered that a mutation in *dPdxk* gene displayed a cytological phenotype consisting in chromosome aberrations (CABs) (Marzio et al., 2014). Subsequent studies, described in detail below, allowed to

establish, for the first time, a correlation between vitamin B6, diabetes and DNA damage.

1.3.1 *dPdxk*^{1} mutations impair genome integrity and glucose homeostasis

It has been demonstrated that mutations in the *pyridoxal kinase* gene (*dPdxk*) cause CABs in *Drosophila* brain cells (Marzio et al., 2014). Consistently, also the treatment of wild type cells with PLP inhibitors such as 4-deoxypyridoxine (4-DP), cycloserine, isoniazid, and penicillamine (Marzio et al., 2014) produces CABs suggesting a role for PLP in chromosome integrity maintenance. This role seems to be conserved because RNAi-mediated silencing of *PDXK* in human cells or treatments with PLP inhibitors resulted in chromosome breakage (Marzio et al., 2014). In addition, studies performed by Kanellis et al., 2007 in yeast indicated that mutations in the *BUD16* gene (the counterpart of *PDXK*) result in gross chromosomal rearrangements.

Vitamin B6 is involved in the one carbon metabolism, an essential pathway for DNA synthesis and repair. In particular, PLP is a cofactor of serine hydroxymethyltransferase (SHMT), whose folate-dependent reaction plays a pivotal role in the synthesis of thymidylate. Thus, it is conceivable that a decreased activity of SHMT can lead to chromosome breakage by causing the misincorporation of uracil in DNA (MacFarlane et al., 2011; Paone et al., 2014; Giardina et al., 2018). Despite this mechanism is at the basis of the chromosome rearrangements observed in *BUD16* mutants (Kanellis et al., 2007), it does not seem to explain the origin of CABs in *Drosophila dPdxk*^{1} mutants. It has indeed been shown that although *dPdxk*^{1} mutants displayed an increased dUTP/dTTP ratio, they did not show any increased sensitivity to hydroxyurea (HU), a drug able to interfere with replication process (Marzio et al., 2014). This finding led to exclude replication failure as main mechanism at the basis of CABs in

Drosophila. Alternatively, it has been proposed that hyperglycemia may be the main cause of CABs in PLP depleted *Drosophila* cells (Marzio et al, 2014). In line with the aforementioned involvement of PLP in diabetes, it has been shown that *dPdxk*^{1} mutants, besides CABs, exhibit an increased glucose content in the larval haemolymph due to insulin resistance, accompanied by a conspicuous accumulation of AGEs (Marzio et al., 2014). Given that the acid alpha lipoic, a strong antioxidant compound, was able to rescue both AGEs and CABs it has been proposed that in *dPdxk*^{1}cells CABs are largely generated by hyperglycemia. In particular, high glucose content caused by reduced PLP levels would trigger AGE production which in turn would increase the oxidative stress responsible for CAB formation (Marzio et al., 2014) (Fig.9).

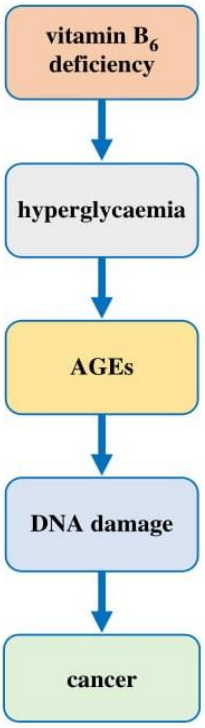


Figure 9. Effects of vitamin B6 deficiency inferred from studies carried out in Drosophila (Contestabile et al., 2020).

1.3.2 PLP as a potential cancer risk factor in diabetes

Studies on diabetic PLP-deficient flies suggested that decreased PLP availability could represent a cancer risk factor for diabetic patients (Merigliano et al., 2018). It is well known that diabetic patients have an increased risk to develop different cancers compared to healthy population (Vigneri, 2009; Noto et al., 2011; Dankner et al., 2016). Despite the underlying mechanisms need to be still clarified, it has been proposed that in addition to hyperinsulinemia, DNA damage might mediate the link between these two pathologies, given that hyperglycemia causes oxidative stress (Contestabile et al., 2020).

It has been shown that in two *Drosophila* models of diabetes the treatment with 4-DP caused severe chromosome damage resulting in 60-80% of CABs (vs 20% in controls) and enhanced AGE accumulation. Additionally, treatment with alpha-lipoic acid rescued both AGEs and CABs, confirming that CABs are largely produced by AGEs (Merigliano et al., 2018) Consequently, extrapolated to humans, this finding indicates that low PLP levels might contribute to increase the cancer risk in diabetic patients. It is possible, in fact, to envisage that in an oxidative environment in which antioxidant defences and DNA repair are weakened, a decline in the capability to counteract AGEs and ROS induced by low PLP levels, might amplify genotoxic effects, and increase the cancer risk.

1.4 *Drosophila melanogaster* as a cancer model

1.4.1 First observations

Bridges was the first to describe dark spots on the body of the *lethal(1)7* mutant larvae which died before to reach the adult stage

(Bridges, 1916). Stark expanded these studies by analysing size, number and timing of appearance of these spots and identified them as 'cellular growths somewhat resembling the tumors of vertebrates' (Stark, 1918). She tried unsuccessfully to transfer the masses into healthy flies but her experiments were inconclusive although they represent the first attempt at tumor transplantation in *Drosophila*. After Stark's studies other tumor-bearing fly lines were discovered by Wilson (1924). The description of new hereditary tumors in *Drosophila* made it clear, therefore, that flies can develop cancer and that Stark's observations were not isolated cases. Later it has been discovered that not just genetic features but also environmental cues are involved in tumor development (Russell,1940). However many years have passed before *Drosophila* was considered a good model also for cancer.

1.4.2 Isolation of the first tumor suppressor gene

In the 1950s Elizabeth Gateff working on *lethal(2)giant larvae* (*l(2)gl or lg*) mutation, previously isolated by Bridges, discovered the first tumor suppressor. She observed that *lgl* mutations resulted in tumors with a genuine malignant phenotype. These tumors occurred in the brain and in the epithelia of the imaginal discs and when transplanted into wild type larvae induced metastasis. Given that they emerged in homozygous individuals, *lgl* was classified as a tumor suppressor gene (Gateff and Schneiderman, 1967).

1.4.3 Clonal Analysis

The employment of genetic mosaics has been instrumental for studying mutation in cancer genes causing early lethality in homozygous condition. In a period throughout the 1980s and 1990s novel genetic strategies, such as the UAS/Gal4 (Brand and Perrimon, 1993) and FLP-FRT (Golic and Lindquist, 1989; Xu and Rubin, 1993) systems, allowed to generate cancer tissues

formed by wild-type and oncogenic mutant clones. The FLP/FRT system is a site-directed recombination technology based on the targeting of a recombination enzyme (flipase - FLP) to specific DNA regions designated as flipase recognition target (FRT) sites; a highly efficient "mitotic recombination system" thus, allows to knockout defined genetic function in specific cells, tissues and organs. A considerable improvement of the FLP-FRT system was the development of the MARCM (Mosaic Analysis with Repressible Cell Marker) technique (Lee and Luo 1999). Prior to the introduction of MARCM, homozygous mutant cells were identified by the absence of a visible marker such as GFP or lacZ in comparison to the surrounding heterozygous environment and the wild type "twin clone". By using the MARCM technique the homozygous clones can be positively marked using e.g. GFP or RFP, which can be of particular importance for the analysis of single cells in a disease model.

1.4.4 Cooperative tumorigenesis models

At the beginning of the new century, whole genome sequencing revealed the most cellular pathways implicated in development and tumorigenesis have been evolutionarily conserved in flies (Adams et al., 2000; Lander et al., 2001). MARCM strategy resulted crucial to discover several developmental signalling cascades at the basis of tumorigenesis. Pagliarini and Xu (2003), created a model of tumor invasion and metastasis which marked the beginning of *Drosophila* as a model organism for cancer research. These authors showed as the expression of oncogenic mutants, such as ras^{G12V}, combined to mutations which disrupt cell polarity such as *scrib* or *discs large (dlg)*, generated invasive tumors in imaginal discs.

About ten years later tumors have also been cooperatively produced by combining Ras activation and mitochondrial dysfunction. These studies revealed that Ras activation

cooperates to mitochondrial dysfunction to trigger the production of reactive oxygen species (ROS), thus activating JUN N-terminal kinase (JNK) signalling. The cooperation of oncogenic RAS with JNK inactivates the Salvador–Warts–Hippo pathway, leading to the upregulation of the interleukin-6 and WNT homologues Unpaired (UPD) and Wingless (WG) (Ohsawa, et al., 2012) These studies were relevant also by considering that mitochondrial function is frequently impaired in human cancers (Wallace, 2012).

1.4.5 Genetic screenings

Drosophila helped us to understand cancer by the approach of carrying out genetic screens that interrogate the entire genome for tumor suppressor functions. Pioneering research based on this approach has generated dozens of tumor types of different grades that range from benign hyperplasia to severe malignant neoplasia (Gonzalez 2007; Watson et al., 1994).

A different approach to the same goal of searching for new functions involved in cancer, has been a genome-wide, (RNAi) screening that allowed to the identification of more than 600 genes controlling *Drosophila* neuroblasts self-renewal, including brain tumor suppressors. Homologues of these genes have been found working as tumor suppressors in mammals balancing self-renewal and stem cell differentiation (Neumuller et al 2011).

More recently (Zoranovic et al 2018) a genome-wide genetic screen using a transgenic RNAi library identified multiple known tumor suppressor pathways as well as many novel genes and pathways potentially involved in the suppression of RasV12-driven epithelial tumorigenesis. Remarkably a large number of human genes orthologous to fly genes identified, were strongly downregulated in human cancers, confirming that genes

controlling epithelial growth, fate and integrity are highly conserved across phyla.

1.4.6 Screening of drugs and avatar flies

The demonstration that drugs can efficiently block a tumor phenotype in flies (Vidal et al., 2005) opened the gate to in vivo screening platforms for anti-cancer drug discovery (Gladstone and Su, 2011; Gonzalez, 2013). Similar studies led Willoughby et al (2013) to identify the glutamine analogue acivicin (a chemical with activity against human tumor cells) as a potent and specific inhibitor of *Drosophila* tumor formation.

Finally, avatar flies carrying specific mutations of cancer patients are currently used to define specific anti-cancer drug cocktails, with a view to develop personalized medicine (Kasai and Cagan, 2010; Sonoshita and Cagan, 2017).

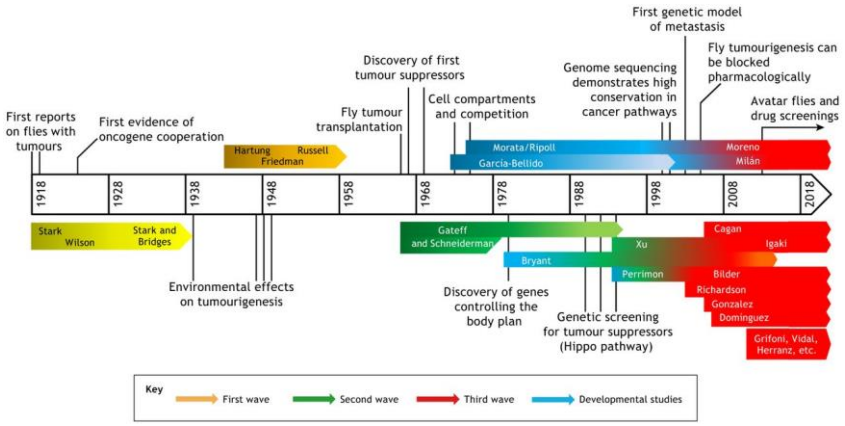


Figure 10 Diagram of the temporal evolution of cancer studies in Drosophila over 100 years from the year 1918 to 2018 (Villegas, 2019).

1.4.7 Relationship between diabetes and cancer in *Drosophila*

MARCM strategy was also a precious system to explore the relationship between diabetes and cancer in *Drosophila*. Hirabayashi and collaborators (2013) showed that a sugar rich diet can transform Ras/Src-cancer tissues into aggressive tumors able to produce metastases. Remarkably, the authors found that whereas most tissues displayed insulin resistance, Ras/Src tumors retained insulin pathway sensitivity, displayed an enhanced glucose uptake, and counteracted apoptosis. It was thus proposed the mechanism that the high sugar diet induces an increased Wingless/Wnt pathway activity, which in turn upregulates the expression of insulin receptor gene and consequent insulin sensitivity. These results indicated that *Drosophila* represents a suitable model to future studies aimed at gaining insights on the relationship between diabetes and cancer.

1.5 *aims of the project*

In eukaryotes the active form of vitamin B6, the pyridoxal 5' phosphate (PLP) is produced in the salvage pathway by concerted action of two enzymes: pyridoxal kinase (PDXK) and pyridoxamine / pyridoxine oxidase (PNPO). PDXK phosphorylates PN, PM and PL into PNP, PMP and PLP respectively, while PNPO oxidizes PNP and PMP into PLP. It has been previously demonstrated the mutations in *Drosophila dPdxk* gene result in chromosome aberrations (CABs) and raise the glucose content in the hemolymph (the *Drosophila* blood). These results suggested that vitamin B6 may be involved in chromosome integrity maintenance as well as in glucose homeostasis. Interestingly, the expression of the *PDXK* human gene in $dPdxk^{1}$ mutant flies is able to rescue both CABs and hyperglycemia suggesting evolutionary conservation of these functions.

The first aim of this work has been to give further support to the hypothesis that PLP is involved in genome integrity and glucose homeostasis. To this purpose, we investigated whether also the impairment of *PNPO* function (*sgll* in *Drosophila*) affects glucose metabolism and results in DNA damage. Then, we wanted to establish whether hyperglycemia and chromosome damage are linked by a cause-effect relationship also in Sgll depleted cells, as occurs in $dPdxk^{1}$ mutants.

The second aim has been to test four human PDXK variants for their effects on DNA damage, to establish whether they could be considered predictors of cancer risk. To this purpose, we generated and inserted in $dPdxk^{1}$ mutant flies the constructs

containing the four mutant forms of PDXK. Their impact on chromosome damage has been evaluated and an attempt was also made to relate the effects of these variants with their biochemical characteristics.

The third aim has been to verify whether vitamin B6 impacts on cancer also in *Drosophila*. To this purpose, we investigated whether low PLP levels can be able to increase malignancy in two *Drosophila* cancer models: Ras and Ras/Src, generated by MARCM strategy.

The choice of these models was determined by the fact that

(1) Ras and Scr are deregulated in several human cancers
(2) Ras cancers in flies are currently used in anti-cancer drug testing screening
(3) Ras /Src cancer have been already used in works aimed at studying the link between cancer and metabolism in *Drosophila*.

Altogether the goal of this work consisted in elucidating the complex relationship existing between vitamin B6, diabetes and cancer in *Drosophila*, by exploiting the advantages that this organism offers for the systematic dissection of biological processes.

2. RESULTS

Part1

2.1 Role of *sugarlethal (sgll)* in genome integrity and glucose homeostasis.

The active form of vitamin B6 (pyridoxal 5' phosphate, PLP) is synthesized through the *salvage pathway* by two enzymes: PDXK that phosphorylates PN, PM and PL and PNPO that produces PLP by oxidizing PNP and PMP (Figure 1). Previous studies showed that mutations in the *Drosophila* gene encoding *pyridoxal kinase* (*dPdxk*) caused chromosome aberrations (CABs) in larval neuroblasts exacerbated by glucose treatment (Marzio et al., 2014). In addition $Pdxk^{1}$ mutations caused hyperglycemia due to insulin resistance (Marzio et al., 2014). The first part of the work is aimed at confirming the role of PLP in genome integrity and glucose homeostasis maintenance and at investigating underlying mechanisms. To this purpose we verified whether blocking the salvage pathway by inactivating PNPO function results in the same phenotypes elicited by $dPdxk^{1}$ mutations.

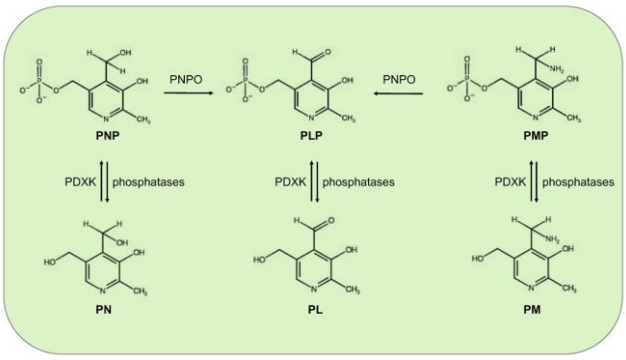


Figure 1. Schematic vitamin B6 salvage pathway. PLP, pyridoxal 5'-phosphate; PNP, pyridoxine 5'-phosphate; PMP,pyridoxamine 5'-phosphate; PL, pyridoxal; PN, pyridoxine; PM, pyridoxamine; PDXK, pyridoxal kinase; and PNPO pyridoxine/pyridoxamine oxidase (Mascolo and Vernì, 2020)

2.1.1 Sgll depletion causes chromosome aberrations

The fly ortholog of the human PNPO enzyme is encoded by *sugarlethal* (*sgll*) gene (Chi et al., 2014). We silenced *sgll* gene by RNA interference and after have checked that *sgll* mRNA levels were descreased (Figure 2A), we examined the resulting phenotypic traits. First of all we noticed that $sgll^{RNAi}$ individuals reached the adult stage, but they displayed a reduced survival rate compared to wild type flies (Figure 2B). To test whether Sgll depletion caused chromosome damage, we incubated in colchicine the brains from $sgll^{RNAi}$ larvae and examined chromosomes in DAPI-stained preparations.

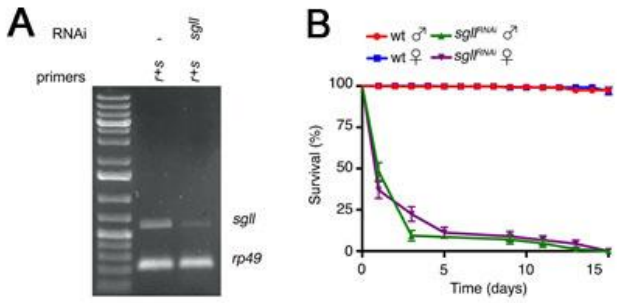


Figure 2 RNA interference against sgll and survival rate of $sgll^{RNAi}$ flies. A RT-PCR showing that RNAi against the sgll gene disrupts the corresponding mRNA. The rp49 mRNA, encoding the Ribosomal protein 49 served as an internal control. The primers used are reported in Materials and Methods and abbreviated as follows: r, rp49; s sgll. Amplification products are: 599 bp with sgll primers; 224 bp with rp49 primers. B Kaplan–Meier survival curve of sgllRNAi and wt flies.

As shown in figure 3 *sgll*^{RNA} neuroblasts exhibited a CAB frequency significantly higher than wild type (2.80% vs 0.3%). An approximately 6-fold increase was observed when *sgll*^{RNAi} brains were incubated in a solution containing 1% glucose, suggesting that Sgll depleted cells were sensitive to glucose. Remakably, the glucose treatment increased not only the frequency of aberrations but also their complexity, giving rise to cells exhibiting more than one break. In addition about 3% of cells showed extensive chromosomal fragmentation (Figure 3A). Since it is difficult to establish the correct number of CABs in cells with multifragmented chromosomes, we decided arbitrarily to assign five CABs to each of these cell. Consequently, the percentage of CABs reported in Fig 3 B represents an underestimation. These data suggest that the amount of PLP produced in *sgll*^{RNAi} cells is not sufficient to protect them from chromosome damage.

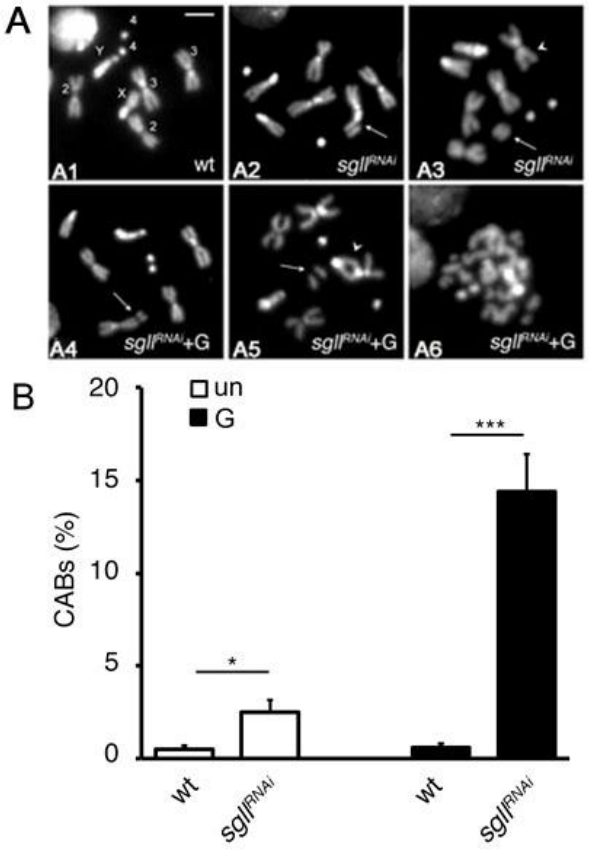


Figure 3 Sgll depletion causes CABs in larval neuroblasts. A Examples of CABs in untreated and 1% glucose (G) treated larval brains. A1 wild-type male metaphase; A2 chromatid deletion of a major autosome (arrow); A3 isochromatid deletion (arrow) of a major autosome (arrowhead); A4 isochromatid deletion of a major autosome; A5 dicentric chromosome (arrowhead) with corrispective acentric fragments (arrow); a6 metaphase with multifragmented chromosomes. Scale Bar = 5 µm. B Quantification of CABs. Each bar represents the mean value ± SEM obtained by scoring at least 5 brains (~800 cells) for genotype. *, Significantly different in Student's t test with p < 0.05 and < 0.001 respectively.***

We also found that PLP as well as PL supplementation rescued CAB frequency in both untrated and 1% glucose treated *sgll*^{RNAi} brains (Figure 4). In contrast, and as expected, supplementation with PN or PM did not significantly reduce CAB frequency (Figure 4) confirming that CABs represent the specific consequence of Sgll reduced activity.

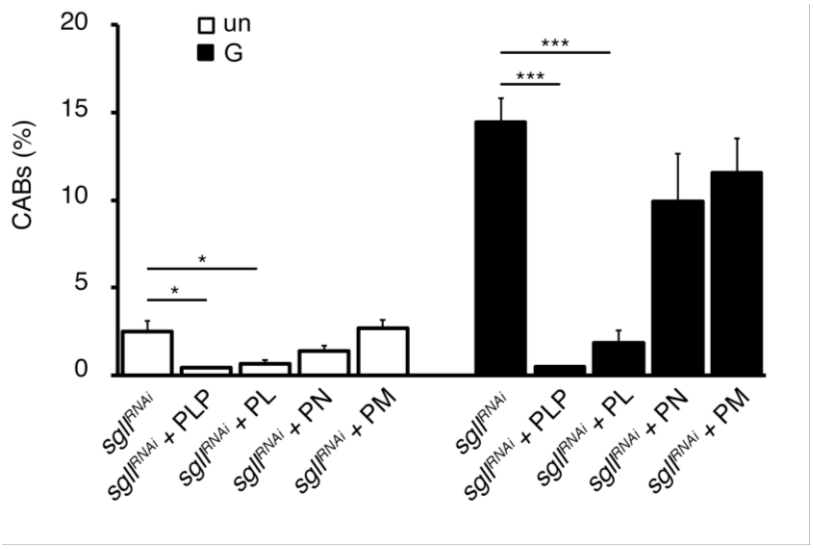
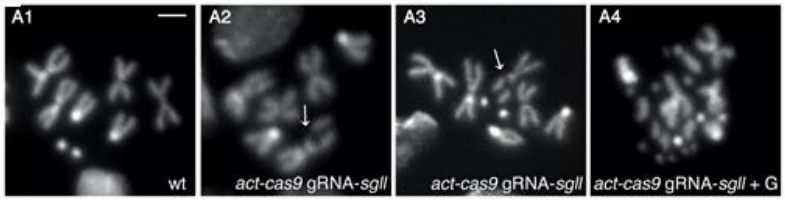


Figure 4 Effect of PLP precursor supplementation on CAB frequency. Each column represents the mean value ± SEM obtained by scoring at least five brains (~800 cells) for treatment. *, Significantly different in Student's t test with p < 0.05 and p < 0.001 respectively.***

To further confirm the specificity of CAB phenotype in *sgll*^{RNAi} cells we examined neuroblasts from larvae in which *sgll* gene was mutagenized at somatic level by *in vivo* CRISPR Cas9 system (Port et al., 2014; https://fgr.hms.harvard.edu/using-tripcrispr-lines) finding about 6% of CABs (Figure 5). We also investigated whether 1% glucose treatment enhanced CAB frequency in these larvae. Due to the mosaic nature of gene inactivation in this system, we observed a wide variability of CAB frequencies in the

different examined brains. However, the presence of cells with multiple chromosome breakage events (never observed in untreated brains) confirmed the sensitivity to glucose of cells depleted of Sgll.

A



B

Genotype/treatment	N. scored brains	N. scored cells	CABs (%)
act-cas9	2	419	1.19
gRNA-*sgll*	3	367	0.82
act-cas9 gRNA-*sgll*	7	1256	6.13†

Figure 5 Mutations in sgll gene induced by CRISPR Cas9 system cause CABs. A Examples of CABs in act-cas9 and act-cas9 gRNA-sgll larval brains. A1 wild type (wt) female metaphase; A2 chromatid deletion of a major autosome (arrow); A3 isochromatid deletion (arrow) of a major autosome; A4 metaphase with multifragmented chromosomes. G= 1% glucose. Scale Bar 5μm. B CAB frequencies in act-cas9 gRNA-sgll. Note that a small but significant percentage of CABs was observed in brains from act-cas9 larvae, however CAB frequency displayed by act-cas9 gRNA-sgll larval brains was about 5 times higher. † Significantly different in the Student's t test with p < 0.05 compared to act-cas9.

2.1.2 DNA double strand breaks (DSBs) are at the basis of CABs in *Sgll* depleted neuroblasts.

CABs originates from unrepaired or misrepaired DNA double strand breaks (DSB) (Obe et al., 2002). To verify whether DSBs were at the basis of CABs in $sgll^{RNAi}$ brain cells we tested them

for the presence of γ-H2Av foci in immunofluorescence experiments, using the pS137 anti-phospho-histone antibody. γ-H2Av (the counterpart of mammalian γ-H2AX) marks the DSBs and represents the first step in recruiting and localizing DNA repair proteins. At cytological level γ-H2Av foci are detected as discrete spots, named γ-H2Av foci (Merigliano et al., 2017; Merigliano et al., 2018; Verni and Cenci, 2015). As showed in Figure 6, $sgll^{RNAi}$ untreated brains exhibited a percentage of γ-H2Av positive cells slightly higher than wild type cells. 1% glucose treatment enhanced the frequency of positive cells, which increased up to 12%. In contrast, glucose treatment did not influence γ-H2Av in control cells. This data suggest that DSBs are at the basis of CABs in Sgll depleted cells and that glucose is involved in the DNA damage observed in these cells.

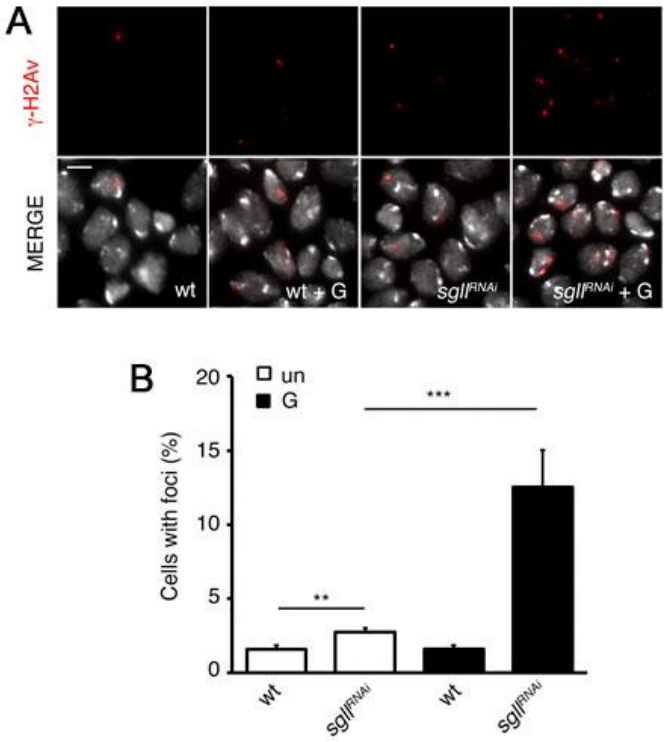


Figure 6 Sgll depleted cells exhibit spontaneous γ-H2Av foci. A Examples of γ-H2Av foci in untreated and 1% glucose treated wt and sgll^{RNAi} neuroblast nuclei. Scale Bar = 5 μm. B Quantification of γ-H2Av positive nuclei. Each bar represents the mean value ± SEM obtained from three independent experiments by scoring at least 1,000 cells in four brains. **, *** significantly different in Student's t test with p < 0.01 and p < 0.001.

2.1.3 *sgll^{RNAi}* flies display diabetic hallmarks

Based on the finding that glucose treatment enhanced CABs frequency in Sgll depleted cells (Fig. 3 A,B) and that *Drosophila dPdxk*^{1} mutants exhibit hyperglycemia (Marzio et al., 2014) we asked whether also Sgll depletion could impair glucose

homeostasis. Thus we measured the glucose content in the hemolymph of *sgll*^{RNAi} individuals finding an increased glucose content with respect to the wild type strain. In addition we found that only PLP or PL treatment rescued the hyperglycemia, while PN and PM did not show any effect (Fig 7).

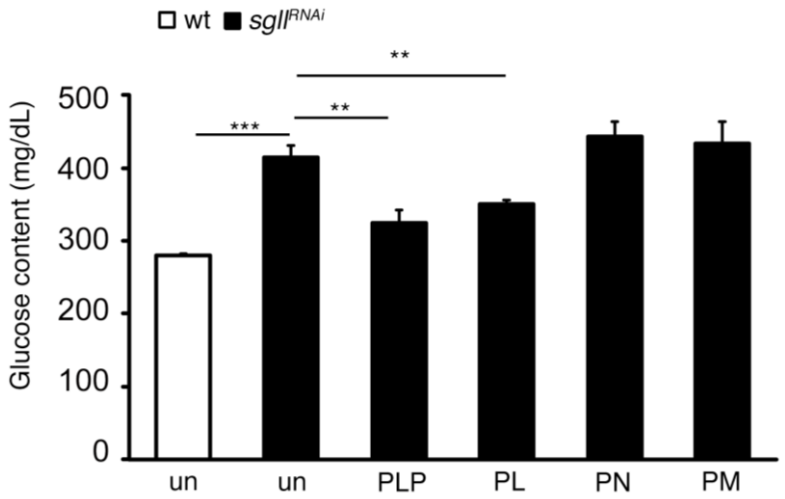


Figure 7 Sgll depletion causes hyperglycemia. Glucose content in hemolymph from wild-type and sgll^{RNAi} larvae reared on standard medium or on a medium supplemented with PLP and PLP precursors (1 mM). Columns are the means of five independent sample measurements ± SEM (each sample = hemolymph extracted from 20 larvae). **, significantly different in Student's t test with p < 0.01, and 0.001 respectively.***

sgll^{RNAi} flies can reach the adult stage, differently from *dPdxk*^{1} mutants (Marzio et al., 2014). This allowed us to examine the adult body size of these individuals. As shown in figure 8 we found that, similar to other *Drosophila* models of diabetes (Musselmann at al., 2011; Merigliano et al., 2018), *sgll*^{RNAi} flies displayed a significant size reduction also observed in flies bearing CRISPR-induced *sgll* mutations (Figure 8). We next asked whether this phenotype was due to a decreased number of cells or instead to a reduced cell size. Single *Drosophila* wing

cells produce a hair, named a trichome, therefore the number of thricomes on a specific area corresponds to the numer of cells contained in that region. Thus, we counted the number of trichomes in a 10,000 μm^2 area immediately posterior to the posterior cross vein (PCV, fig Ab2 arrow) on $sgll^{RNAi}$ wings to evaluate cell density and size. As reported in figure 8, $sgll^{RNAi}$ flies showed a reduction of approximately 20% of cell size and a slight reduction of wing area (13.5%). these results suggest that overall body size reduction cause by Sgll depletion may be caused by a decreased cell volume.

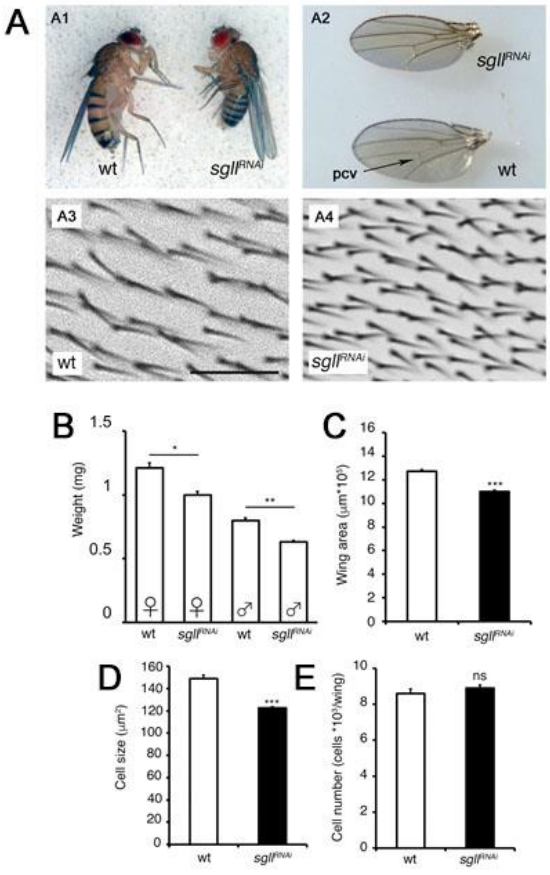


Figure 8 Diabetic hallmarks in sgllRNAi individuals. A (A1) sgll^{RNAi} adults display a smaller body size compared with wild type. (A2) Example of wing with a reduced area from a sgll^{RNAi} female. (A3,4) Trichome density in wings from sgll^{RNAi} and wild-type individuals. B Body weight measurement in wild-type and sgll^{RNAi} males and females. Each column represents the mean weight (±SEM) of single flies obtained by weighing 5 to 6 samples of 15 individuals for each genotype. C Wing area has been measured in 15 females of each genotype and expressed as the mean value ± SEM. D Cell size mean value (±SEM) represents the reciprocal value of mean cell density assessed by counting number of wing hairs (trichomes) on the dorsal wing surface in a 10.000 µm2 area just posterior to the PCV. E Total cell number expressed as mean value (±SEM) has been generated by multiplying mean cell density by mean wing area. *,**, significantly different in Student's t test with p < 0.05, 0.01, and 0.001 respectively.***

In diabetes insulin resistance is often accompanied by obesity (Merigliano et al 2018). To investigate whether Sgll depletion might alter lipid metabolism we examined the fat bodies (the counterpart of human adipose tissue and liver) from *sgll^{RNAi}* larvae. Fat body contains vesicles named lipid droplets (LD) where flies store fats. Nile Red staining revealed larger LD in Sgll-depleted larvae v respect to control (Figure 9) indicating that Sgll depletion impairs lipid storage.

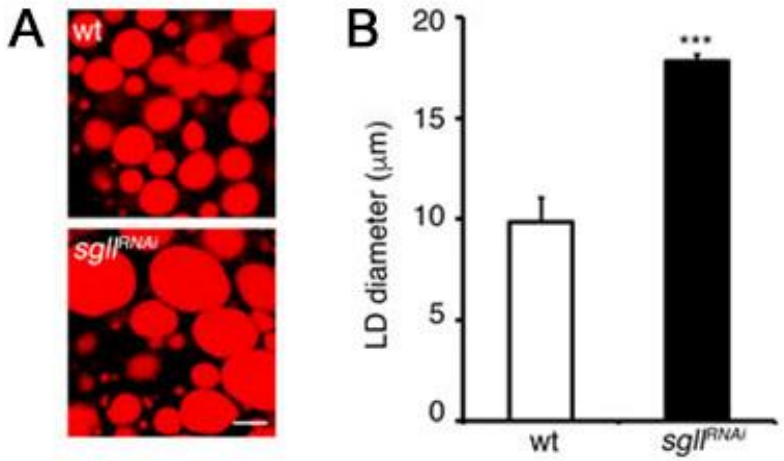


Figure 9 sgll^{RNAi} larvae display larger lipid droplets (LD) with respect to control. (A) Nile Red staining of control and sgll^{RNAi} larval fat bodies. Bar = 10 µm. (B) The size of 100 individual lipid droplets was measured in each preparation and the mean lipid droplet diameter ± SEM was calculated. *** significantly different in Student's t test with p<0.001.

2.1.4 Sgll depleted cells accumulate AGEs

Established that DNA damage is sensitive to glucose in Sgll depleted cells, we hypotesized that sugar might be involved in CAB formation. To elucidate the mechanism that promotes CABs we took into consideration the formation of AGEs, as these compunds have been associated to PLP, diabetes, and DNA damage. AGEs are genotoxic compounds, associated with senescence, whose accumulation rises in diabetic complications (Brownlee, 2001; Thorpe and Baynes, 1996; Vlassara and Palace, 2002). AGE formation starts with nonenzymatic glycation reactions triggered by high glucose which reacts with amino groups of proteins and DNA. Remarkably, during AGE metabolism are produced ROS, which are well recognized mediators of DNA damage (Sharma et al., 2018). PLP is able to counteract AGEs by sequestering 3-deoxyglucosone, an intermediate compound of the pathway (Nakamura et al., 2007). To investigate whether Sgll depletion caused AGE accumulation we immunostained *sgll^{RNAi}* neuroblasts and evaluated the frequency of AGE-positive cells. As showed in figure 10, Sgll-depleted brains accumulated AGEs whose frequency was increased by 1% glucose treatment and was rescued by PLP treatment.

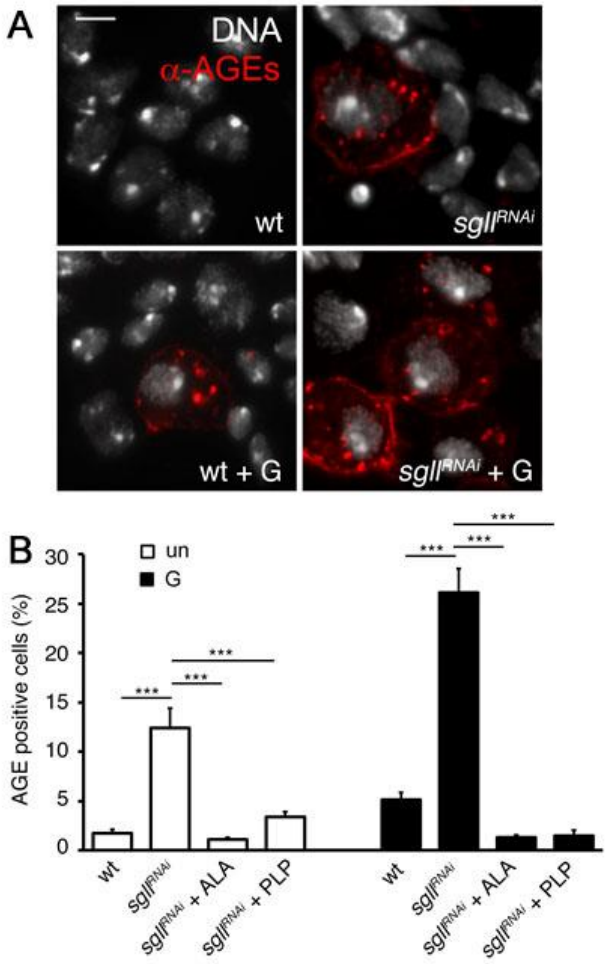


Figure 10 sgll^{RNAi} neuroblasts accumulate AGEs. A Examples of neuroblasts from untreated and 1% glucose treated wt and sgll^{RNAi} brains stained with an anti-human AGE antibody. Scale Bar = 5 µm. B Frequencies of AGE-positive cells in wild type and sgll^{RNAi} brains untreated and exposed to 1% glucose with or without α-lipoid acid (ALA) or PLP. Note that either PLP and ALA treatment rescues AGE accumulation. Bars in the graph represent the mean frequencies of AGE-positive cells (±SEM) in three independent experiments by scoring at least 1,000 cells in four brains. *** Significantly different in Student's t test with p < 0.001.

In addition alpha lipoic acid (ALA), an antioxidant compound able to counteract AGE formation rescued the frequency of AGE-positive cells in both untreated and 1% glucose treated *sgll*^{RNAi} cells (Figure 10B). Remarkably, ALA was also able to reduce CAB frequency and γ-H2Av foci in *sgll*^{RNAi} neuroblasts (Figure 11) suggesting that in Sgll depleted cells DNA damage is largely caused by AGEs, which are in turn triggered by high glucose.

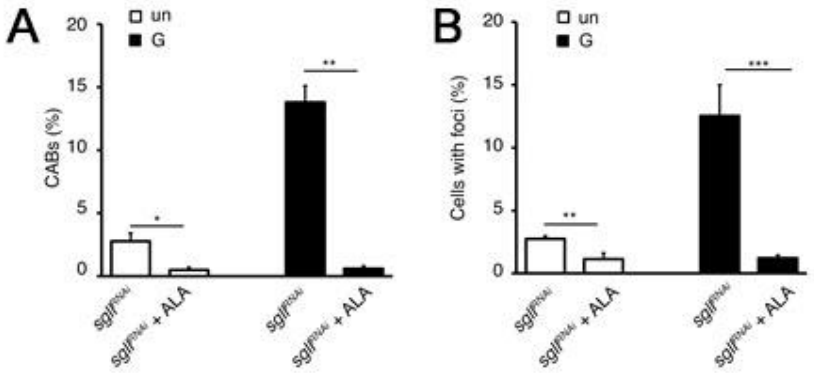


Figure 11: Effect of alpha lipoic acid (ALA) on DNA damage induced by sgll silencing. A ALA treatment rescues CAB frequency in sgll^{RNAi} neuroblasts. Each bar represents the mean value ±SEM obtained by scoring at least five brains (~800 cells) for genotype. *, ** significantly different in Student's t test with p < 0.05 and p < 0.01 respectively B ALA treatment rescues γ-H2Av positive cells in sgll^{RNAi} brains. Each bar represents the mean value ±SEM from three independent experiments by scoring at least 1000 cells in four brains. **, *** significantly different in Student's t test with p < 0.01 and < 0.001 respectively.

Part 2

2.2 Studies on pyridoxal kinase human variants

In this second part of the work we wondered whether human PDXK variants, present in population, impact on DNA integrity and might be considered predictive of an increased cancer risk. For this purpose, we focused on four PDXK human variants that carry missense mutations; three of them were picked up from databases and one was isolated in a screening of diabetic patients. We generated and expressed these variants, into *dPdxk*^{1} mutant flies and evaluated their impact on CABs, glucose content and AGE accumulation. Moreover, the group of the professor Roberto Contestabile (Department of Biological Science "Rossi Fanelli" Sapienza University of Rome), carried out the biochemical characterization of these variants.

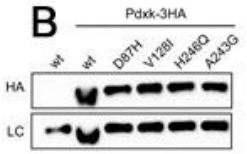
2.2.1 Genetic and biochemical basis of the study

It has been previously demonstrated that the silencing of human *PDXK* gene induce the formation of CABs in fibroblasts and HeLa cells (Marzio et al., 2014). Moreover, the PLP inhibitor 4-deoxypyridoxine (4-DP) enhanced the formation of 53BP1 repair foci in HeLa cells (Kanellis et al., 2007). These findings prompted us to speculate that humans carrying mutations in the *PDXK* encoding gene could have an increased predisposition to accumulate chromosome aberrations and a consequent increased risk to develop malignancies. To evaluate this hypothesis, we decided to express human PDXK loss-of-function variants in flies homozygous for the *dPdxk*^{1} mutation and test them for their effects on CABs. The rationale of this strategy comes from previous data indicating that a wild type copy of the human PDXK gene is able to rescue CABs when expressed in *dPdxk*^{1} mutants (Marzio et al., 2014). We analysed four human PDXK variants: Asp87His (D87H), Val128Ile (V128I), His246Gln (H246Q) and Ala243Gly (A243G). The first three have been picked up from the Exome variant server (Exome variant server, http://evs.gs.washington.edu/EVS/) which contains many human

PDXK variants not yet associated to any diseases. The variants have been chosen on the basis of their putative damaging effects predicted in silico by thePolyPhen-2 software and of the evolutionary conservation of each mutated residues in *Drosophila*. In particular D87H displays the highest damaging score (1.0) and concerns a conserved position in *Drosophila* and human PDXKs; V128I and H246Q carry mutations in invariant positions (http://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl) and in addition display high damaging scores (0.99 and 0.98 respectively) (Fig. 12). These variants are very rare in the population (their frequency ranging from 2.84e-5 to 7.97e-6; https://gnomad.broadinstitute.org/) and are carried in heterozygous condition. A243G (Fig. 12) is not present in major databases, displays a damaging score of 0.72 and has been found in an ongoing genetic screening in patients with gestational diabetes.

A

Human residue	*Drosophila* residue	Human variant	rs_ID	Damaging probability (*in silico* prediction)
D87	N93	D87H	rs148548270	1.00
V128	V130	V128I	rs377592729	0.99
H246	H240	H246Q	rs372098146	0.98
A243	A237	A243G	-	0.72



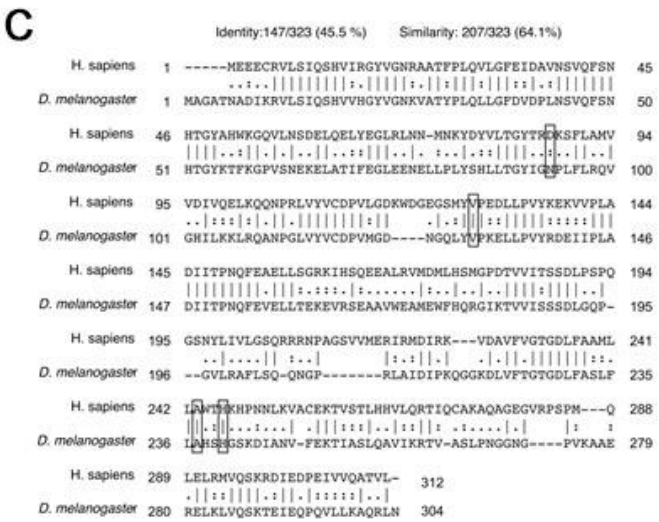


Figure 12 PDXK variants studied. A Table listing human PDXK variants, studied in this work, present in Exome variant server (D87H, V128I, H246Q) or found at genetic screening (A243). B Western blot showing protein expression of PDXK human variants in brains from dPdxk[1] Drosophila mutants. An antibody anti-HA was used to detect PDXK proteins. α-tubulin served as loading control (LC). C Clustal Omega pairwise sequence alignment of Drosophila melanogaster Pdxk (NP_996031.1) with that of Homo sapiens (NP_003672.1). Dash indicates the identical residues, colon indicates the conserved residues. Mutated residues carried by variants examined in this study are boxed.

2.2.2 PDXK human variants fail to rescue CABs in *dPdxk[1]* mutant flies

By site-directed mutagenesis, we generated four constructs of human HA-tagged PDXK cDNA, each containing a variant, and

introduced them in flies by germline mediated transformation. We validated the expression of these constructs by western blot analysis using an anti HA antibody (Fig 12 B). By performing crosses described in Material and Methods, we introduced these variants into *dPdxk*^{1} mutant flies and tested them for CABs in DAPI stained brain preparations from third instar larvae. As reported in figure 13 the D87H, V128I, H246Q and A243G variants were unable to completely rescue CABs unless the larvae were reared in a medium containing PLP (1 mM). The expression of either wild type or variant PDXK forms in a *dPdxk*^{1}/+ background did not produce CABs, allowing us to exclude any dominant negative effect. Taken together, these findings suggest that all tested variants behave as loss of function alleles which impact on genome integrity.

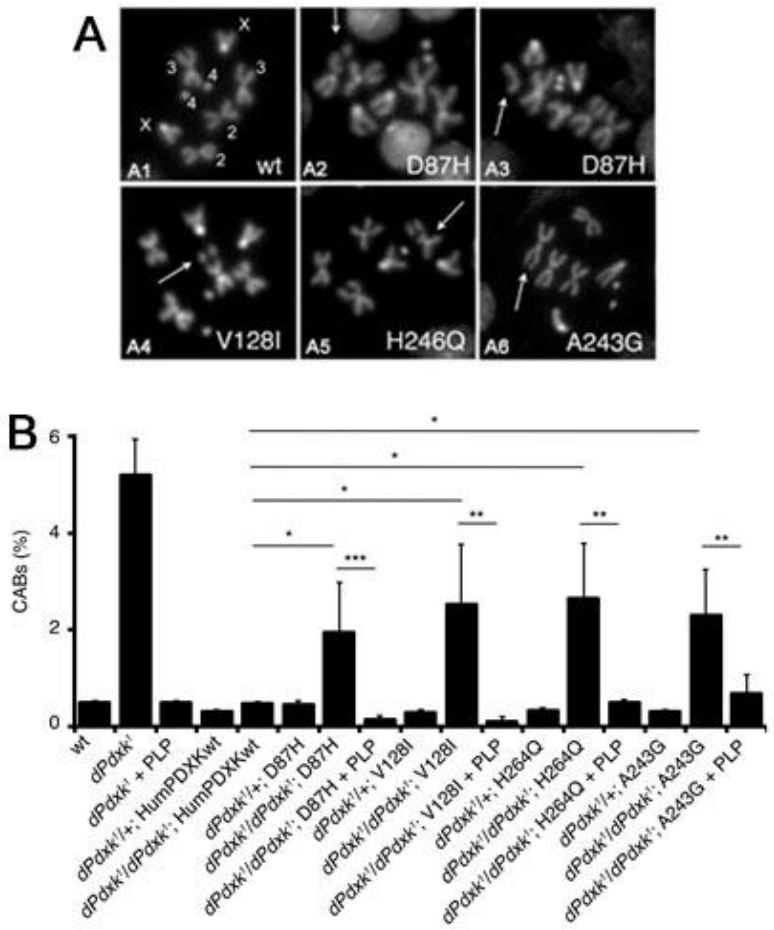


Figure 13 Human PDXK variants expressed in dPdxk1 flies do not rescue CABs. (A) Examples of chromosomeaberrations in neuroblasts from larvae expressing PDXK variants in dPdxk1 background. A1 wild typemetaphase; A2 and A6 autosomal chromatid deletion (arrows); A4 and A5 isochromatid deletion (arrowed); A3 isochromatid deletion at the level of centromere (arrows). Scale Bar 5 µm. (B) Quantification of CABs.Each bar represents the mean value ± SD obtained by scoring at least 5 brains (~800 cells) for genotype.*,**,Significantly different in the Student's t test with p < 0.05, 0.01 and 0.001 respectively. (dPdxk1 with respect to wt and to dPdxk^{1} + PLP is <0.001, not reported in the graph).***

2.2.3 PDXK human variants expressed in *dPdxk*^{1} flies impact on glucose metabolism.

The results obtained in the first part of this work provided a strong support to the hypothesis that hyperglycaemia is one of main inducers of CABs, through the AGE metabolism, in PLP depleted cells. Thus, we asked whether the four PDXK variants could impact on genome integrity by the same mechanism. For this purpose we tested whether the expression of D87H, V128I, H246Q and A243G variants affected glucose homeostasis in

dPdxk^{1} larvae. This analysis showed (Fig. 14) that none of the variants was able to significantly reduce hyperglycemia caused by *dPdxk*^{1} mutation, which was instead rescued by PLP treatment (Fig.14). In addition, by immunostaining experiments using an human anti-AGE antibody we demonstrated that *dPdxk*^{1} cells expressing D87H, V128I, H246Q and A243G variants displayed an accumulation of AGEs, exacerbated by 1% glucose treatment (Fig. 14). These results indicate that AGEs can mediate the formation of CABs induce by the PDXK human variants.

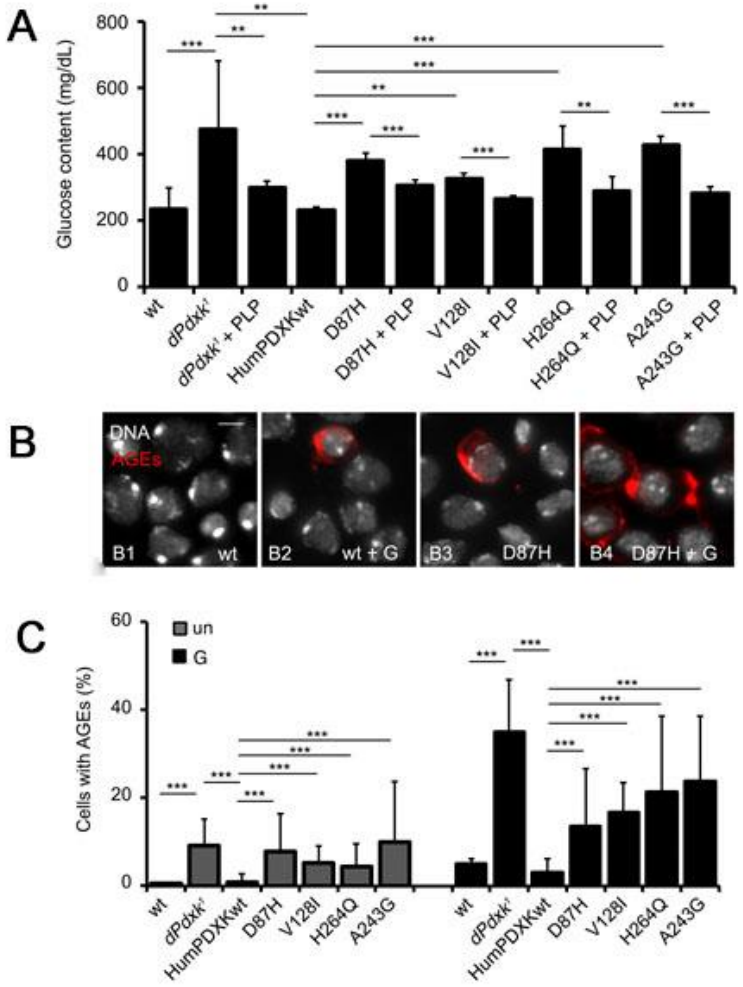


Figure 14 Human PDXK variants expressed in dPdxk[1] flies do not rescue hyperglycemia. A Glucose content in hemolymph from larvae expressing either wild type or PDXK variants, reared on standard medium with or without PLP (1 mM) supplementation. Columns are the means of 5 independent sample measurements ± SD (each sample = hemolymph extracted from 20 larvae). **, ***Significantly different in the Student's t test with p < 0.01 and <0.001 respectively. B Examples of neuroblasts from untreated and 1% glucose treated brains expressing wt and D87H PDXK

stained with an anti-human AGE antibody. Scale Bar 5 µm. G = glucose. C Frequencies of AGE-positive cells in brains untreated (un) and exposed to 1% glucose (G). Bars in the graph represent the mean frequencies of AGE-positive cells (±SD) in three independent experiments by scoring at least 1000 cells in 4 brains. *** significantly different in the Student's t test with p < 0.001.

2.2.4 PDXK variants respond differently to PLP precursors.

To clarify the reason why PDXK variants failed to rescue CABs in *dPdxk*^{1} background, we tested the effect of PLP precursors (PL, PM and PN) on CAB frequency in brains from larvae expressing D87H, V128I, H246Q and A243G variants. As reported in Fig. 15 PLP precursors were added to growth medium at two different concentrations (0.5 and 1 mM). The results obtained showed that each PDXK variant behaved differently towards each different tested vitamer. In brains expressing D87H, PL treatment did not rescue CABs, while a weak rescue was obtained with PM (1 mM). In contrast, PN reduced significantly CAB frequency but only at 1 mM concentration. In brains expressing V128I, neither 0.5 nor 1mM PL concentrations were able to rescue CABs, whereas both PM and PN rescued CABs at 1 mM concentration. In H246Q expressing cells PL and PM reduced CAB frequency only at 1 mM concentration, whereas PN reduced CAB frequency also at 0.5 mM concentration. In A243G expressing neuroblasts all precursors rescued CABs but only at 1 mM concentration. Remarkably, we found that PLP treatment rescued CABs at both concentrations in all tested variants. These data suggest that the replacement of H246, V128, D87 and A243 amino acids with those found in the respective variants can impact on PDXK functionality by altering in different ways its capability to phosphorylate the substrates.

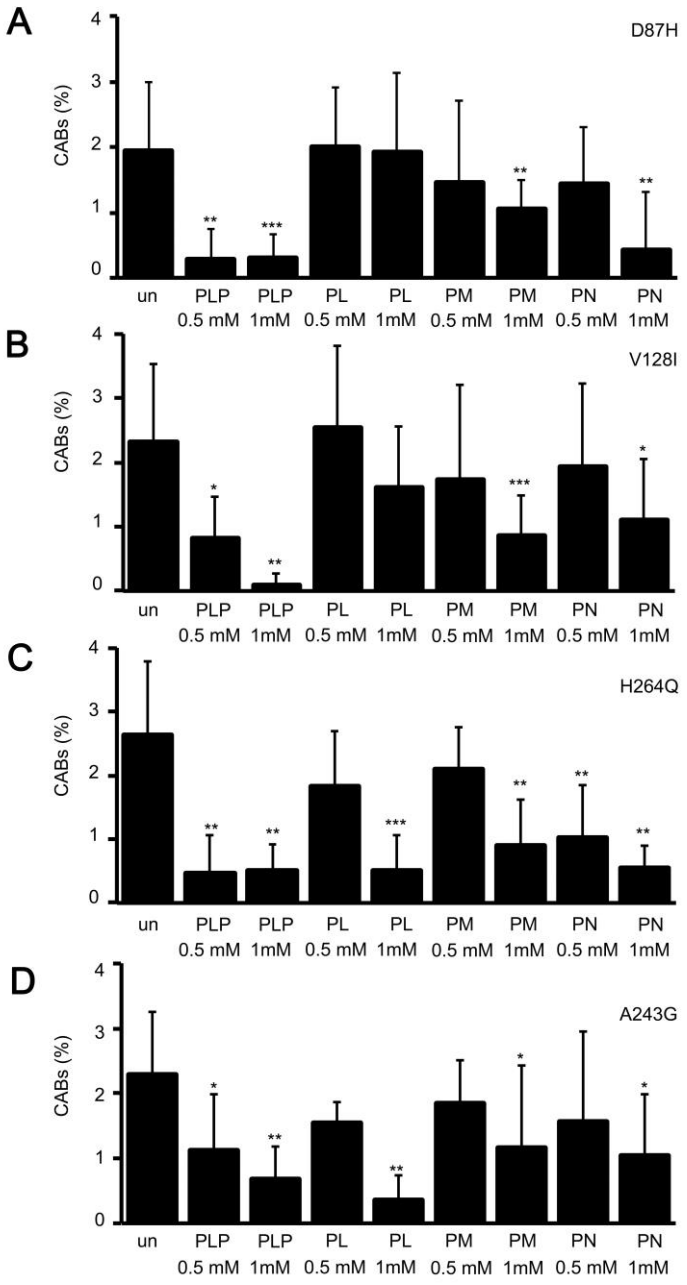

A
D87H
B
V128I
C
H264Q
D
A243G
CABs (%)
0
1
2
3
4
un
PLP 0.5 mM
PLP 1mM
PL 0.5 mM
PL 1mM
PM 0.5 mM
PM 1mM
PN 0.5 mM
PN 1mM

Figure 15. Effect of PLP precursors (PL, PM and PN) on CAB frequency in brains from larvae expressing D87H (A), V128I (B), H246Q (C) and A243G (D) variants. Each bar represents the mean value ± SD obtained by scoring at least 8 brains (~1000 cells) for genotype. *, **, *** significantly different in the Student's t test with p < 0.05, 0.01 and 0.001 respectively.

2.2.5 Biochemical characterization of PDXK variants

In order to explain the different behaviour showed by the PDXK variants towards PLP precursors, in collaboration with the group of the professor Roberto Contestabile (Department of Biochemical Science "Rossi Fanelli", Sapienza University of Rome) the four variant PDXK enzyme forms were recombinantly expressed in *Escherichia coli*, purified to homogeneity and characterized with respect to their catalytic properties with PL, PN and PM as substrates. This analysis revealed altered kinetic parameters in all the examined enzymes (Table 1).

	PL as substrate			PN as substrate			PM as substrate		
PDXK	[a]K_M^{PL} (μM)	[a]K_M^{ATP} (μM)	[b]k_{cat} (min^{-1})	[a]K_M^{PN} (μM)	[a]K_M^{ATP} (μM)	[b]k_{cat} (min^{-1})	[a]K_M^{PM} (μM)	[a]K_M^{ATP} (μM)	[b]k_{cat} (min^{-1})
WT	189 ± 27	407 ± 65	58 ± 3	7.0 ± 0.9	104 ± 24	19 ± 2	5.0 ± 0.5	46.2 ± 8.2	6.9 ± 0.2
D87H	2090 ± 173	377 ± 48	40 ± 1	34.1 ± 0.4	125 ± 16	21 ± 3	26.5 ± 6.3	55.3 ± 9.1	7.2 ± 0.4
V128I	3839 ± 507	3096 ± 555	67 ± 5	24.8 ± 0.2	113 ± 6	20 ± 4	51.5 ± 6.3	56.3 ± 7.2	8.5 ± 0.2
H246Q	141 ± 10	901 ± 164	35 ± 1	11.5 ± 2.1	126 ± 1	17 ± 4	34.6 ± 7.1	76.8 ± 15.7	6.3 ± 0.3
A243G	177 ± 10	1024 ± 81	28 ± 1	9.2 ± 0.6	132 ± 23	18 ± 2	31.9 ± 4.7	34.1 ± 7.0	5.0 ± 0.1

Table 1 Kinetic parameters of PDXK variant enzyme forms. All values are the average ± standard deviation of at least three independent determinations. [a]Determined varying the concentration of the related substrate, while keeping the other fixed and saturating. [b]Determined with ATP as fixed, saturating substrate. Values of kcat determined with the vitamer as fixed, saturating substrate were very similar and are not reported for simplicity

In particular, the D87H mutation strongly increased KM for PL, and to a lesser extent also increased K_M for PN and PM, leaving K_M for ATP and k_{cat} almost unaltered. This finding is consistent with the location of the Asp87 residue on an active site loop that plays a pivotal role in the B6 vitamer substrate binding. The

V128I mutation enhanced K_M for PL and K_M for ATP with this vitamer, whereas it did not affect k_{cat}. This result was expected, based on the role of the active site loop containing Val128 in substrate binding and specifically in the interaction with ATP (Fig 16). K_M for PN and PM were also increased, although with these vitamers K_M for ATP and k_{cat} were unaltered. This latter observation is difficult to explain on the basis of the available observed results; however, it suggests that binding of B6 vitamers different from PL may affect the modality of ATP binding by the loop containing Val128. The H246Q mutation somewhat reduced the affinity for ATP when using PL as substrate and the affinity for PM, while it did not affect K_M for PL and PN. In addition, it showed the effect to halve k_{cat} with PL. Finally, A243G mutation displayed a behaviour very similar to that showed by the H246Q mutation. Given the distance of Ala243 and His246 from the active site, the observed alteration of the kinetic parameters, although relatively mild, testifies that the mutation of these residues is somehow transmitted to the active site of the enzyme. Taken together, these findings indicate that the specific changes introduced in the four examined variants reduce the PDXK functionality with the consequent impact on both genome integrity and glucose homeostasis.

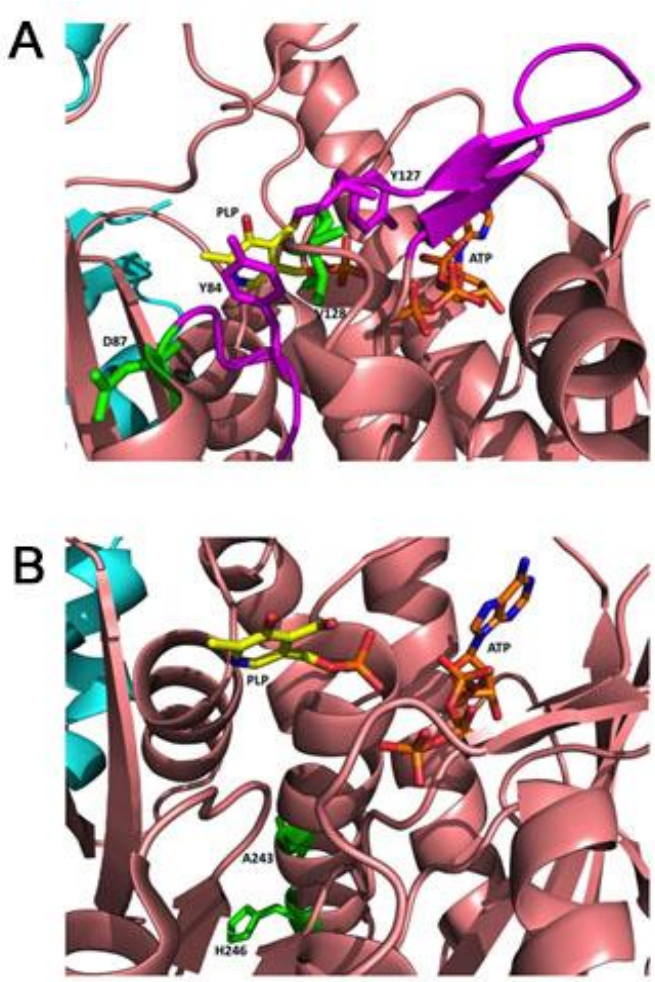


Figure 16 Active site view of human pyridoxal kinase in complex with ATP and PLP. A Active site view of human pyridoxal kinase in complex with ATP and PLP (PDB code 3KEU) showing the position of residues Asp87 and Val128 (in green). The two subunits of the enzyme are shown in salmon and cyan, respectively. The crystal structure represented in this figure was obtained with both PLP and ATP bound at the active site (unpublished structure deposited in the Protein Data Bank; PDB code: 3KEU). The position occupied by PLP is believed to correspond to the site where unphosphorylated B_6 vitamers (PL, PN and PM) also bind. PLP and ATP

are in yellow and orange, respectively. The active site loops involved in binding of vitamin B_6 (left-hand side of the figure) and ATP (right-hand side), as explained in the text, are shown in magenta. B Active site view of the same human pyridoxal kinase structure shown in panel A, from a different perspective, showing the location of residues Ala243 and His246. As explained in the text, the N-terminus of the α-helix to which Ala243 and His246 belong to is positioned at the active site and interacts with the PLP phosphate group.

Part 3

2.3 PLP and cancer in *Drosophila melanogaster*

It is well known that chromosome aberrations are strictly linked to cancer (Bonassi et al., 2004).
The finding that low PLP levels are inversely related to several human cancers (Contestabile et al., 2020) and that PLP deficiency causes CABs in *Drosophila* and human cells (Marzio et al., 2014) prompted us to speculate that one of the mechanisms through which low PLP levels can impact on cancer is by promoting DNA damage. However, before to test this hypothesis, the third aim of this work has been to verify whether low PLP levels could have an impact on cancer in *Drosophila*.
The study of cancer in *Drosophila* largely relies on the use of genetic mosaics in which the over expression of an oncogene such as *ras*, is restricted only to a specific larval body part (e.g. imaginal discs). Advanced tools made it possible to mark cancer cells with GFP protein to allow tumors to be easily detected in vivo. To test the effects of PLP deficiency on cancer we decided to use two different *Drosophila* cancer models (Ras and Ras/Src) which express the oncogenes in the eye antennal disc. Since both cancers give rise only rarely to metastasis, we established to investigate whether PLP deficiency could transform these tumors in more aggressive forms. Interestingly, malignancy of Ras/Scr

tumors was found increased in larvae grown in a medium supplemented with high glucose concentrations (Hyrabahashi et al 2013). Given that low PLP levels cause hyperglycemia, this finding prompted us to envisage that Ras/Scr tumors could be a suitable model for our purposes.

2.3.1 Mosaic analysis with a repressible cell marker (MARCM) strategy

By performing crosses (described in materials and methods section) we generated Ras and Ras/Src cancer models in which GFP-marked tumour cells were specifically produced in larval eye-antennal discs. The molecular mechanism that give rise to these tumours is based on the activity of the flippase (eyFLP) expressed in eye-antennal disc cells which mediates the mitotic recombination at the level of FRT sites. Then, each cell division generates two kinds of cells: GFP-marked tumor cells and non-GFP non-tumor cells (Figure 17). Tumour cells are produced when the mitotic recombination removes Gal80, a repressor of Gal4 (Lee and Luo, 1999); this event enables Gal4 to induce the ectopic expression of both UAS-GFP and UAS-Ras^{G12V} transgenes. Non-tumor cells are instead produced when both copies of Gal80 are segregated into the same daughter cell. In addition, Gal4 is under the control of a *flip out* construct. Between the actin promoter and the Gal4 gene there is a cassette flanked by FRT sites which contains transcriptional termination signals. Thus, only after that this cassette is removed by eyFLP, Gal4 protein can be produced (Ito et al., 1997). Ras/Src tumors are generated in larvae carrying both UAS-Ras^{G12V} construct and the $csk^{Q156Stop}$ mutation (in heterozygous condition), which prevents the expression of Csk, a negative Scr regulator. Ras/Src cells originates, thus, when mitotic recombination segregates both csk^{-} alleles into the same cell (Fig 17).

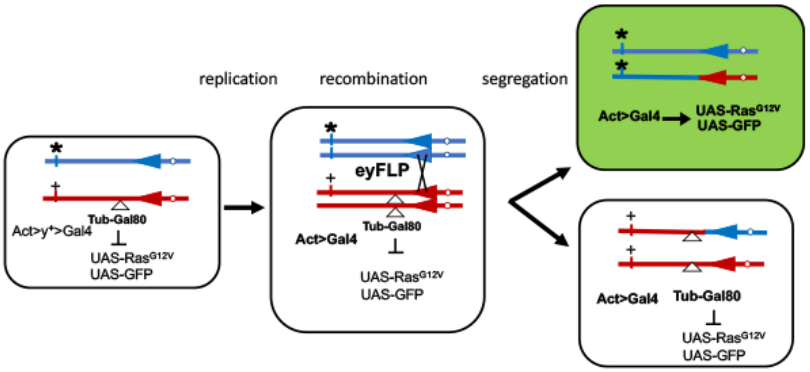


Figure 17 A Drosophila genetic model for studying tumor progression. The expression of the FLP recombinase in the developing eye (eyFLP) mediates mitotic recombination at FRT sites. Mitotic segregation generates two kinds of clones: GFP-marked Ras tumor clones which lose the Gal80 repressor and express both UAS-GFP and UAS-RasV12 constructs, and non-GFP, non-tumor cells that retain Gal 80. Gal 4 is under the control of "flip-out" construct lying between actin promoter and Gal4 gene (Act>y+>Gal4). Ras/Src tumors are produced when mitotic recombination occurring in cells bearing a csk mutation (*) in heterozygous condition generates homozygous csk⁻ clones.

2.3.2 PLP depletion increases the aggressivity of Ras cancers

Before testing the effects of PLP depletion in above described models, we made sure that in our models mitotic recombination occurred only in the eye-antenna disc cells, to exclude any ectopic expression of the flippase. For this purpose we checked that control larvae (generated using MARCM strategy) bearing GFP clones in the eye-antenna disc did not express GFP elsewhere, also when they were growth on the different media indicated below.

To induce PLP deficiency in Ras and Ras/Src cancer models we used the PLP antagonist 4-deoxypyridoxine (4-DP) dissolved into the growth medium at 1mM concentration. Give that sugar

exacerbates the effects of PLP deficiency on DNA damage, we tested 4-DP also in combination to a sugar rich diet (HSD).

HSD medium and, to a lesser extent, 4-DP medium, induces a delay in larval development (about 4 and 2 days respectively). Therefore, in all the experiments, we compared 13 days-old larval progenies from all crosses performed in HDS (or 11 days-old from crosses performed in 4-DP medium) to 7-8 days-old larvae from crosses performed in control growth medium.

To evaluated the effects of PLP deficiency on primary and secondary tumors in ras^{GV12} and ras^{GV12}; $csk^{-/-}$ larvae we measured the GFP-marked cephalic area and examined the GFP-labeled spots on larval cuticle at distant sites from primary tumors. As shown in figure 18 we found that ras^{GV12} larvae grown in the standard medium, displayed cephalic tumors which occupied about 5% of the total larval body. In contrast, larvae grown in 4-DP medium displayed more enlarged cephalic areas (9.4%). Larvae reared in HSD plus 4-DP did not exhibit substantial differences with respect to 4-DP fed individuals, suggesting that, in this context, high sugar levels and 4-DP do not seem to cooperate in exacerbating malignant phenotypes. We found that about 20% of ras^{GV12} larvae grown in 4-DP medium displayed secondary tumors in different body parts. HDS diet did not induce significant metastasis, and the frequency of secondary tumors in larvae reared in 4-DP plus HSD medium was not different from that found in 4-DP feed larvae. These results suggest that low PLP levels impact on Ras cancer by both increasing primary tumors and triggering metastasis.

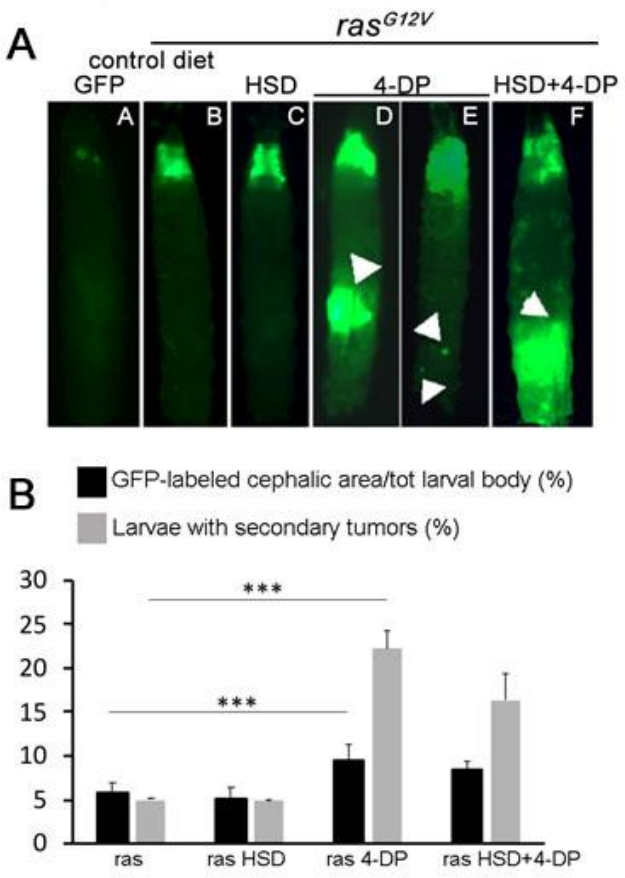


Figure 18 PLP deficiency increases malignant phenotype of Ras tumors. A Examples of larvae with metastasis (A) Control larvae, grown in standard medium, expressing only GFP. (B-F) Larvae grown in the indicated media. Arrows indicate secondary tumors. B Measurement of GFP-labeled cephalic area (the tumor area) in relation to the total body area and percentage of larvae with metastases. Each column represents the mean value ± SEM calculated on 100 larvae analysed in five different experiments. Student's t test and Chi-squared test have been respectively used to calculate significance of head areas and secondary tumors. *** significant with p <0.001.

We found that *ras*^{GV12}*; csk*$^{-/-}$ larvae reared on control diet displayed a reduced GFP cephalic area (2.9%) with respect to *ras*^{GV12} larvae (Fig 19). This is consistent with the fact that Src overexpression promotes apoptosis and *ras*^{GV12} is unable to counteract this process (Hirabayashi et al 2013). In contrast we observed that 4-DP diet induced a significant increased proliferation of the primary tumor (up to 5.5%). The same effect has been observed in *ras*^{G12V} *csk* $^{-/-}$ larvae grown in HSD, accordingly to Hyrabayashi et al., (2013). In addition we found a significant further increase of cephalic area in larvae growth in 4-DP plus HSD with respect to larvae reared in either 4-DP or HSD diet. Interestingly 23% of 4-DP fed larvae also displayed secondary tumors and a similar frequency was found in HDS-fed larvae according to Hyrabayashi et al., (2013). Remarkably although we found that rearing larvae in 4-DP plus HSD exacerbated the effect on primary tumors, we did not observe the same effects for secondary tumors, suggesting that there may be different mechanisms at the basis of primary and secondary tumors.

Taken together these results indicate that PLP depletion impacts on both primary and secondary tumors in Ras/Src model and in addition that PLP deficiency may cooperate to HDS in the development of the primary tumors.

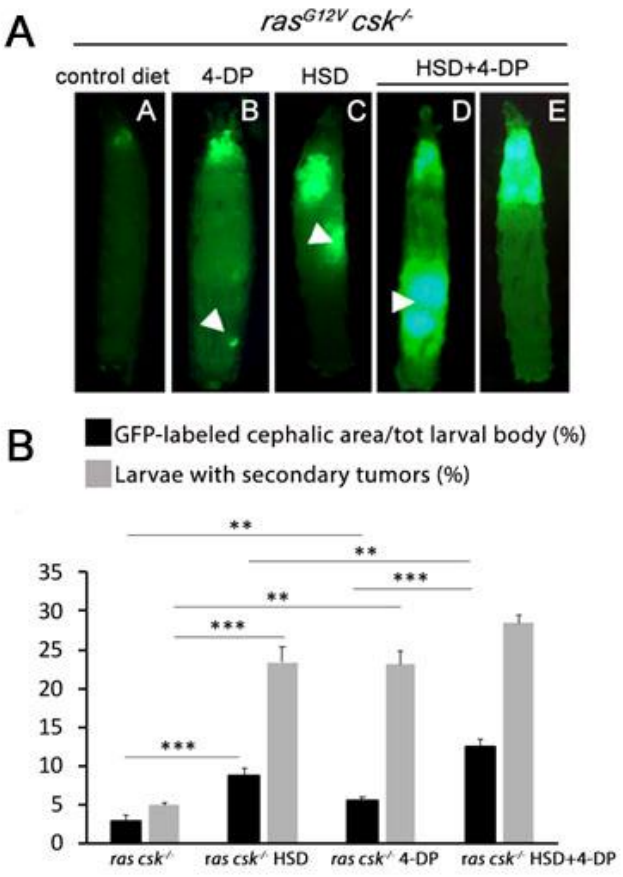


Figure 19 PLP deficiency increases malignant phenotype of Ras/Src tumors. A Examples of metastasis in larvae grown in the indicated media. Arrows indicate secondary tumors. B Measurement of GFP-labeled cephalic area (the tumor area) in relation to the total body area and percentage of larvae with metastases. Each column represents the mean value ± SEM calculated on 100 larvae analyzed in five different experiments. Student's t test and Chi-squared test have been respectively used to calculate significance of head areas and secondary tumors. **, *** significant with p <0.01and <0.001 respectively,

2.3.3 Low levels of PLP promote tumor cells migration into the hemolymph

We found that *ras*^{G12V} as well as *ras*^{G12V};*csk*$^{-/-}$ larvae, reared in 4-DP, consistently displayed GFP-labeled cells scattered in their hemolymph (Fig. 20). In contrast, we never found GFP cells in the hemolymph of GFP control larvae. This finding represents a first indication that secondary tumors induced by PLP deficiency are true metastasis generated by cells that detach from primary tumors and float throughout the hemolymph before to colonize distant sites. Remarkably the frequency of larvae with GFP cells floating in the hemolymph does not perfectly match to the frequency of larvae which show metastasis (Fig 20B). This is probably due to the fact that not all tumor cells have the chance to colonize distant sites.

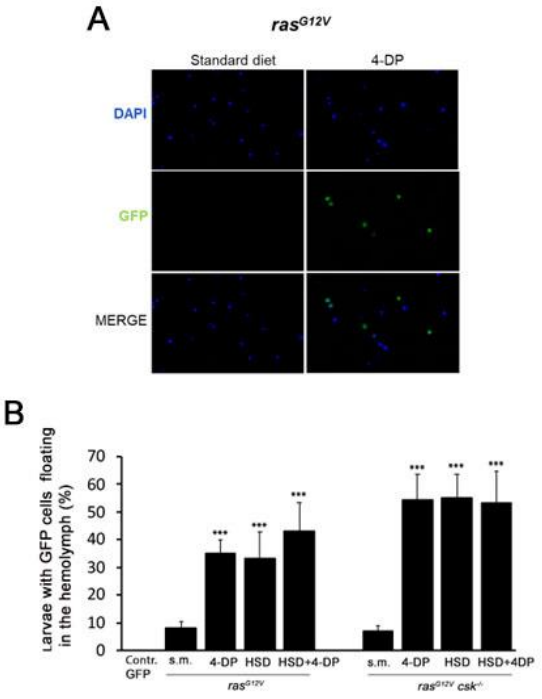


Figure 20 GFP cells floating in the hemolymph of 4-DP treated larvae. A Examples of GFP cells floating in the hemolymph of rasG12V cells. B percentage of larvae reared in the indicated media with GFP cells in the hemolymph

2.3.4 Rescue of secondary tumors in Ras/Src cancers

To confirm that reduced PLP levels can promote the formation of secondary tumors, we tested whether PLP supplementation was able to reduce metastasis in ras^{G12V} $csk^{-/-}$ larvae reared on HSD. As shown in figure 21 we found that PLP significantly reduced the frequency of secondary tumors from 23% to 10%. Interestingly, a similar result has been obtaned by treating same larvae with the antioxidant compound ALA (10mM). Since ALA is able to rescue the CABs induced by PLP deficiency, the reduced frequency of secondary tumors in ALA-treated larvae suggests that PLP deficiency might in part to impact on cancer by promoting DNA damage.

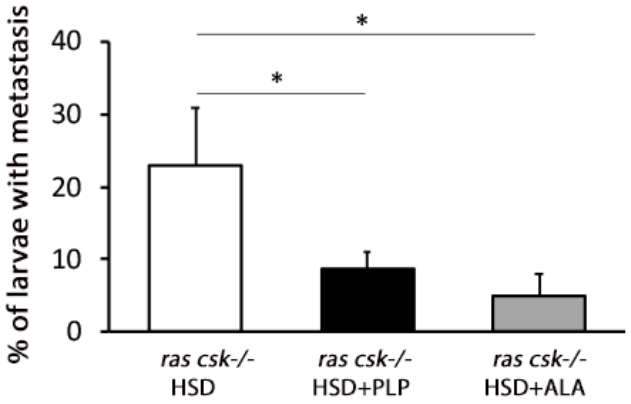


Figure 21 Treatment with PLP as well as ALA reduces secondary tumors in ras^{G12V}; $csk^{-/-}$ larvae. Each column represents the mean value ± SEM calculated on 50 larvae analyzed in 3 different experiments. Significance was calculated using the Chi-squared test. * significant with p <0.05

3. DISCUSSION

3.1 Role of *sugarlethal (sgll)* in the maintenance of genome integrity and glucose homeostasis

The study of micronutrients is a topic of great interest due to beneficial effect of vitamins and minerals on several pathologies. In contrast, detrimental effects produced by vitamin deficiencies can be comparable to those induced by common mutagenic agents (Contestabile et al., 2020). Nevertheless, the knowledge of how vitamins exert their protective role is still in its infancy. Epidemiological and intervening studies have been useful to associate some vitamins to specific diseases, but the employment of model organisms represents the best choice to shed light on cellular e molecular underlying mechanisms. In the last decade *Drosophila melanogaster* has emerged as precious model organism also for studies concerning metabolic diseases. This has been made possible by the discover that flies possess the major pathways that regulate cellular metabolism in humans (Owusu-Ansah and Perrimon, 2014; Padmanabha and Baker, 2014; Teleman et al., 2012).

In *Drosophila*, as well as in humans, the active form of vitamin B6 (PLP) is produced by the concerted action of a kinase (PDXK) and an oxidase (Sgll) which, recycle B6 vitamers such as PL,PM and PN from food, in the salvage pathway.

It has been demonstrated that mutations in *dPdxk* gene cause CABs and hyperglycemia and, in addition, that these two phenotypes are related (Marzio et al., 2014). From these studies emerged a new model to explain how low PLP levels can impact on DNA. Besides to the hypothesis that low PLP levels could impact on genome integrity due to the PLP role as cofactor in reactions regarding DNA metabolism, results obtained in *Drosophila* revealed another possible mechanism pinpointing hyperglycemia as responsible for CABs.

This hypothesis was the starting point for the research carried out in this thesis. First of all we verified whether impairing the salvage pathway by blocking *sgll* function would result in the same phenotypes caused by $dPdxk^{1}$ mutations. It should be noted that the lack of *sgll* function does not prevent PLP to be formed directly from PL by the action of Pdxk. However, in the *Drosophila* standard medium PL is present only in traces, suggesting that most of PLP production involves the activity of Sgll. The silencing of *sgll* gene resulted in CABs and diabetic phenotype. Interestingly $sgll^{RNAi}$ individuals reach the adult stage differently from $dPdxk^{1}$ mutants. This allowed us also to detect other diabetic features typical such as reduced body size, that further confirmed the causative role of low PLP levels in diabetes onset. Regarding the involvement of PLP in diabetes there are some evidence showing that diabetes can lower PLP levels, but only a few direct proofs that, instead, low PLP levels can trigger diabetes onset (Mascolo and Vernì, 2020). Results obtained in flies depleted of salvage enzymes, represent a strong direct evidence that low PLP levels can cause diabetes. In addition, we also found that $Sgll^{RNAi}$ accumulate AGEs which are genotoxic metabolites induced by hyperglycemia. The finding that the antioxidant alpha lipoic acid (ALA) was able to reduce both AGEs and CABs strongly reinforced the model that CABs are a byproduct of hyperglycemia induced by low PLP levels. Interestingly same results have been found in PDXK depleted human cells, suggesting that the relationship between PLP diabetes and DNA damage relies on mechanisms conserved throughout the evolution.

3.2 PDXK human variants impact on genome integrity and glucose homeostasis

In the second part of this work *Drosophila* has been used to validate the effects of four human *PDXK* variants on genome integrity and glucose metabolism. We previously showed that

silencing *PDXK* gene in human cells results in CABs. In addition low expression levels of *PDXK* have been correlated to lung cancer (Galluzzi et al., 2012) and more recently also to insulin resistance which leads to type 2 diabetes (Moreno-Navarrete et al., 2016). However in literature, data are lacking associating specific PDXK variants to diseases; only a recent work reported the association of two biallelic mutations of *PDXK* to polyneuropathy (Chelban et al., 2019).

Databases contain *PDXK* variants (from heterozygous carriers), some of which carry changes in amino acid positions conserved in *Drosophila*. Here we used *Drosophila* to validate the effects of four human variants (D87H, V128I, H246Q reported in the Exome variant server and the novel A243G variant) of *PDXK* on genome integrity and glucose metabolism. We found that none of the human variants expressed in *dPdxk*^{1} mutant flies was able to rescue CABs, differently from wild type *PDXK* (Marzio et al., 2014). This finding reinforces the notion (suggested by in silico analysis) that the examined variants are loss-of-function alleles. The expression of all these variants did not rescue hyperglycemia caused by *dPdxk*^{1} mutation nor the accumulation of AGEs supposed to be largely responsible for CABs in *dPdxk*^{1} flies. By considering that the A243G variant has been isolated during an ongoing screening on diabetic patients (carried out in collaboration with prof. Fabrizio Barbetti, Tor Vergata University, Rome), the impaired rescue of hyperglycemia displayed by this variant is particularly interesting and could be a preliminary indication of the association of *PDXK* gene to diabetes.

The kinetic characterization of the mutant enzymes showed that all mutations affected the catalytic activity of PDXK, although with different modalities. We found that the effect of both D87H and V128I mutations were more drastic with respect to H246Q and A243G, according to the location of the former couple of

residues in a more critical region of the enzyme. Also, it is worth noting that D87H and V128I behave similarly, and also variants H246Q and A243G display similar biochemical defects, in agreement with the relative proximity of these residues. Interestingly, PLP vitamer supplementation impacted in different ways on CAB frequency induced by the different variants. This behaviour is consistent with the kinetic parameters of mutant enzymes. In particular, the higher K_M for PL (about 10-fold than wild type) displayed by D87H explains why this mutant protein did not respond to PL. Differently, K_M values for PM and PN about 5 times higher than wild type explain the rescue observed only at 1 mM concentration. The very high K_M for both ATP and PL found in the V128 mutant protein explains why PL failed to rescue CABs, whereas PN and PM reduced CAB frequency but only at the higher concentration. The H246Q variant, whose mutant enzyme form has normal kinetic parameters with PN, responds to both concentrations of this vitamer. The same mutant, displaying slightly altered kinetic parameters for ATP (when PL is used as substrate) and PM, responded positively to these precursors but only at the higher concentration. The A243G mutant enzyme displayed kinetic parameters very similar to those showed by H246Q. Similarly to H246Q, this variant responded to PL and PM; however, 0.5 mM PN was unable to reduce CAB frequency. Such different effects of *PDXK* mutations on the catalytic properties of PDXK are very interesting, since they are related to structure-function relationships of the enzyme. However, their full understanding is not possible on the basis of the available data and is postponed to future investigations.

In *Drosophila* heterozygous *dPdxk*^{1} mutations do not result in CABs. Thus, we expect the same behaviour also in humans. However, it is reasonable to envisage that in certain contexts in which PLP levels are low, a reduced functionality of PDXK can be particularly critical. For example, it is known that during pregnancy an increased PLP demand to support foetal

development causes a drastic reduction of PLP levels that, in some cases, can contribute to gestational diabetes onset (Mascolo and Vernì, 2020). Thus, we can envisage that in pregnant women *PDXK* variants, also carried in heterozygous condition could be dangerous. Analogously, *PDXK* variants in heterozygous condition could also impact on genome integrity in either patients treated with drugs that reduce PLP levels or also in patients affected by pathologies such as celiac disease and diabetes which *per se* decrease PLP levels (Clayton, 2016; Okada et al., 1999; Nix et al., 2015).

3.3 PLP deficiency impacts on cancer in *Drosophila*

Growing evidence associates vitamin B6 to cancer, however, this relationship is rather complex and based on apparently contrasting data. It has been reported that *PNPO* overexpression is associated to several cancers (Chen et al., 2017; Zhang et al., 2017; Ren et al., 2019). In line with this finding, *PDXK* knockdown blocks cell proliferation in myeloid leukemia cells (Chen, 2020). However, in contrast, elevated levels of *PDXK* have been shown to constitute a good prognostic marker in patients affected by non-small cell lung carcinoma (NSCLC) (Galluzzi et al., 2012). Consistently an inverse relationship has been observed between PLP serum levels and development of many kinds of cancer including pancreas and lung cancers (Kayashima et al., 2011; Gylling et al., 2017; Zuo et al., 2019). Galluzzi et al., (2013) tried to correlate these contrasting data in a model according to which tumors can be sensitive to either high levels or low levels of vitamin B6 depending on the developmental stages in which they are. In the early stages, high levels of vitamin B6 are required to sustain high rate of proliferation. The excessive consumption of this vitamin leads, however, to the establishment of a status of vitamin B6 deficiency that may promote tumor progression for following reasons: (1) vitamin B6 is involved in immune response (2) vitamin B6 is

involved in one-carbon metabolism, essential for genomic stability maintenance; and (3) defects in vitamin B6 metabolism, such as those ensuing the downregulation of *PDXK* compromise the ability of cancer cells to die in response to stress conditions such as nutrient deprivation and hypoxia (Galluzzi, 2013).

In this work we examined the effect of reduced PLP levels in larvae in which tumors were already developed, thus in the stage sensitive to a decrease of this vitamin according to aforementioned model. In the last decade *Drosophila* has become a precious model to dissect cancer biology thanks to the discover that flies share with humans most of the signalling pathways involved in carcinogenesis. Interestingly many crucial pathways involved in the regulation of human cancers were discovered first in flies (Mirzoyan et al., 2019).

The rationale behind the use of Ras and Ras/Src cancer models in this work is based on several reasons: (1) The overexpression of *ras* (due to G12V mutation) has been one of most used systems to perform several screenings aimed both at isolating genes involved in cancer, and also at testing antineoplastic drugs (Gonzalez 2013) (2) The Ras/Scr model has been chose because it has been shown to be sensitive to a sugar rich diet. In particular Ras/Src transformed cells are more aggressive when larvae are reared on a sugar rich medium (HDS) (Hyrabayashi et al., 2013). This discover is extremely interesting because a growing body of evidence indicates that the risk of specific cancers increases in patients with metabolic dysfunction, including obesity and diabetes (Giovannucci et al., 2010). Given that low PLP levels have a significant impact on glucose metabolism we reasoned that Ras/Scr model might be an useful system for our purposes (3) Moreover, the oncogenes *ras* and *scr* are deregulated in several human cancers (Fernández-Medarde and Santos, 2011; Shields et al. 2011) thus the results obtained in *Drosophila* can be possibly exported to humans.

To test the effect of PLP deficiency on these tumor models we employed the 4-DP, a PLP antagonist which has been proved to be the most effective PLP inhibitor in inducing CABs in wild type *Drosophila* neuroblasts (Marzio et al., 2014). Our analysis revealed that 4-DP treatment increased the proliferation of primary tumors occurring in eye-antennal discs and also triggered the formation of metastasis. We also found that a sugar rich diet (HDS) did not increase the overproliferation of ras^{G12V} cells and that HSD plus 4DP diet did not exacerbated the effect showed by larvae grown only on 4-DP. This suggested that PLP and HSD do not cooperate to induce ras^{G12V} cancers and that PLP do not impact on this cancer through glucose metabolism. To explain the effect of 4-DP on ras^{G12V} cells we can envisage two scenarios. In the first, as a consequence of the reduced PLP availability induced by 4-DP, the coenzyme is preferentially delivered to the PLP-dependent enzymes involved in cellular metabolism. This would cause a reduced activity of PLP- dependent enzymes involved in DNA metabolism such as serine hydroxymethyltranferase (SHMT) and glycine decarboxylase (GLDC). This situation would increase the UTP/TTP ratio resulting in DNA damage. Alternatively, PLP would be preferentially delivered to SHMT and GLDC enzymes; thus would be ensured a faster replication to sustain tumor overgrowth.

Ras /Src cancers resulted, instead, sensitive to HSD, in line with results obtained by Hyrabayashi (2013). We also found that 4-DP treatment as well as HSD treatment resulted in primary and secondary tumors. Interestingly ras^{G12V} $csk^{-/-}$ larvae grown on 4-DP plus HSD medium displayed more enlarged GFP-positive eye field than tumors from larvae grown in either PLP or HSD media. In contrast the observed increase of secondary tumors did not result statistically significant. This finding suggests that PLP may impact on primary and secondary tumors through different mechanisms, and that only in primary tumors the mechanism may involve sugar metabolism. Interestingly also Hyrabayashy et al.,

(2013) hypothesized the existence of different mechanisms for primary and secondary tumors. These authors demonstrated that HDS promotes overgrowth of *ras*^{G12V} *csk*$^{-/-}$ primary tumors by impacting on insulin signalling pathway, but they did not explored in detail the mechanisms underlying cell migration. They provided evidence that HSD-induced insulin resistance triggers the overexpression, in eye disc cells, of the Insulin receptor (INR) causing insulin sensitivity, which in turn increases glucose flux into the cells. In addition, that elevation of insulin pathway signalling through HDS leads to elevated Diap1 which blocks apoptotic cell death. Mutations overexpressing INR produce overgrowth of primary tumors analogously to HSD and are sufficient to elevate *diap1* expression. Remarkably, INR mutations do not promote secondary tumors, suggesting that primary and secondary tumors in HSD fed larvae are produced by different mechanisms (Hyrabayashi et al., 2013). Given that 4-DP plus HSD medium exacerbated the overgrowth of primary tumors we can envisage that 4-DP can impact on these tumors by inducing insulin resistance and, as a consequence, by impacting on INR receptor. However we obtained preliminary data (not shown) indicating that 4-DP treatment increases apoptosis in wild type eye disc cells suggesting that reduced PLP levels do not promote tumor overgrowth by counteracting apoptosis. An alternative hypothesis is that 4-DP may impact on Ras/Src primary tumors by promoting DNA damage.

Interestingly the treatment of *ras*^{G12V} *csk*$^{-/-}$ cells with PLP reduced the frequency of metastasis and a similar effect was observed after ALA treatment. Given that ALA was shown to be able to rescue CABs induced by PLP deficiency, these results suggest that in part the effect of PLP on Ras/Src cancer model may be mediated by DNA damage. We obtained preliminary encouraging data (not included in the thesis) showing the 4-DP induces CABs in these tumors. These data will be expanded in a future research which

will investigate even whether PLP impacts on cancer as cofactor of enzymes involved in DNA metabolism, as antioxidant or both.

4. MATERIALS AND METHODS

4.1 Stocks and crosses

sgll ^{v105941} line was obtained from the Vienna Drosophila Resource Center (VDRC) stock center. To generate *sgll* ^{RNAi} individuals we crossed females from the *sgll* ^{v105941} line to *actin Gal4/CyO-GFP* males and selected non-*GFP* larvae. To mutagenize *sgll* using the *in vivo* CRISPR Cas9 system (https://fgr.hms.harvard.edu/using-trip-crispr-lines) we crossed *y sc v P{TKO.GS01766} attP40* females to *Act5C-Cas9* males and examined the brains of the larval offspring (named *act-cas9* gRNA-*sgll*). *dPdxk*^{1} mutation was previously described in Marzio et al., 2014. To introduce the transgenes carrying the PDXK variants (*PDXK*^{VAR}) in a mutant *dPdxk*^{1} background we crossed *PDXK*^{VAR}*/ CyGFP; MKRS/TM6B* females to *CyGFP/Sco; dPdxk*^{1} */TM6B* males. The progeny of this cross, *PDXK*^{VAR} */CyGFP; dPdxk*^{1} */TM6B*, was crossed inter se to obtain a stable stock. From this stock larvae *PDXK*^{VAR}*; dPdxk*^{1} */ dPdxk*^{1} selected for their non-*Tubby* phenotype have been analyzed.

To test *PDXK*^{VAR} *on a dPdxk*^{1} /+ background we analyzed larvae *PDXK*^{VAR}*/Cy; dPdxk*^{1} */TM6B* from the same stock.

To generate Ras cancer models we crossed:

UAS ras^{G12V} */ UAS ras*^{G12V}*; FRT82B / FRT82B* males to *yw eyFlp; Act> y +> Gal4 UAS GFP; FRT82B, Tub Gal80* females and the *yw* e*yFlp*; *UAS ras*^{G12V} */ Act> y +> Gal4 UAS GFP; FRT82B / FRT82B, Tub Gal80* larval progeny has been examined.

To generate Ras/Scr cancer model we crossed:

UAS ras^{G12V}; FRT82B csk^{Q156} / TM6B males to *yw eyFlp; Act> y +> Gal4 UAS GFP; FRT82B,* Tub Gal80 females and the non-Tubby larval progeny has been examined.

To generate controls we crossed:

CyGFP / Sco; FRT82B / TM6B males to *yw* e*yFlp*; *Act> y +> Gal4 UAS GFP; FRT82B, Tub Gal80* females and non-Tubby, non-*CyGFP* larvae were examined.

The *Oregon R* strain was used as control. All stocks were maintained and crosses were made at 25°C on standard *Drosophila* medium (prepared from cornmeal, sucrose, brewer's yeast, agar, water and treated with propionic acid) or supplemented media (see below). The balancers and the genetic markers used in these crosses are described in detail in FlyBase (http://fybase.bio.indiana.edu/).

4.2 Chromosome preparations and immunostaining

Colchicine-treated *Drosophila* metaphase chromosome preparations for CAB scoring were obtained as previously described (Gatti and Goldberg, 1991). For immunostaining brain preparations from third instar larvae were carried out according to (Bonaccorsi et al., 2000). Preparations were rinsed in phosphatebuffered saline 0.1% Triton (PBST), incubated overnight at 4°C with primary antibodies diluted in PBST, rinsed in PBST, and then incubated for 1 hr at room temperature with the secondary antibody. The primary antibodies used were: rabbit anti-histone H2AvD pS137 (1:100 in PBST; Rockland code #600-401-914) and rabbit anti-human AGE antibody (1:200 in PBST, ab23722; Abcam,UK). Both antibodies were detected with Alexa-Fluor-555-conjugated antirabbit antibody (1:300 in PBST; Molecular Probes, Eugene, OR). All fixed preparations were mounted in Vectashield H-1200 with 4,6 diamidino-2-

phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to stain the DNA. To quantify cells positive to γ-H2Av or to AGE immunostaining at least 1,000 cells were analyzed for each genotype. All cytological preparations were examined with a Carl Zeiss (Thornwood, NY) Axioplan fluorescence microscope, equipped with an HBO100W mercury lamp and a cooled charged-coupled device (CCD camera; Photometrics CoolSnap HQ).

4.3 Treatments of larvae and isolated brains

To evaluate the effects of PLP precursors on CAB frequency and hyperglycemia we reared larvae on a standard medium supplemented with PLP, PL, PN or PM 1 mM (or 0.5mM). To test the in vitro effects of glucose and α-lipoic acid (ALA) on CABs, AGEs, and γ-H2Av foci, and the effect of PLP on AGEs, brains were dissected from third instar larvae and incubated in 2 ml of saline supplemented with 10% fetal bovine serum (Corning) for 4 hr with or without addition of 1% glucose, 10 mM ALA or 1mM PLP. For chromosome preparations 1 hr before fixation brains were treated with colchicine. For γ-H2Av foci and AGE detection brains were treated according to the above-described procedure for immunostaining.

4.4 Glucose measurement and weight analysis

Glucose concentration in hemolymph from third instar larvae was measured using the Infinity Glucose Hexokinase reagent (Thermo scientific). Hemolymph collection and glucose measurement were done as described in Marzio et al.2014.

For weight analysis, 5 to 6 samples of 15 flies each were weightedwith a precision weight scale (Gibertini E42; range 0.1 mg-120 g). Flies were reared under the same growth conditions and were cage matched (2 days old) before weighing.

4.5 Wing measurement

Wings were dehydrated in isopropanol and mounted on a slide in Canada Balsam (Carlo Erba # 321553) Cell density was assessed by counting the number of thricomes on the dorsal wing surface in a 10.000 μm^2 area just posterior to the posterior cross vein. Cell size was the reciprocal value of cell density. The approximate number of cells in the whole wing was calculated by multiplying the cell density by wing area (the alula and the costal cells were excluded).

4.6 Lipid droplets measurement

For LD staining, 3rd instar larvae were dissected in PBS. Extracted fat bodies were fixed in 3,7% formaldehyde for 30 min at room temperature. Tissues were then rinsed twice with PBS and incubated for 30 min in a 1:1000 dilution with PBS of 0.5mg/ml Nile Red. After rinsed twice with PBS fat bodies were mounted in Vectashield Antifade Mounting Medium and analyzed to a confocal microscope Zeiss LSM 780. Slides were imaged at 63x magnification. ImageJ software was used to quantify droplet size.

4.7 Life span test

Flies were anesthetized briefly and transferred into a standard medium. Fifteen flies were grouped into a vial and scored daily for lethality. Flies were transferred to fresh vials every 2-3 days. Five replicate vials were examined for genotype and each experiment was repeated three times.

4.8 Analysis of tumors in larvae

Larvae from crosses described above were selected and placed into a 96-well cell culture plate containing 1x PBS and left at 4 ° C for at least 2 hours. This treatment was carried out to

immobilize the larvae so that they could be easily scored. The larvae were then placed on a slide and examined at the microscope to visualize GFP-labeled primary and secondary tumors.

4.9 Hemolymph analysis

Larval hemolymph was examined to highlight the presence of GFP cells by inspection of the larval body directly under the microscope or in hemolymph preparations extracted in mineral oil.

4.10 Microscopy

Cytological and hemolymph analysis were performed using a Zeiss III RS microscope with a 100W HBO lamp and a Nikon TE 2000 inverted fluorescence microscope connected to a CCD (Charge Coupled Device) camera for fluorescence analysis. CoolSnap (RoperScientific) software was used for the acquisition of digital images which have been processed using Adobe Photoshop 7.0. To detect larval GFP-labeled tumors, a Zeiss Axioskop2 microscope with 2.5x magnification was used and the pictures were taken by a digital camera mounted on the microscope. The GFP larval head area was measured and analyzed using ImageJ software.

4.11 Picture acquisition

Pictures of adult flies were taken using a Nikon D5200 digital camera mounted on a stereomicroscope (Nikon SMZ-1). Pictures were taken using a 1/6 second exposure, and 800 iso.

4.12 Site directed mutagenesis

D87H, V128I, H246Q and A243G PDXK variants were generated by introducing mutations (by PCR based site-directed

mutagenesis, QuikChange II XL Site-Directed Mutagenesis Kit, Agilent) into the wild type HA-tagged PDXK gene.

Primers used are:

D87H F gccaggaacgacttgtgcctcgtataacctgtg

D87H R cacaggttatacgaggcacaagtcgttcctggc

V128I F gcgaaggctcgatgtacatcccggaggacc

V128I R ggtcctccgggatgtacatcgagccttcgc

H246Q F gttattggggtgcttctgtgtccacgccagg

H246Q R cctggcgtggacacagaagcaccccaataac

A234G F cttgtgtgtccaccccaggagcatggc

A243G R gccatgctcctggggtggacacacaag

PDXK genes carrying the mutations were then cloned into a pCaSpeR-tubulin vector35. The correct generation of the variants was verified by Sanger sequencing and recombinant plasmids were introduced in flies by germline transformation (Best Gene Inc. Service, USA).

4.13 Western blotting

Extracts for Western blotting were prepared by lysing samples of 20 brains in 150mM NaCl, 50 mM Tris-HCl, pH 7.5, 30 mM NaF, 25 mM b-glycerophosphate, 0.2 mM Na3VO4, Triton X-100 1%, and complete Protease Inhibitor Cocktail (Roche). Extracts were immunoblotted according to Somma et al., 2002 blotted proteins were detected using an antibody against HA tag (Anti-HA-

Peroxidase 12013819001 Roche). Anti-alpha tubulin (SIGMA) was used as loading control. Primary antibodies were detected using HRP conjugated anti-mouse and anti-rabbit IgGs and the ECL detection kit (all from GE Healthcare). Chemiluminescent blots were imaged with the ChemiDoc MP imager (Bio-Rad). Band intensities were quantifed by densitometric analysis with Image Lab sofware (Bio-Rad).

4.14 Nucleic acid extraction

Preparation of fly RNA, PCR, RT-PCR and agarose gel electrophoresis were performed with standard procedures. RNA extraction was performed with the RNeasy Mini Kit (Qiagen, Hilden, Germany). For RT-PCR we used 1 μg of RNA to synthesize complementary DNAs (cDNAs)using the Quantitect Reverse transcription kit (Quiagen cod # 205311) For cDNA amplification were used the following primers:

sgll F	GCACTTCGTCTGAAGTACTGT
sgll R	ACCTCGTCTAAAGCGGATGCG
rp49 F	TACAGGCCCAAGATCGTGAA
rp49 R	ACGTTGTGCACCAGGAACTT

4.15 Statistical analysis

Results are expressed as means ± SEM; probability values < 0.05 were considered statistically significant. Statistical analysis of the data was done with the two-tailed Student's t-test or with the χ^2-test.

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LIST OF PUBBLICATIONS

1. Merigliano C., **Mascolo E.**, La Torre M., Saggio I., Verni F. (2018). Protective role of vitamin B6 (PLP) against DNA damage in Drosophila models of type 2 diabetes. Sci. Rep. 8, 11432.

2. Merigliano C., **Mascolo E.**, Burla R., Saggio I., Verni F. (2018). The relationship between vitamin B6, diabetes and cancer. Front. Genet. 9, 388.

3. Merigliano C., **Mascolo E.**, Cesta A., Saggio I., Vernì F. (2019). A New Role for Drosophila Aurora-A in Maintaining Chromosome Integrity. Chromosoma 128, 41-52.

4. **Mascolo E.**, Barile A., Mecarelli L.S., Amoroso N., Merigliano C., Massimi A., Saggio I., Hansen T., Tramonti A., Di Salvo M.L., Barbetti F., Contestabile R., Vernì F. (2019). The expression of four pyridoxal kinase (PDXK) human variants in Drosophila impacts on genome integrity. Sci. Rep. 9, 14188.

5. **Mascolo E.**, Amoroso N., Saggio I., Merigliano C., Verni F. (2020). Pyridoxine/pyridoxamine 5'-phosphate oxidase (Sgll/PNPO) is important for DNA integrity and glucose homeostasis maintenance in Drosophila. J. Cell. Physiol. 235, 504–512.

6. **Mascolo E.**, Verni F. (2020). Vitamin B6 and Diabetes: Relationship and Molecular Mechanisms. International Journal of Molecular Sciences. 21, E3669.