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Kevin Legent, Magali Mas, Annie Dutriaux, Solange Bertrand, Domenico Flagiello, et al.. in vivo analysis of *Drosophila* deoxyribonucleoside kinase function in cell cycle, cell survival and anti-cancer drugs resistance.. *Cell Cycle*, 2006, 5(7), pp.740-49. hal-00019320

HAL Id: hal-00019320

<https://hal.science/hal-00019320v1>

Submitted on 20 May 2006

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***in vivo* analysis of *Drosophila* deoxyribonucleoside kinase function in cell cycle, cell survival and anti-cancer drugs resistance**

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Keywords

Drosophila, deoxyribonucleoside kinase, dNK, antifolate resistance, apoptosis, proliferation, growth, dE2F1

Acknowledgements

The authors acknowledged the excellent technical assistance of B. Legois and M-C. Gendron and English corrections by A. Kropfinger. They are grateful to C. Carré, B. Edgar, H. Richardson, J.P., W. Knecht and S.Carroll and the Bloomington *Drosophila* stock center for fly strains and antibodies. This work was supported by an ‘Action Thématique concertée (ATC) vieillissement’ grant from the Institut National pour la Santé Et la Recherche Médicale (INSERM) and the ‘Association Française contre les Myopathies’ (AFM). K.L. is supported by the Fondation pour la Recherche Médicale (FRM)

Abbreviations

deoxyribonucleoside kinase (dNK), thymidine (dT), thymidine kinase (TK), aminopterin (AM), methotrexate (MTX), retinoblastoma-family protein (RBF), loss of function (LOF), gain of function (GOF), Zone of Non-proliferating Cells (ZNC)

Abstract

in vitro studies have shown that *Drosophila melanogaster* has a highly efficient single deoxyribonucleoside kinase (dNK) multisubstrate enzyme. dNK is related to the mammalian Thymidine Kinase 2 (TK2) group involved in the nucleotide synthesis salvage pathway. To study the dNK function *in vivo*, we constructed transgenic *Drosophila* strains and impaired the nucleotide *de novo* synthesis pathway, using antifolates such as aminopterin. Our results show that *dNK* over-expression rescues both cell death and cell cycle arrest triggered by this anti-cancer drug, and confers global resistance on the fly. Moreover, we show that fly viability and growth depend on the exquisite ratio between *dNK* expression and its substrate thymidine (dT) in the medium, and that increased dT concentrations trigger apoptosis and a decrease in body mass when *dNK* is mis-expressed. Finally, *dNK* expression, unlike that of *TK2*, is cell cycle dependent and under the control of CyclinE and the dE2F1 transcription factor involved in the G1/S transition. dNK is therefore functionally more closely related to mammalian TK1 than to TK2. This strongly suggest that *dNK* plays a role in cell proliferation in physiological conditions.

Running title

in vivo study of *Drosophila* dNK

Introduction

In the cell, the building blocks of DNA biosynthesis (deoxyribonucleotides) are provided through both *de novo* and salvage pathways. The mammalian deoxyribonucleoside kinases are deoxycytidine kinase (dCK), thymidine kinase 1 (TK1) and 2 (TK2) and deoxyguanosine kinase (dGK). These enzymes phosphorylate deoxyribonucleosides and thereby provide an alternative to *de novo* synthesis of DNA precursors. The salvage pathway is a complementary route for providing cells with DNA precursors, primarily utilizing uptake of deoxyribonucleosides from the extracellular space, derived from nutrients or degraded DNA (see Fig 1). These deoxyribonucleosides enter the cell membrane by facilitated diffusion *via* a nucleoside carrier protein with a wide specificity.¹ Nevertheless, the salvage pathway is not a prerequisite for cell survival in replicating mammalian cells, since the *de novo* synthesis can, in principle, provide all the DNA precursors necessary for cell growth. Under normal culture conditions, neither the viability nor the growth of cells seems to require the uptake of exogenous deoxynucleosides. Nevertheless, the salvage pathways do have important roles.² In mammals, TK1 is cell cycle regulated, whereas TK2, a mitochondrial enzyme, is no.³ Moreover, mutations in genes of the salvage pathway are responsible for various human diseases. Several TK2 mutations have been linked to severe skeletal myopathy in infancy due to mitochondrial DNA depletion.⁴ In addition, Herpes Simplex Virus TK (HSV-TK) acts as activator of various pro-drugs.⁵

In *Drosophila* and other insects, only one member of the deoxyribonucleoside kinase family (*dNK*) that displays broad substrate specificity as well as a very high catalytic rate can be found.⁶⁻⁸ The *Drosophila* enzyme can indeed phosphorylate the four deoxynucleosides and many therapeutical cytotoxic nucleoside analogs with a higher turnover than other known viral and mammalian kinases (see Fig 1).^{7, 9, 10} Hence, these features make the *Drosophila* enzyme a unique member (EC 2.7.1.145) of the nucleoside kinase enzyme family. As for its

phylogeny, *dNK* belongs to the *TK2*, *deoxycytidine kinase (dCK)*, *deoxyguanosine kinase (dGK)* and the Herpes viral thymidine kinase family, and is not so closely related to *TK1*.⁶

The exact functions *in vivo* of the *Drosophila* enzyme, however, are still unknown, especially those in proliferation and apoptosis. Here we show that *dNK* expression is associated with proliferating cells and under the control of the *dE2F1* transcription factor. To understand the function of *Drosophila* *dNK in vivo*, we constructed transgenic *Drosophila* strains that allow ectopic or over-expression of *dNK*. We observed that *dNK* over-expression, when the *de novo* pathway is inhibited by antifolates such as methotrexate (MTX) and aminopterin (AM), rescues cell death induced by the drugs in the wing imaginal disc. Moreover, *dNK* over-expression confers on the flies global resistance to these anti-cancer drugs. We show that this is caused by both a decrease in cell death and a stimulation of cell cycle progression. In addition, the viability of the flies depends on the exquisite ratio between *dNK* expression and thymidine (dT) concentration in the medium. This nucleoside induces cell death when *dNK* is over- or under-expressed and a drastic body mass decrease in this latter genotype.

Materials & Methods

Drosophila strains and media

The Wild type Canton S, *w¹¹¹⁸*, *engrailed-GAL4 (en-GAL4)*, *patched-GAL4 (ptc-GAL4)* and *daughterless-GAL4 (da-GAL4)* strains come from the Bloomington *Drosophila* stock center (Indiana University). The *vg-GAL4* strain that drives GAL4 expression according to part of the *vg^{BE}* enhancer was provided by S. Carroll. The *UAS-CycE*, *UAS-RBF1* and *UAS-dE2F1*, *UAS-DP*, *UAS-P35* strains were provided by B. Edgar.¹² The *UAS-dNK* strain was generated in our laboratory. The deficiency covering *dNK* comes from the Bloomington center (Df(3R) n°5600). Aminopterin (AM), Methotrexate (MTX) or Thymidine (dT) (Sigma) were added to the medium depending on the experiment.

Histology

Images were acquired with a Leica TCS-SP confocal microscope or a Leica DMR microscope equipped with a DXM 1200 (Nikon) digital camera, and processed with Adobe Photoshop or Image J softwares. Dissections and immunochemistry were performed according to⁴². 5-Bromo-2'-deoxyuridine (BrdU) and 4'-6-Diamidino-2-phenylindole (DAPI) labeling of imaginal discs was performed as described.¹⁵ Digoxigenin (DIG)-labeled antisense or sense RNA probes were generated with T3 or T7 RNA polymerase (Promega) and DIG-UTP (Roche), from cloned cDNAs of *dNK*. These probes were used for whole mount *in situ* hybridization of fixed larval imaginal discs. The DIG-labeled RNA probes were detected with the aid of an anti-DIG antibody coupled with alkaline phosphatase (Roche) and NBT/BCIP as substrate.⁴³ Stainings with TUNEL Reagent (Roche), rabbit polyclonal anti-dNK antibodies (1:1000) (gift from W. Knecht & J. Piskur)¹¹, mouse monoclonal (n°8B10) anti-CyclinE antibody (1:1) (gift from H. Richardson)⁴⁴ and rabbit polyclonal CM1 anti-activated caspases antibodies (1:5000) (BD Pharmingen) were performed according to standard protocols.

Fluorescence-activated cell sorter (FACS) experiments

Staged larvae (120 h AED) derived from 2-3h egg collections and raised at 25°C were dissected in PBS for each genotype. Twenty wing discs were washed twice in PBS and incubated with gentle agitation in trypsin (Invitrogen) and Hoechst 33342 (Sigma) for 150 min according to Neufeld *et al*¹². About 20 000 GFP+ and GFP- cells were analyzed for each genotype. An Elite Beckman Coulter FACS was used and data were analyzed using the Multicycle Software.

Quantitative RT-PCR analysis

RNA from third instars larvae were extracted with Gibco BRL trizol reagent kit. First strand cDNA was synthesized with oligo-dT primer and AMV reverse transcriptase according to the

manufacturer's instructions (Pharmacia). RT-PCR amplification mixture (10 μ l) contained 1/20 of cDNA product, 10X SIBR Green I Master Mix buffer and 100 ng forward and reverse primers. Reactions were run on a Light Cycler PCR apparatus (Roche) Cycle condition were 10 min at 95°C and 45 cycles at 95°C for 10 sec, 60°C (*RP49*, *dE2F1*) or 65°C (*dNK*) for 10 sec and 72°C for 10 sec. Each assay included a standard curve of 3 serial dilution points of wild type third instar larvae cDNA (ranging from 100 ng to 1 ng). All PCR efficiencies were above 95% . The expression of *dE2F1* and of *dNK* was quantified relative to *rp49*. Each point was repeated three times. Primers for *rp49* amplification were : forward primer 5'-CCGCTTCAAGGGACAGTATCTG, reverse primer: 5'-CACGTTGTGCACCAGGAAGCTT, primers for *dE2F1* amplification were : forward primer 5'-CAATGACTATCCGTATGCGCTGA, reverse primer: 5'TTCGTGGACGTCTCCTCTTAGG, primers for *dNK* amplification were : forward primer 5'-TAGTCCTCGATGCCGATCTGA, reverse primer: 5'-GATAATGGTTATCTGGCGACCCT. Differences in *dNK* and *dE2F1* cDNA concentrations were adjusted by normalizing to *RP49*. For each gene, values were averaged over at least three independent measurements. Three independent RNA isolation experiments were performed for all genotypes and means were calculated.

Results

***dNK* is expressed in proliferating cells**

First, the *dNK* pattern of expression was assessed in the wing and eye imaginal discs by *in situ* hybridization, and immunochemistry with anti-dNK antibodies.¹¹ Imaginal discs provide useful model systems for the study of developmental control of proliferation.¹² In late 3rd instar wing discs, proliferation occurs uniformly, but in the Zone of Non-proliferation Cells (ZNC) along the Dorsal/Ventral boundary, where cells undergo precocious cell cycle arrest, prior to differentiation.¹³⁻¹⁶ In mid-late 3rd instar wing imaginal discs, a quite uniform dNK expression (both transcripts and proteins) was observed (Fig 2A,C). A few hours later, in late 3rd instar wing discs, once the ZNC is completely established, dNK expression is clearly

down-regulated in ZNC arrested cells that no longer incorporate the S-phase marker : BrdU (Fig 2D). In the eye disc, dNK expression (both transcripts and proteins) is mainly associated with the cells immediately posterior to the morphogenetic furrow that are about to enter the S phase of the second mitotic wave (Fig 2B,E). We further confirmed this using anti-cyclinE antibodies that enable to distinguish the morphogenetic furrow (Fig 2E). These observations suggest that *dNK* expression may be cell cycle-regulated and involved in cell proliferation under normal conditions, whereas its closest mammalian homologue TK2 is believed not to be proliferation-dependent.¹

Mammalian deoxyribonucleoside kinases have been identified in distinct sub-cellular compartments of the cells. *Drosophila* dNK might have both mitochondrial and nuclear import signals.¹⁷ *dNK* transfection experiments in mammalian cells with hybrid protein constructs, indicate a nuclear localization for the enzyme. Indeed, nuclear addressing sequences have been identified in the C-terminal domain of the protein.⁹ Surprisingly, in *Drosophila* wing discs, only a cytoplasmic localization was observed by immunocytochemistry (Fig 2C). These discrepancies suggest that *dNK* transfection experiments in heterologous cells represent specific conditions that may only partly correspond to the physiological localization of the protein. Double staining experiments using the mito-trackerTM reagent or *UAS-mito-GFP* transgenic strains (see reference 18) and dNK antibodies in the salivary gland or wing disc peripodial cells of third instar larvae, where mitochondria are easily visualized, did not show any clear overlap (data not shown). Since no specific mitochondrial localization has been identified so far, it is still unclear whether the salvage enzymes are present in insect mitochondria or whether mtDNA replication and repair rely on the import of deoxyribonucleoside mono-, di- and/or tri-phosphates from cytoplasm.

***dNK* is under the control of the *dE2F1* transcription factor**

The broad substrate specificity and sequence analysis both show that the *Drosophila* enzyme

is more closely related to human mitochondrial TK2 than to the other mammalian deoxyribonucleosides kinases.^{6, 17} But since *Drosophila dNK* seems to be associated with proliferating cells, we hypothesized that, like TK1 in mammalian cells, *dNK* is developmentally regulated by *E2F1*, the transcription factor that regulates expression of different genes involved in G1/S transition and DNA replication.^{3, 19} Therefore, in order to test this postulate, we expressed *dE2F1* ectopically using the UAS-GAL4 system described in.²⁰ In this system, two transgenic strains are crossed, one expressing the yeast GAL4 transcription factor in a specific pattern and the other carrying UAS sequences upstream of the gene of interest. The binding of GAL4 on UAS sequences will trigger the ectopic expression of the gene of interest in this tissue. We expressed *dE2F1* according to the *patched* (*ptc*) domain in the wing disc, using the *UAS-dE2F1*, *UAS-DP*, *UAS-P35* strain, which makes *dE2F1-DP* expression possible without triggering apoptosis.¹² The *ptc-GAL4* driver enables strong expression along the Anterior-Posterior compartment boundary. *In situ* hybridization revealed a strong induction of *dNK* transcripts according to the *ptc* pattern, indicating that *dNK* is regulated by *dE2F1* in *Drosophila* (Fig 3A). This observation was confirmed at the protein level (Fig 3B, where the *ptc* domain is labelled by GFP expression). Similar results were also observed with *cyclinE*, which positively regulates *dE2F1* activity (Fig 3C). To confirm that *Drosophila dNK* is under the control of the *dE2F1* transcription factor, we ectopically expressed the retinoblastoma protein (RBF1), a cell cycle progression inhibitor, according to the *ptc* domain. RBF1 binds the *dE2F1* protein, inhibiting transcriptional activation of *dE2F1* target genes involved in proliferation.²¹ *dNK* down-regulation in response to RBF1 expression was observed (Fig 3D), indicating that *dE2F1* function is required for *dNK* expression. Altogether, these results indicate that, unlike the TK2 enzyme, *dNK* expression is associated with proliferating cells and is under the control of *dE2F1* and is therefore functionally more closely related to mammalian TK1.

***dNK* expression and *de novo* biosynthesis inhibition**

To understand the role of *dNK* in the *Drosophila* salvage pathway we decided to impair the *de novo* pathway using antifolates such as AM or MTX that inhibit dihydrofolate reductase (DHFR) activity and thus the *de novo* thymidylate (dTMP) synthesis. MTX is widely used in chemotherapy, mainly in the treatment of childhood acute lymphoblastic leukemia. MTX-induced inhibition of DHFR depletes the tetrahydrofolate pool, thus decreasing purine and pyrimidine nucleotide biosynthesis and contributing to a proliferation arrest and cell death (see Fig 1).²² In mammals, no rescue of the effects of MTX administration by TK1 or TK2 has been reported, probably because an imbalance of purine and pyrimidine nucleotides triggers cell death. Indeed, rescue from folate depletion by addition of both purine and pyrimidine nucleosides have been reported in megaloblastic anemia.²³

In *Drosophila*, however, there exists a unique deoxynucleoside kinase that can phosphorylate all nucleosides. In order to assess the role of *dNK* in the salvage pathway, we generated transgenic strains allowing ectopic or over-expression of *dNK*. The *Drosophila dNK* cDNA was cloned in the P-Casper vector and the resulting *UAS-dNK* construct injected into *Drosophila* embryos. Nine independent transgenic *Drosophila* lines were generated. *e.g.* *dNK* over-expression in the wing disc, according to the *ptc-GAL4* driver, is shown in Fig. 3E. These strains made it possible to perform Gain Of Function experiments (GOF). In addition, a heterozygous deficiency encompassing the *dNK* gene was used to unravel Loss Of Function (LOF) phenotypes, since no mutants are as yet available. This hypomorphic strain displays normal viability, but the *dNK* transcriptional level, assessed by quantitative RT-PCR, is decreased by 25% (data not shown). To ascertain that the observed LOF phenotypes were due to *dNK* decrease and not from another gene deleted in the deficiency, we constructed a *UAS-dNK, df(dNK)/+* strain, in order to rescue the phenotypes observed with the LOF strain.

We decided to use the wing imaginal disc to assess the putative role of *dNK* when the *de novo* pathway is inhibited. As previously mentioned, the *Drosophila* wing disc is a highly

proliferating tissue that provides a relevant model for genetic studies.^{12, 15} Therefore, we over-expressed *dNK* in the *vestigial* (*vg*) expression domain that determines the future wing margin, and looked at the wing phenotype when control or *df(dNK)/+* flies were reared on normal or AM-containing medium (1mg/kg). This latter compound induces cell death and decreases cell proliferation in the wing disc.²⁴ Since AM blocks the *de novo* synthesis pathway, any functional interaction between addition of AM and impairment of the salvage pathway (*df(dNK)/+* deficiency) should specifically result from down-expression of the salvage enzyme dNK. When reared on AM, control flies displayed nicks in their wings (Fig 4A). The wings of *df(dNK)/+* flies were even more often affected, demonstrating the deleterious effect of *de novo* and salvage pathways concomitant inhibition. Nevertheless, the wing phenotypes observed in both the control and the *df(dNK)/+* *Drosophila* were greatly rescued by *dNK* over-expression along the wing margin (Fig 4A). None of these flies displayed any abnormal wing phenotype on normal medium. These results demonstrate that the wing phenotype of *df(dNK)/+* flies reared on AM is greatly dependent on the specific down-expression of *dNK* in this strain.

Since dNK is also specifically expressed in the cells of the eye that are entering S phase (Fig 2B,E), we looked for eye phenotypes, adding AM to the medium during fly development. AM treatment did not impair eye development in the control strain, but specifically reduced eye size in the *df(dNK)/+* strain (Fig 5A,B,D,E). Moreover, this phenotype was completely rescued when *dNK* was expressed under the control of the ubiquitous *daughterless-GAL4* (*da-GAL4*) driver (Fig 5C,F). Similar results were obtained with another antifolate : MTX (data not shown). This suggests that dNK is required for eye growth when the *de novo* pathway of nucleotide synthesis is inhibited.

At this stage, it was not possible to determine whether *dNK* over-expression prevents drug-induced apoptosis or stimulates cell proliferation, since both cell death and lack of proliferation induce nicks in the wings and abnormal eyes.²⁴⁻²⁶ To test the effect of *dNK* on

apoptosis induced by AM, we looked initially for apoptosis, using (i) anti-activated-caspases antibodies, (ii) TUNEL experiments, in wing discs of flies grown on AM, where *dNK* was over-expressed under the control of the *engrailed-GAL4* (*en-GAL4*) driver. This strain allows gene expression in the posterior (P) compartment of the wing disc, as visualized using a *UAS-GFP* reporter construct (Fig. 4B). In the *en* domain, we observed almost no cell death using TUNEL staining (Fig 4C). This was confirmed with anti-activated caspase antibodies staining, indicating a strong rescue of apoptosis in the P compartment marked by GFP, while caspase activation was clearly observed in the anterior (A) part of the disc (Fig 4B). This indicates a strong rescue of apoptosis where *dNK* was over-expressed. This observation is striking when looking at the wings of *en-GAL4 ; UAS-dNK* adult flies grown on AM. Nicks at the level of the wing margin could be observed in the A compartment, while the P compartment displayed a wild type phenotype due to *dNK* over-expression (Fig. 4D). Together, these results demonstrate that *dNK* over-expression *in vivo* rescues wing cells from antifolates-induced apoptosis.

***dNK* over-expression confers resistance to AM and MTX on the whole organism**

Next we wished to investigate whether the effect observed in proliferating tissues such as the wing or eye discs could be extended to the whole organism. Using the same *da-GAL4* driver, which allows ubiquitous expression in all tissues from embryogenesis to the adult stage, we tested the survival of *UAS-dNK* and *df(dNK)/+* flies compared to control, when reared on normal or AM-containing medium (Fig 4E). Our results clearly showed that AM treatment reduced the viability of the control strain. The viability of *df(dNK)/+* flies was even further reduced. Nevertheless, *dNK* over-expression conferred resistance to AM on both control and *df(dNK)/+* strains. We obtained similar results using another DHFR inhibitor : MTX (2mg/kg) (Fig 4E).

To assess whether survival of *dNK* over-expressing flies on AM is due to resistance to

apoptosis and/or to an increase in cell proliferation, we chose to evaluate, by real time quantitative RT-PCR in third instar larvae, a major gene controlling cell proliferation : *dE2F1*, assuming that increased proliferation and an increased level of *dE2F1* are correlated as previously reported.¹⁹ Interestingly, we found that AM dramatically repressed *dE2F1* expression in the control strain (Fig. 6A). This result is quite consistent with the recent demonstration of MTX-induced down-expression of many cell-cycle genes such as *cyclinA* or *cyclinE* in ovaries²⁷. *dNK* over-expression using the *da-GAL4* driver had no effect on *dE2F1* expression compared to control on normal medium (Fig 6A), suggesting that, in normal conditions, *dNK* over-expression cannot induce over-proliferation, at least in larvae. Interestingly, *dE2F1* down-regulation in response to AM treatment was significantly weaker when *dNK* was over-expressed using the same driver (Fig. 6A). The fact that *dE2F1* was less repressed in the *dNK* over-expressing strain reared on the drug, suggests that *dNK* induces some proliferation in larvae fed with the drug. This may explain, at least partly, AM-resistance at the level of the whole organism. Nevertheless, these experiments did not assess any post-transcriptional modulation of *dE2F1*, which has been previously described as an additional mechanism for *dE2F1* regulation.²⁸ We also investigated whether AM feeding had an effect on *dNK* expression. No difference in the *dNK* mRNA levels were observed in control *Drosophila* in presence or absence of AM (data not shown).

Proliferation is commonly assessed by looking at S and M phases cells in tissues, by means of BrdU pulse experiments and anti-PhosphoHistone3 antibodies labeling, respectively. In our experiments, these methods did not give clear results. Indeed, both apoptosis and proliferation are probably involved in the process making it difficult to distinguish the role of *dNK* in proliferation when using this method.

Nevertheless, to strengthen the previous results, we investigated the role of *dNK* on the cell cycle by means of cell sorter experiments using the wing disc model.¹² *GFP* alone or together with *dNK* were over-expressed in the P compartment of the wing disc using the *en-GAL4*

driver. Cell cycle profiles of the P and A compartments cells were compared in control *en-GAL4*, *UAS-GFP* discs when larvae were reared on normal or AM-containing medium (Fig 6B₁,B₃). On normal medium the cell cycle profile was similar in both compartments. On AM, significant changes in the overall cycle were observed with, as expected, a dramatic decrease in the number of G2/M cells and an increase in the number of S phase arrested cells, albeit without significant differences between the A and P compartments. In addition, a significant amount of apoptotic nuclei could be observed.

In a second experiment, *dNK* was over-expressed in the P compartment (Fig 6B₂,B₄). On normal medium, the content of S phase cells in the P compartment was slightly increased compared to that of the A compartment of the discs. It suggests that *dNK* over-expression can alter the normal cell cycle to some extent. When larvae were grown on AM, a significant shift towards G2/M was observed in the P compartment where *dNK* was over-expressed. The percentage of G2/M phase cells was even higher than on normal medium. Moreover, the quantity of apoptotic nuclei, as detected by DNA profile, was reduced. We also observed a partial cell cycle profile rescue in the adjacent A compartment of the disc. This suggests that *dNK* expression may have some non-autonomous effects on cell proliferation. One explanation may be that input of proliferation induced by *dNK* over-expression in the P compartment in turn activates morphogenetic events that control proliferation of both A and P compartments.

Our results indicate that over-expression of *dNK* changes the cell cycle profile not only on normal but especially on AM-containing medium. It strongly suggests that *dNK* over-expression allows flies to survive when reared on antifolate drugs, by decreasing apoptosis and allowing the cell cycle to proceed. These data show that *dNK* plays a key role when normal proliferation is perturbed in *Drosophila*. It also suggests that drugs inhibiting the dTMP pool such as AM and MTX, can dramatically enhance the potential activities of the *Drosophila* dNK enzyme.

dT concentration and *dNK* expression play a role in fly viability, growth and cell survival

It has been shown that, among dNK substrates, only addition of the nucleoside thymidine (dT) can rescue the effect of AM-induced lethality, although this drug depletes both pyrimidine and purine nucleotide biosynthesis.²⁹ We decided to evaluate the capacity of dT to rescue the effect of a high concentration of AM, which completely arrests the nucleotide *de novo* synthesis pathway. Therefore, we measured viability of flies on AM with increasing concentrations of dT during development. Low concentrations of dT (0.1 or 0.5g/kg) strongly rescued the viability of *Drosophila* reared on AM (10mg/kg) (Fig 7A). This underlines the importance of this nucleoside when the *de novo* biosynthesis is inhibited. At higher concentrations of dT (≥ 1 g/kg), the rescue was lost. Moreover, *Drosophila* viability progressively decreased. Lethality was also observed upon addition of dT (≥ 2 g/kg), even without AM treatment (Fig 7A). This clearly demonstrates the toxicity of dT *per se* at high doses, although a slight synergistic effect of the two compounds (AM + dT) was visible (Fig 7A).

To date, our knowledge of the relationship *in vivo* between the enzyme (dNK) and its substrate (dT) is only slight. We wondered whether dNK could be regulated by this substrate in flies. Strikingly, both *dNK* over-expression (Fig 4,5) or dT addition can rescue the effect of AM on *Drosophila*. We decided to measure the effect of dT addition on *dNK* expression. Quantitative RT-PCR were performed and showed an increase in *dNK* transcripts in response to dT (Fig 7B), suggesting that the *dNK* promoter is inducible in the presence of the nucleoside. The maximum *dNK* induction (# x2) was obtained at 2.5g/kg without any further increase, at higher dT concentrations (5,6,10 g/kg, data not shown).

We therefore assessed viability in *Drosophila* genetic contexts providing different *dNK* expression levels (LOF, control, GOF). In response to dT, even at a low dose (1g/kg),

df(dNK)/+ *Drosophila* displayed reduced viability, whereas the control strain remained almost unaffected until 2g/kg (dT) (Fig 7C). In addition, these *df(dNK)/+* flies display 75% of the normal level of *dNK* transcripts, and only very weak *dNK* induction was observed in response to dT (Fig. 7B). One explanation could be that the remaining copy of the *dNK* promoter is not sufficient to support induction by dT and may already be fully activated in these *df(dNK)/+* flies.

In *dNK* over-expressing flies (*da-GAL4, UAS-dNK*), viability on dT was also assessed. Unexpectedly, they displayed a reduced survival rate compared to the control (Fig. 7C). Thus, in response to dT, both *dNK* LOF and GOF genotypes show similar viability profiles ; a developmental delay was also observed (data not shown). In these flies, accumulation of dT or intermediate dTTP synthesis products are probably toxic. As a whole, since *dNK* expression is coordinately increased in response to dT addition in the control strain (Fig. 7B), *dNK* mis-expression in LOF or GOF genotypes, may make the flies unable to adjust accurately *dNK* level to dT concentration.

To understand the developmental delay and lethality observed in GOF and LOF flies compared to controls, we looked for possible alteration of a proliferating tissue, the wing disc. Some cell death was observed in third instar wing discs of the LOF genotype when grown on dT (Fig. 7D). A similar and more visible effect was detected in *vg-GAL4, UAS-dNK* wing discs (GOF), while almost no cell death was observed in wild type larvae in the same conditions (Fig. 7D). At this point, it was difficult to know whether this cell death induction was responsible for the lethality observed in LOF and GOF flies reared on dT-containing medium.

dT concentration and *dNK* expression, however, bear a striking connection to fly tissue growth. Our results show a significant weight decrease in *df(dNK)/+* emerging adults compared to controls when reared on increasing dT concentrations. At dT (2g/kg), weight was even reduced two fold (Fig 7E). This was confirmed when the pupae surface of the two

genotypes was estimated at different concentrations of dT (Fig 7F). The results are highly significant, and similar to those observed in mutants for cell growth regulators such as *InR*, *chico* and *S6K* (see references 30-32), indicating that major cellular events took place in the *df(dNK)/+* heterozygotes and led to the reduction of body mass.

Discussion

Several studies demonstrate the crucial importance of deoxyribonucleotide regulation. The necessity for precisely regulated deoxyribonucleoside kinase function is emphasized by the fact that mutations in some genes of the salvage pathway in humans cause severe diseases. Indeed, in human, TK2 mutations are responsible for mitochondrial DNA depletion associated myopathy.⁴ Deoxyribonucleoside kinases are also key enzymes for the use of nucleoside analogs, such as fluorouracil (FU) and fluorodeoxyuridine (FudR) in chemotherapy (see Fig. 1).³³ Moreover, deoxyribonucleoside kinases are used as activators for drug therapy in cancer and AIDS. For instance, HSV-TK makes cancer cells sensitive to the pro-drug ganciclovir and *dNK* transfection in cancerous lines increases cell sensitivity to cytotoxic nucleoside analogs, suggesting that this enzyme, like HSV-TK, could be used as a suicide gene in chemotherapy.^{9, 34} Only a few studies, however, have evaluated the role of these genes *in vivo*. Our goal was to use *Drosophila* as a model organism to study the relationship between the *dNK* enzyme and antifolates, and their influence on cell survival and proliferation. The study of *Drosophila* *dNK* function is also an important issue to understand how *de novo* and salvage pathways harmoniously achieve DNA replication.

Antifolates resistance

In order to shed light on the *dNK* function we decided (i) to construct transgenic strains over-expressing *dNK*. (ii) We also used a heterozygous deficiency as a hypomorphic *dNK* context.

Moreover, *dNK* over-expression rescued the *df(dNK)/+* phenotypes, confirming that they are specific to *dNK* loss of function. (iii) In addition, we inhibited the *de novo* nucleotide synthesis pathway using antifolates (AM, MTX). In mammals, this is commonly done in cancer treatment to stop proliferation and induce apoptosis of cancerous cells. Resistance to these drugs has been regularly reported and extensively studied.³³ Resistance mechanisms vary in different cancers. Mainly, transport, efflux and polyglutamination of the drugs are the most often described. An increase in DHFR activity due to gene amplification or mutations in DHFR that decrease its affinity for the drug, have been observed in cell lines and also in tumors.³³ The involvement of any one of the four different deoxyribonucleoside kinases, however, has never been reported in MTX or AM resistance. This may be due to nucleotides imbalance, a major cause of DNA alteration and cell death.^{35, 36} Activation of only one of the several deoxyribonucleoside kinases would trigger this phenomenon in mammals. In *Drosophila*, *dNK* over-expression, without addition of nucleosides, rescued the effect of AM in the wing disc, both by decreasing apoptosis and restoring cell cycling. Indeed, under these circumstances, it is possible that *dNK* phosphorylates efficiently all the nucleosides required for functional DNA replication. This sheds light on the difference between mammals and *Drosophila* in the regulatory mechanisms used to achieve normal DNA replication through the *de novo* and salvage pathways.

In the fruit fly, it has been previously reported that the effects of AM can be rescued by addition of dT in the medium with no further need for purine nucleosides.²⁹ The reason for this could be either that AM blocks only or mainly the pyrimidine nucleotides biosynthesis in flies or that dT concentration is limiting compared to purine deoxyribonucleosides. This also implies that *dNK* activity increases when necessary. Moreover, we also showed that dT induces *dNK* expression, possibly strengthening the rescue from AM.

In mammals, FUdR and FU (but not MTX) are rescued by addition of dT alone. Indeed, these two drugs inhibit thymidylate synthetase – this latter converts dUMP into dTMP - and do not

impair any other pathway (see Fig 1). However, so far, no resistance due to enhanced mammalian Thymidine Kinase (TK) activity has been observed in tumors. Interestingly, not only did *dNK* over-expression in *Drosophila* confer resistance to AM and MTX in the wing and eye discs, it also rescued the viability of the whole animal throughout its development. To our knowledge, this is the first time such a resistance has been described in an entire organism. Our data show that *dNK* over-expression enables AM-treated cells to proliferate and rescues these cells from apoptosis. Similar observations have been reported in heterologous HSV-TK transfection experiments in the mammalian interleukin-3 dependent pre-B cell line BAF3, where a delay in apoptosis induced by MTX has been observed.³⁷

dNK-dT relationship in Drosophila

It has been shown recently that *dNK*, like TK1, is inhibited in a feedback loop by dTTP, a final product of the pathway (see Fig. 1)^{38, 39}, confirming that *dNK* enzymatic activity is actively controlled. Our data show that the deleterious effects of high concentrations of AM are rescued by addition of low doses of dT, a *dNK* substrate. This confirms the importance of this nucleoside and its efficiency in the rescue of *de novo* pathway inhibition. On the other hand, higher concentrations of dT are toxic for the fly, for reasons that are still unclear but which probably involve apoptosis. This toxicity is also enhanced when *dNK* is mis-expressed. Normal development therefore requires a precise correlation between the level of this nucleoside in the medium and a specific level of *dNK* expression.

Moreover, our results demonstrate that dT induces expression of the *dNK* gene in the wild type strain suggesting that the *dNK* promoter is responsive to dT. Given the inhibition of *dNK* activity by the final product dTTP, these results suggest that *dNK* expression and activity are indeed finely tuned in a homeostatic loop.

Consistently, in heterozygous *df(dNK)/+* *Drosophila*, *dNK* is barely activated by dT ; this accurate regulation is lost. In a control strain, roughly a two fold maximum *dNK* induction is

obtained in response to dT. Nevertheless, the LOF strain contains only one copy of the *dNK* promoter that supports 75% of normal *dNK* transcription. This promoter is probably already greatly activated and cannot be induced any further. This may account for the very weak responsiveness of *dNK* heterozygotes.

In addition, *df(dNK)/+* phenotypes were enhanced by dT addition ; we therefore considered them to be *dNK*-specific. They reveal functional interactions between dT and *dNK* loss of function. We observed apoptotic cells in the wing discs of heterozygotes reared on increasing concentrations of dT. An excessive accumulation of unprocessed nucleoside may be responsible for the toxicity and cell death. *dNK* over-expression also induced lethality and significant cell death when flies were reared on dT. Therefore, both *dNK* over- and under-expression trigger similar effects confirming that exquisite *dNK* regulation plays a key role in physiology of *Drosophila* during development.

Moreover, in LOF *Drosophila*, even at low concentrations, addition of dT induced a high lethality rate associated with a two fold decrease in adult body mass. This latter effect is reminiscent of those observed in *InR*, *chico* and *S6K* mutants.³⁰⁻³² Additional studies will be required to discover whether this striking effect is due to apoptosis, diminution of cell growth and/or cell proliferation. Particularly, the relationship between dT metabolism and pathways involved in growth regulation, like *InR/FOXO*, needs to be evaluated in wild type and in *df(dNK)/+* flies.

Potential role of *dNK* in cell proliferation

In *Drosophila*, it is difficult to evaluate whether the salvage pathway is required for normal DNA replication or whether it is merely activated when the *de novo* pathway is inhibited. The wing and eye phenotypes observed in *df(dNK)/+* flies grown on AM are, however, rescued by *dNK* over-expression. This argues in favour of a role for *dNK* in cell proliferation, in specific conditions when the *de novo* pathway is inhibited. When available, the use of a stronger *dNK*

mutant or *RNAi*, and the study of viability and phenotypes on a nucleoside free-medium should help to elucidate this point in the future. (i) Nevertheless, our results show that *dNK* is expressed in proliferating cells. (ii) It is also positively regulated during development by *dE2F1* which controls almost all genes involved in DNA replication and G1/S transition. (iii) Moreover *dNK* is down-regulated by *RBF1*. (iv) We also note that, in late 3rd instar wing discs, *dNK* expression is down-regulated at the ZNC (see Fig. 2D). Since *dE2F1* has been shown to be lately inactive in this region, this might reflect the requirement of *dE2F1* for *dNK* expression in the wing disc.¹⁴ Therefore, our data strongly suggests that *dE2F1* function is necessary and sufficient for *dNK* expression and that *dNK* may be an actor of cell proliferation under normal conditions. Interestingly, before the *dNK* gene was characterized, it had been shown that ‘thymidine kinase’ activity in *Drosophila* Schneider cells is cell cycle regulated, with a peak activity during S phase, like human TK1.⁴⁰ Nevertheless, TK1 activity is regulated at the (post)-translational level and is degraded during mitosis, while its transcription remains stable in proliferating cells.⁴⁰

A recent study in which *RNAi* experiments were carried out on S2 cultured cells in order to elucidate the potential role of the *dE2F* and *RBF* gene families by DNA chip, identified *dNK* as a target of *dE2F1*, *dE2F2* and *RBF1*. Chromatin immunoprecipitation revealed binding of *dE2F1* to the *dNK* promoter.⁴¹ We are thus able to emphasize the developmental and physiological significances of this regulation in normal conditions and the role for *dNK* when the *de novo* pathway is inhibited.

Biochemical and phylogenetic studies have suggested that the insect progenitor was likely to contain several deoxyribonucleoside kinases genes. However, all genes but one (*dNK*, which encodes a TK2-like kinase) have been lost during evolution.⁶ Apparently, this enzyme has undergone a retrograde evolution, from a specialized TK2-like enzyme toward an enzyme with a broad specificity. Here we show that, unlike the TK2 enzyme, *dNK* is cell cycle regulated, and under the control of the *dE2F1* transcription factor, like mammalian TK1. This

suggests that, like broad substrate specificity, cell cycle regulation of the *dNK* gene is also subjected to positive adaptative selection in *Drosophila*.

As a whole, our study of the functions of dNK in *Drosophila* highlights its role in proliferation and anti-apoptotic processes, when the *de novo* pathway is impaired. Moreover, our data emphasize the importance of dT in cell survival and mass accumulation during development, since over- or under-expression of *dNK* renders flies very sensitive to the nucleoside and prone to cell death. Finally, our results point out the tight link between the *de novo* and salvage pathways *in vivo* in *Drosophila*. We demonstrate that *dNK* over-expressing transgenic flies are rescued from the effects of antifolate treatment. Strikingly, we describe a novel mechanism of resistance to antifolates, involving *dNK* gain of function.

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Figure legends

Figure 1 : Schematic representation of both *de novo* and salvage pathways of purine (dATP, dGTP) and pyrimidine (dTTP, dCTP) nucleotides synthesis in metazoan.

Figure 2 : Imaginal discs *dNK* expression pattern

(A-B) are *dNK in situ* hybridization of third instar wing and eye-antennae imaginal discs respectively. (A) Mid-late 3rd instar wing imaginal disc showing quite uniform *dNK* expression. (B) Eye-antennae imaginal disc displays specific *dNK* expression (arrowheads) in the late G1-S cells of the disc, posterior to the morphogenetic furrow (MF) (open arrowheads). (C) Mid-late 3rd instar wing imaginal disc staining with anti-*dNK* antibodies (left) and the DAPI nuclear dye (middle) where a uniform *dNK* protein expression can be observed as in (A). Insets clearly show a cytoplasmic localization for *dNK* (merge picture). (D) Late 3rd instar wing imaginal discs showing that *dNK* expression is down-regulated (arrowheads) in the ZNC cells along the Dorsal/ventral boundary. These cell cycle arrested cells do not incorporate the S phase marker : BrdU (inset). (E) Eye-antennae imaginal disc stained with anti-*dNK* (left) and anti-Cyclin E (middle) antibodies that marks cells entering S phase. Empty arrowheads indicate the MF ; white arrowheads indicate *dNK* expression. *dNK* is up-regulated in CyclinE-expressing cells (merge picture).

Figure 3 : *dNK* expression is regulated by the *dE2F1-DP* factor

(A) Ectopic expression of the *dE2F1-DP* factor and the caspase inhibitor *P35*, according to *ptc-GAL4*, triggers *dNK* expression (arrowheads) in the wing disc, as visualized by *dNK in situ* hybridization. (B) As in (C), *dE2F1-DP* and *P35* expression trigger *dNK* protein

expression in the *ptc* domain marked by GFP (arrowheads), as visualized by anti-dNK antibodies. Insets show that, in this experiment also, dNK is localized in the cytoplasm. (C) *ptc-GAL4*, *UAS-CycE*, *UAS-GFP* (green) disc stained with anti-CycE antibody (blue), anti-dNK antibodies (red) and DAPI. As for dE2F-1, dNK expression is induced in response to CyclinE. (D) Over-expression of *RBF1* according to *ptc-GAL4* represses dNK expression (arrowheads). RBF1 inhibits proliferation suggesting that *dNK* expression is involved in this phenomenon. (E) Wing discs over-expressing *dNK* according to the *ptc-GAL4* driver display a strong dNK protein induction in the *ptc* domain marked by GFP (arrowheads). Insets show that, even when over-expressed, dNK is localized in the cytoplasm.

Figure 4 : *dNK* expression rescues antifolates-induced cell death

(A) Comparison of wing phenotypes in control flies (*vg-GAL4* driver), *df(dNK)/+* flies and *dNK* over-expressing flies according to this driver that marks the future wing margin. Wing phenotypes of flies reared on AM (1mg/kg) are subdivided in two classes : [+] or [notched]. AM-treatment induces wing nicks in the control strain. This phenotype is enhanced in *df(dNK)/+* flies. *dNK* over-expression greatly rescues these wing nicks at the margin of both control and *df(dNK)/+* strains. (n : number of wings observed). (B) *en-GAL4*, *UAS-dNK*, *UAS-GFP* wing disc reared on AM (1mg/kg) and stained with anti-activated caspases antibodies and DAPI. GFP expression marks the posterior (P) compartment of the disc. Caspases were activated only in the anterior (A) compartment of the disc. (C) *en-GAL4*, *UAS-dNK* wing disc stained for apoptosis with TUNEL when larvae were reared on AM (1mg/kg). Strong staining in the A compartment of the disc is observed, whilst almost no apoptosis is detected in P cells rescued by *dNK* over-expression. (D) Upper wing : control *en-GAL4* flies reared on AM (1mg/kg) which induces apoptosis and scalloped wings. Lower wing : *dNK* is over-expressed according to the *en-GAL4* driver, in the posterior (P) compartment of the wing disc. The P half of the wing displays a normal structure suggesting that defects induced by the drug are

antagonized by *dNK*. **(E)** Percentage of viability on AM (3mg/kg) and MTX (2mg/kg). The viability is calculated as the ratio of the number of adult flies hatched over the number of eggs deposited in the medium (n). The diagram shows the relative *Drosophila* viability when reared on the drug compared to the viability on normal medium. For both drugs the control strain displays a viability. Heterozygous flies for a *dNK* deficiency are even more severely impaired. *dNK* over-expression with the ubiquitous *da-GAL4* driver confers strong resistance to antifolates, in both control (*da-GAL4*) and *df(dNK)/+* strains.

Figure 5 : *dNK* expression rescues antifolate-induced eye phenotype

Drosophila were grown on a medium containing AM (3mg/kg) **(D-F)** or not. **(A-C)**. Upon AM treatment, the eyes of controls remain comparable to those of controls reared on normal medium **(A,D)**, while *df(dNK)/+* flies display reduced eyes with a frequency of 40% **(B,E)**. This eye phenotype was completely rescued when *UAS-dNK* was expressed under control of the ubiquitous driver *da-GAL4*, in a *df(dNK)/+* genetic background **(C,F)**. The same results were observed with MTX (2mg/kg). (n > 100 eyes observed for every genotype)

Figure 6 : Effect of *dNK* expression on cell cycle transition.

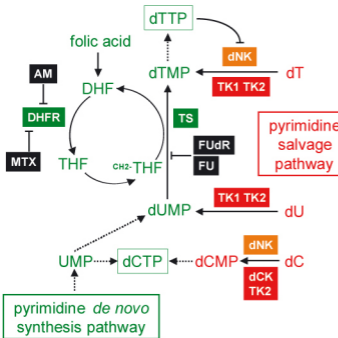
(A) Quantitative 'real-time' RT-PCR analysis performed on whole extracts of third instar larvae. Results show *dE2F1/RP49* transcript levels in control (*da-GAL4*) or *da-GAL4,UAS-dNK* larvae reared on medium containing AM (2mg/kg) or not. Transcript levels were adjusted by normalizing to the ribosomal protein RNA *RP49* and were calculated relative to the wild type level (expression in this genotype was set to 1). *dE2F1* expression decreases in response to AM treatment, but this effect is significantly reduced in *dNK* over-expressing *Drosophila*. **(B)** Staged third instar wing discs over-expressing GFP (*en-GAL4; UAS-GFP*) (1,3) or *dNK* and GFP (*en-GAL4; UAS-GFP; UAS-dNK*) (2,4) in the posterior (P) compartment of larvae, and reared on either normal medium (1,2) or medium containing AM

(1mg/kg) (3,4) were analysed by cell sorter experiments. Discs were dissociated according to¹². Cell cycle profile of the P compartment (red line) was compared to the anterior (A) compartment (green line). The percentages of cells in the different cycle phases in both compartments are indicated. Cells on the bottom lefthand side of the DNA profiles, with less DNA content than G1 ones, are assumed to undergo cell death. Results show that AM treatment significantly decreases the number of G2/M phase cells. This cell cycle phasing alteration is rescued by *dNK* over-expression in the P compartment.

Figure 7 : Relationship between *dNK* and its substrate dT

(A) Diagram showing the percentage of viability of a WT strain on *media* containing a high concentration of AM (10mg/kg) or not. WT flies are highly sensitive to AM10 and display an extremely reduced viability. Increasing concentrations of dT were added to the medium. Low [dT] rescues the flies' viability to a great extent, whereas adding further dT is deleterious. n=500 eggs were deposited on the medium and emerging adults were collected for every experimental condition. **(B)** Quantitative 'real-time' RT-PCR analysis performed on whole extracts of third instar larvae. In the wild type strain, the *dNK/RP49* level of transcripts in larvae reared on medium containing dT (2.5g/kg) is increased when compared to control. Nevertheless, in heterozygous *df(dNK)/+* *Drosophila*, this *dNK* in response to dT addition, is very weak, if indeed at all. **(C)** Diagram showing the percentage of viability of control flies, *da-GAL4 UAS-dNK* (GOF) overexpressing flies, and *df(dNK)/+* (LOF) *Drosophila* reared on increasing concentrations of dT. n=100 eggs. Each condition was repeated 10 times. For the three strains, viability on the control (dT 0g/kg) is similar. Until dT 2mg/kg, almost no effect is observed in the control strain, whereas strong lethality is observed for both the LOF and the GOF genotypes. **(D)** Third instar wing disc of control, *df(dNK)/+* (LOF) and *vg-GAL4, UAS-dNK* (GOF) larvae grown on dT (1,5g/kg) and stained for apoptosis with TUNEL and with the nuclear dye DAPI. Little cell death is detected in wild type wing discs, whereas apoptosis is

clearly observed for the LOF and particularly for the GOF genotypes. **(E)** Diagram showing the average mass (μg) of control and *df(dNK)/+* *Drosophila* reared on increasing concentrations of dT. 50 flies (25 males + 25 females) were pooled together, frozen, dehydrated and weighted. Average mass of a single fly was calculated for each condition and representative pictures are shown. **(F)** Diagram showing the average size (pixels) of control and *df(dNK)/+* *Drosophila* reared on increasing concentrations of dT. 100 late pupae images were analysed and pupae area was measured using ImageJ software. Mean area and standard deviation of pupae were calculated for each condition and representative pictures are shown.



Nucleotide synthesis

dNK : deoxyribonucleoside kinase

- Folic acid cycle

CH₂-THF : methylene tetrahydrofolate
 THF : tetrahydrofolate
 DHF : dihydrofolate
 DHFR : dihydrofolate reductase
 AM : aminopterin
 MTX : methotrexate

- Pyrimidine synthesis

UMP : uridine monophosphate

dTTP synthesis

dU : deoxyuridine
 dUMP : deoxyuridine monophosphate
 dT : deoxythymidine
 dTMP : deoxythymidine monophosphate
 dTTP : deoxythymidine triphosphate
 TS : thymidylate synthetase
 TK : deoxythymidine kinase
 FU : 5-fluorouracil
 FUdR : 5-fluoro deoxyribo uridine

dCTP synthesis

dC : deoxycytidine
 dCMP : deoxycytidine monophosphate
 dCTP : deoxycytidine triphosphate
 dCK : deoxycytidine kinase

- Purine synthesis

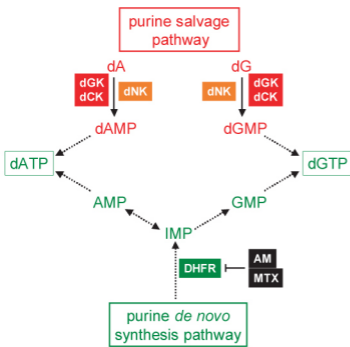
IMP : imidazole monophosphate
 dCK : deoxycytidine kinase
 dGK : deoxyguanosine kinase

dATP synthesis

dA : deoxyadenosine
 dAMP : deoxyadenosine monophosphate
 AMP : adenosine monophosphate
 dATP : deoxyadenosine triphosphate

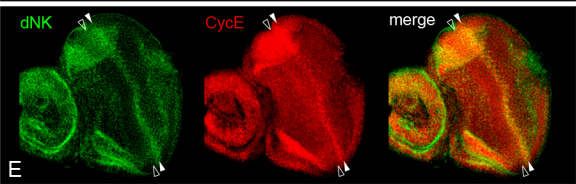
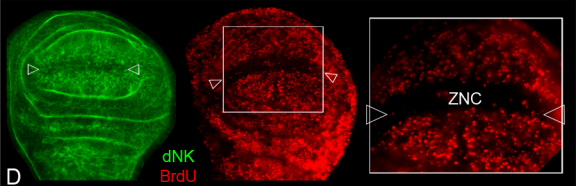
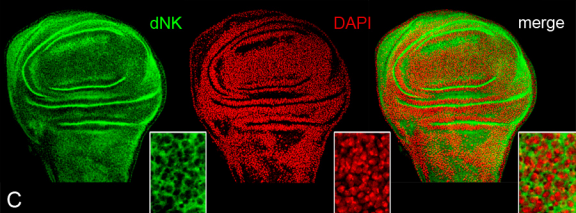
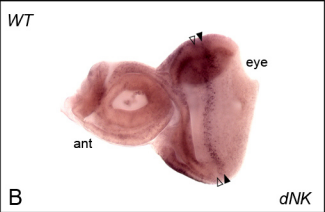
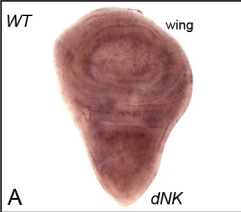
dGTP synthesis

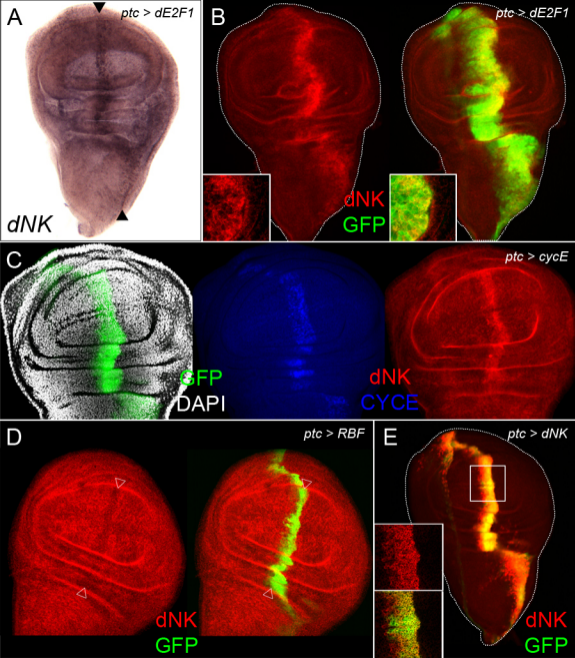
dG : deoxyguanosine
 dGMP : deoxyguanosine monophosphate
 GMP : guanosine monophosphate
 dGTP : deoxyguanosine triphosphate

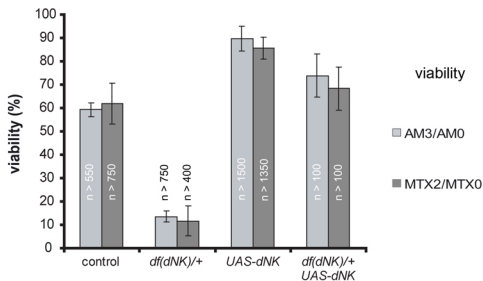
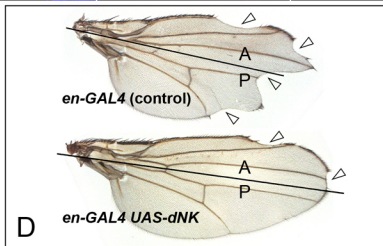
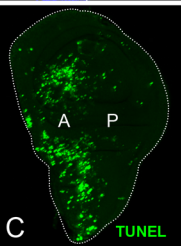
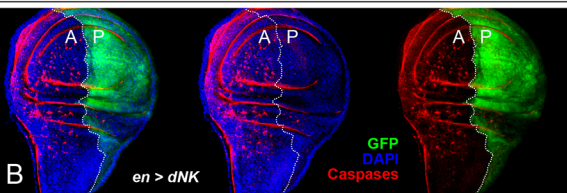
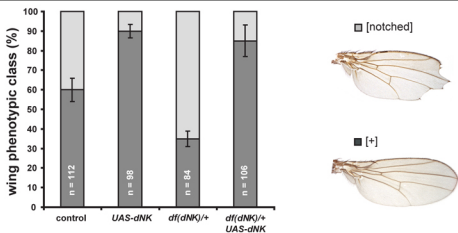


■ *Drosophila* salvage pathway
■ mammalian salvage pathway

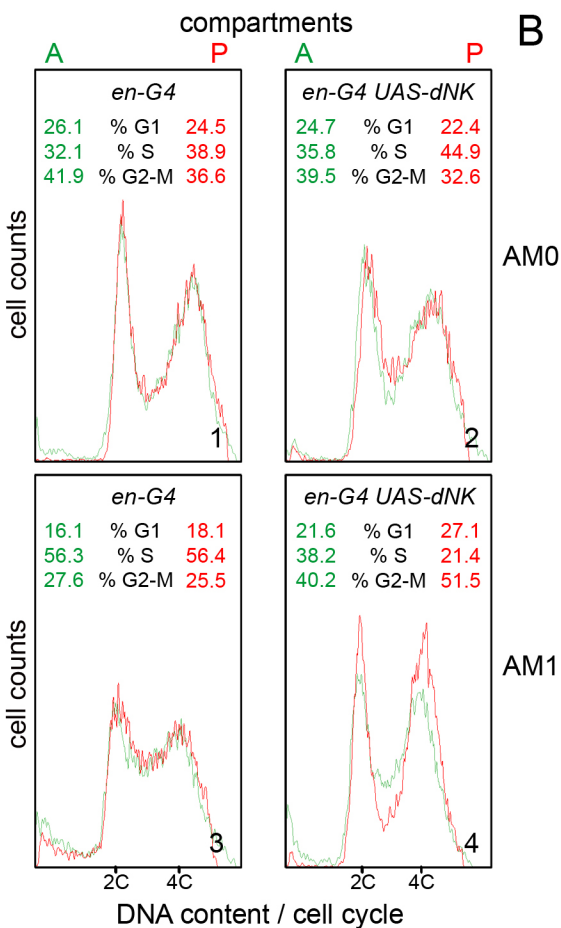
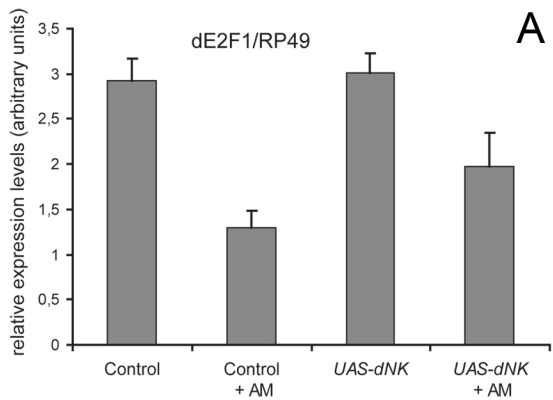
■ *de novo* pathway
■ cytotoxic analogs

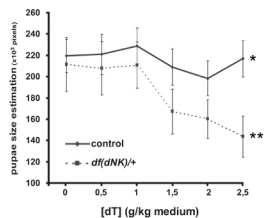
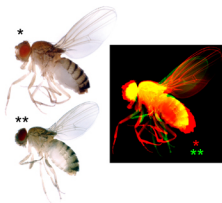
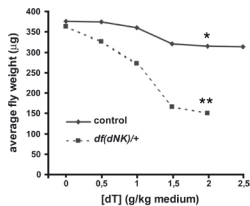
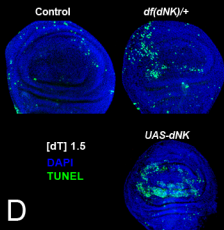
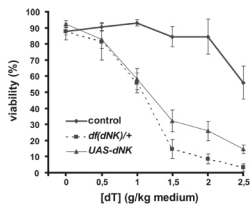
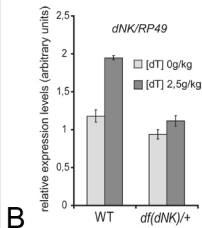
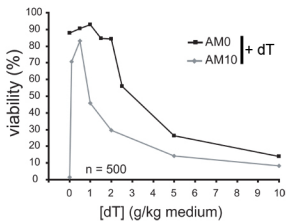


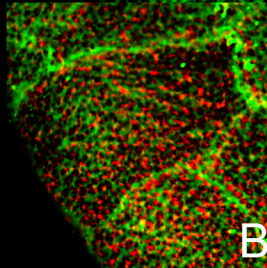
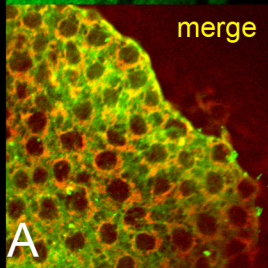
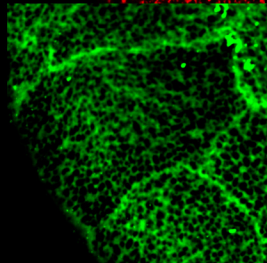
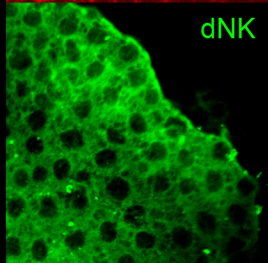
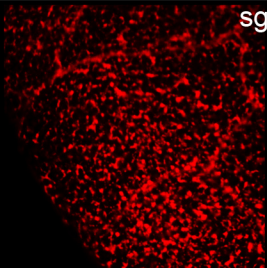
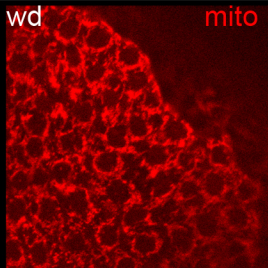








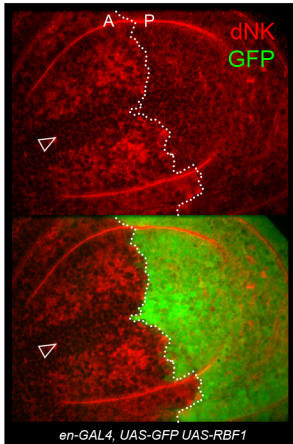




A

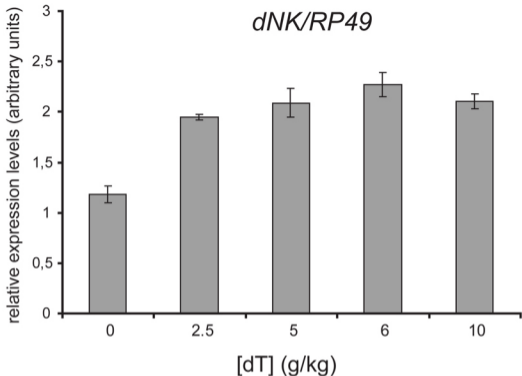
B

adding proof 1 : dNK expression and mitochondria localization
Mitochondria are visualized by mitotracker™ incorporation in living tissues. Once tissues are fixed, dNK is detected using classical immunochemistry. (A) Peripodial membrane of third instar wing disc (wd). mitochondria are mainly localized around nuclei while dNK proteins are present in almost all of the cytoplasm. (B) salivary gland (sg) of third instar larvae. One cell covers the central part of the picture. dNK and mitochondria present only partial overlap with no obvious pattern..



en-GAL4, UAS-GFP UAS-RBF1

adding proof 2 : RBF1 ectopic expression down-regulates dNK Late third instar wing disc ; posterior (P) is to the right. *RBF1* ectopic expression, according to the *en-GAL4* driver, down-regulates dNK protein expression in the P compartment of the disc visualized by GFP. Since RBF1 inhibits dE2F1-dependent transcriptional activation, this strongly suggest that dE2F1 function is required for dNK expression. Note that dNK expression is also decreased in the ZNC of the anterior compartment where dE2F1 is expressed but lately inactive (arrowhead).



adding proof 3 : dT addition induces *dNK* expression

Quantitative 'real-time' RT-PCR analysis performed on whole extracts of third instar wild type larvae reared on increasing dT concentrations Transcript levels were adjusted by normalizing to the ribosomal protein RNA *RP49*. The *dNK/RP49* level of transcripts in larvae reared on medium containing dT (2.5g/kg) is increased when compared to control. Nevertheless, when increasing dT concentrations are added, further *dNK* induction is very weak, if indeed at all. Values were averaged over at least three independent measurements. Three independent RNA isolation were performed for all concentrations and means were calculated.