Dynamic control of DNA precursor synthesis in early embryos

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Abstract

Animal embryogenesis starts with multiple rounds of nuclear divisions. During and shortly after these divisions, the zygotic genome is activated, the body plan of the organism is established, and gastrulation initiates the formation of tissues and organs. For these events to occur, the embryo needs to generate energy and provide metabolic precursors for biosynthesis. In this thesis, we used quantitative mass spectrometry and genetic manipulation techniques to examine how the early Drosophila melanogaster embryo controls the synthesis of DNA precursors.

Early Drosophila embryos undergo 13 rapid and synchronous nuclear division cycles within two hours of fertilization. This exponential increase in the number of nuclei requires massive amounts of deoxynucleoside triphosphates (dNTPs). Surprisingly, despite the breakneck speed at which Drosophila embryos synthesize DNA, maternally deposited dNTPs can generate less than half of the genomes needed to reach gastrulation. The rest of the dNTPs are synthesized “on the go”. The rate-limiting enzyme of dNTP synthesis, ribonucleotide reductase (RNR), is inhibited by endogenous levels of dATP present at fertilization and is activated as dATP is depleted via DNA polymerization. In the absence of inhibition by dATP, dNTP levels increase dramatically and induce embryonic lethality with particularly severe structural defects in the anterior regions.

In conclusion, this thesis demonstrated that dNTP synthesis in early Drosophila embryos is controlled mainly through a single feedback inhibition loop at the end of the dNTP production pathway. In the process, we have also found that misregulation of RNR activity suprisingly confers tissue specific defects. Going forward, this thesis establishes Drosophila development as a platform for mechanistic and quantitative studies of dNTP metabolism.
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To my parents and my brother.
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Chapter 1

Introduction

Embryos develop from a single cell into a multicellular organism through a rapid sequence of complex and coordinated biochemical transformations. Compared to the deep knowledge of the signaling and tissue specification mechanisms that control developmental events, we have very limited understanding of how the metabolic requirements of embryogenesis are met. In this thesis we examined the metabolic properties of early Drosophila melanogaster embryos, a well studied developmental system that continues to provide fundamental insights in cell biology. In the introduction, we will review some basic but important physiological properties of Drosophila embryos.

1.1 Embryos contain large maternal stores of macromolecules

The Drosophila melanogaster embryo is about 10 nL in volume, and develops outside the mother for roughly 24 hours at room temperature[?, ?]. During this time, the number of nuclei increases from 1 to roughly $10^5$ [?]. However, compared to the dramatic increase in DNA content, the majority of the macromolecular components
stay relatively constant. The total protein concentration throughout embryogenesis stays constant \[?\]. The composition of the phospholipidome also remains constant between early (0-1 hours post fertilization (hpf)) and late embryogenesis (15-16 hpf)\[?\, ?\]. Finally, the majority of the RNA present in late embryos (15-16 hpf) is deposited by the mother \[?\]. These data from literature suggest, for instance, that maternally deposited yolk proteins are degraded into amino acids and polymerized into new functional proteins, and that maternal stores of phospholipids are reassembled to form cellular membranes. In other words, embryos reorganize large maternal stores of macromolecules, in contrast to proliferating cells in culture that synthesize enormous amounts of biomass de novo \[?\].

### 1.2 Embryos burn sugar and fat

Embryogenesis requires energy in the form of ATP. Just as any other animal, *Drosophila* embryos must generate ATP by using energy stored in carbon fuel sources. Since embryos rely entirely on the maternally deposited nutrients, we gauged the net energy usage by measuring the depletion of two common fuel sources in animal metabolism, glycogen and triglyceride (TAG). To do this, we pooled \(\sim20\) embryos at the first and last hours of embryogenesis, and measured the glycogen and TAG contents using commercially available biochemical assay kits. We found that both stores of glycogen and TAG are essentially gone by the end of embryogenesis (Figure \[1.1A\]), consistent with previous studies \[?\, ?\].

However, the depleted fuel sources need not have necessarily been completely oxidized to generate ATP. For instance, glucose is a substrate for DNA, chitin, and trehalose. Over the course of embryogenesis, some amount of glycogen is converted into these compounds instead of being oxidized. Similarly, TAG can be converted into membrane lipids. In this case, extensive lipidomics data from early and late embryos
showed that the membrane lipid content does not change over time, which suggests that the depleted TAG was indeed mostly oxidized [?]. To estimate the extent to which the depleted carbon fuel sources were oxidized, we can refer to the amount of oxygen consumed by the embryo.

Based on stoichiometry, six oxygen molecules are required to fully oxidize a glucose molecule.

\[ C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2 \]

To calculate the oxygen required for the combustion of TAG, we need to first approximate the composition of TAG. From the fatty acid composition of whole adult flies obtained by Carvalho et al., [?], we can assume that embryonic TAG are on average tri esters of three palmitates (C16 fatty acid chains) and glycerol (C$_3$H$_8$O$_3$). Palmitate is catabolized by β–oxidation and subsequently the TCA cycle, whereas glycerol is turned into dihydroxyacetone–phosphate and subsequently catabolized by glycolysis and the TCA cycle. Again, based on stoichiometry,

\[ 3(C_{16}H_{32}O_2 + 23O_2) + (C_3H_8O_3 + 3.5O_2) \rightarrow 3(16CO_2 + 16H_2O) + (3CO_2 + 4H_2O). \]

Therefore, roughly 72 moles of oxygen are required to fully oxidize one mole of TAG. Combining these calculations with the glycogen and TAG depletion measurements in Figure 1A, the total oxygen required to fully oxidize the depleted carbon fuel sources is

\[ 6 \times \Delta[\text{glycogen}] + 72 \times \Delta[\text{TAG}] = 22.7 \text{ nmol of } O_2. \]

Total oxygen consumption during Drosophila embryogenesis has been measured multiple times using multiple different respirometers [?, ?, ?]. All of these studies reported similar values for total oxygen consumed during embryogenesis, with the most recent measurement from 1967 using cartesian divers at about 24 nmoles [?]. Therefore, the amount of oxygen consumed throughout embryogenesis is roughly sim-
ilar to the amount required to oxidize the depleted sugar and fat stores (Figure 1.1B). Since combustion of carbon fuels is the dominant pathway for oxygen consumption in animal metabolism, this suggests that embryos burn most of the combined stores of sugar and fat to generate ATP.

1.3 Fly embryogenesis costs 10 mJ

Another way to measure the energy usage of embryos is by calorimetry. To start, we can define the internal energy of the embryo, $U$, as the sum of all chemical bond energies. Importantly, this term is by definition independent from any spatial organization inside the embryo. Since the embryo is a semi-closed system and does not perform work on the environment, the change in internal energy is equal to the heat ($Q$) dissipated over time.

$$\Delta U = Q.$$ 

In general, we can expect a more active embryo to dissipate more heat to the environment. To measure the heat dissipation rate of fly embryos, we used a high precision isothermal calorimeter that has been used previously for embryos of other species \cite{? , ?, ?}. The calorimetry measurements in Figure 1.1C were done with a small number of embryos (n=4-5) enclosed in a humidified closed volume ampoule filled with air gas. The thermal dissipation rate of embryos increased linearly from $\sim$100 nW to $\sim$170 nW throughout embryogenesis, accumulating at about $Q = -10$ mJ by hatching (Figure 1.1C).

How does this compare to the fuel depletion measurements? We can calculate the heat released by oxidation of glucose and TAG by known heats of combustion ($U_{\text{glucose}}^c$ and $U_{\text{TAG}}^c$) \cite{?, ?, ?}. For calculation of $U_{\text{TAG}}^c$, the composition of TAG was approximated by the fatty acid composition of whole *Drosophila* adults as described previously.
\[
U_{\text{glucose}}^c = 2800 \text{ kJ/mol}
\]

\[
U_{\text{TAG}}^c \approx 3 \times U_{\text{palmitate}}^c + U_{\text{glycerol}}^c = 31630 \text{ kJ/mol}
\]

The heat that would have been released by total oxidation of the depleted glycogen and TAG stores is

\[
U_{\text{glucose}}^c \times \Delta[\text{glycogen}] + U_{\text{TAG}}^c \times \Delta[\text{TAG}] = 10.1 \text{ mJ}.
\]

This value closely matches the total heat dissipation measured through calorimetry (10 mJ, Figure 1.1B). If we divide the embryo’s internal energy into fuel and non–fuel portions \(U_{\text{total}} = U_{\text{fuel}} + U_{\text{non–fuel}}\),

\[
\Delta U = \Delta U_{\text{fuel}} + \Delta U_{\text{non–fuel}} = Q.
\]

Since \(\Delta U_{\text{fuel}} \approx Q\),

\[
\Delta U_{\text{non–fuel}} \approx 0.
\]

This is paradoxical at first glance, in that the embryo seems to dissipate all of the energy produced by the fuel sources, instead of “using” some of it to generate new chemical bonds and increase \(U_{\text{non–fuel}}\).

There are two ways to explain this result. In general, a large portion of the energy stored in carbon fuel sources is lost to heat as it is converted to ATP and used to form new bonds [?]. Thus, our approximations based on fuel depletion may not be accurate enough to capture meaningful differences between \(\Delta U_{\text{fuel}}\) and \(Q\). Another way to understand why \(\Delta U_{\text{non–fuel}} \approx 0\) is by considering the physiology of the embryo. At fertilization, the embryo contains large maternal stores of macromolecules. Throughout embryogenesis, the amount of major macromolecular components (pro-
tein, membrane lipids, and RNA) stay relatively constant. Thus, $U_{\text{non-fuel}}$, the sum of all non-fuel chemical bond energies, remains constant as well. This in turn suggests that large portions of the energy derived from fuel sources must be dissipated as heat. Below, we use a specific hypothetical example to demonstrate how this can happen.

Suppose that the early embryo contains exactly $10^5$ identical maternal yolk proteins each 100 amino acid long. We can approximate the internal energy of these proteins $U_{\text{protein}}$ by the sum of all peptide bond energies.

$$U_{\text{protein}} = \sum_{\text{all peptide bonds}} U_{\text{bond energy}}.$$  

As mentioned before, this expression is by definition independent of the spatial distribution of the proteins themselves. The embryo proceeds to degrade all these yolk proteins and synthesizes $10^5$ new proteins by the same size and same amino acid composition. By the end of embryogenesis, each new protein is actively transported to each one of the $10^5$ newly formed cells. This process requires large amounts of ATP to degrade, synthesize, and relocate the proteins. However, the total chemical bond energy $U_{\text{protein}}$ of the $10^5$ maternal yolk proteins is identical to that of the spatially organized $10^5$ new proteins, since the total number of peptide bonds in the system did not change. Of course, some of the energy from ATP is retained in the process of making new bonds. However, since the net internal energy of the system did not change, the equivalent amount to the energy used to drive this process must be dissipated as heat. In sum, since embryos synthesize new macromolecules by breaking down the maternal stores, the equivalent to large portions of the energy used to drive embryogenesis is expected to be dissipated as heat.
1.4 Relative energetic intensity of fly embryos

By combining multiple assays, we now have a confident estimate of 10 mJ for the total energy used during Drosophila embryogenesis. To put this into perspective of everyday physical activities, 10 mJ is equivalent to the work of lifting a tomato (100 g) 1 cm. How does this compare to other biological systems?Normalized for size, Drosophila embryos dissipate roughly ten times the heat compared to Xenopus laevis embryos [?]. We can also estimate the total ATP production of Drosophila embryos from measurements of fuel depletion, since the number of ATPs generated by oxidation of glucose and TAG are well known.

Glucose is catabolized by glycolysis and the TCA cycle, the products of which subsequently generate ATP through the electron transport chain. In total, one mole of glucose generates approximately 30 moles of ATP. TAG is first hydrolized into three palmitates and glycerol. Palmitate is then catabolized through β-oxidation and the TCA cycle. Glycerol consumes one ATP and generates one NADH as it is transformed into dihydroxyacetone-phosphate, which is subsequently catabolized by glycolysis and the TCA cycle. In total, each palmitate and glycerol molecule can generate roughly 106 and 17 ATP molecules, respectively [?]. Therefore, the total ATP generated during embryogenesis by catabolyzing glycogen and TAG is

\[ 30 \times \Delta[\text{glycogen}] + (3 \times 106 + 17) \times \Delta[\text{TAG}] = 108 \text{ nmol of ATP}. \]

Normalized by aqueous volume (7.5 nL [?]), Drosophila embryos use approximately 600 nmol/µL/hr of ATP. This is comparable to 861 nmol/µL/hr used by an immortalized baby mouse kidney (iBMK) cell dividing once every 24 hours [?]. Together, these comparisons suggest that fly embryogenesis is a relatively energy intensive process.
1.5 The energy budget of embryogenesis

While the previous comparisons provide us with a sense of the overall metabolic activity level of embryos, the enormous complexity of cellular activities underlying of embryogenesis prohibits the construction of a quantitative and testable energy budget. We can, however, assign minimal energy requirements to well characterized anabolic events such as protein synthesis. In cells proliferating in culture, such as iBMK cells, increase two-fold in protein content per cell division. Thus, an iBMK cell produces at least one cell equivalent of proteins (∼88 μg/μL) every 24 hours [?].

Assuming the average amino acid mass to be 110 g/mol, an iBMK cell polymerizes roughly 0.033 μmol/μL of amino acids every hour. Since it takes a minimum of four ATP molecule equivalents to polymerize one amino acid, iBMK cells use at least 0.13 μmol/μL/hr of ATP for translation, which is 15% of the total ATP production rate [?].

In Drosophila embryos between 2-6 hours old, the total translation rate has been measured through radio isotope tracing experiments to be ∼6 ng/hr [?]. Following similar calculations done for iBMK cells, we can approximate total ATP usage for translation to be ∼0.028 μmol/μL/hr in embryos. We can estimate the amount of total ATP produced during this time by using heat dissipation measurements (Figure 1.1 C). From the previous section, we know that a total of ∼108 nmol of ATP is generate during embryogenesis, which also dissipates 10 mJ of heat. Between the second and sixth hours of development, the embryo dissipates roughly 1.4 mJ of heat. Assuming that the ratio between ATP production and heat dissipation remained constant throughout embryogenesis, we can infer that a total of 108/10 × 1.4 = 15 nmol of ATP was produced in embryos 2-6 hours post fertilization. Normalized for size, this is equivalent to 0.5 μmol/μL/hr. Therefore, translation consumes 5–6% of the total ATP produced. Importantly, we cannot endow meaning on the specific value of 5–6%. We had to make numerous simplifications to arrive at this value, and therefore
cannot claim to have realistic estimates for the energetic requirements of the complex process of synthesizing new proteins. The limited conclusion we can make based on the above approximations is that translation seems to compose a small portion of the early embryo’s energy budget relative to that of proliferating cells.

1.6 Summary

All living organisms dissipate heat into the environment in the process of creating and maintaining order within [?]. Oviparous embryos that develop with minimal material exchanges with the environment provide a particularly clear example of this thermodynamic property. Here, we reviewed the major metabolic properties of Drosophila embryos that transform from a zygote into a multicellular organism with $10^5$ cells.

By biochemical assays and calorimetry measurements, we found that embryos burn 10 mJ worth of glycogen and TAG to fuel developmental events. The complexity of cellular events comprising embryogenesis prohibits the construction of a comprehensive energy budget. However, we were able to show, with some approximations, that translation, a major biosynthetic activity, composes a relatively small portion of the early embryo’s energy budget. This is in accordance with the fact that embryogenesis starts with a large maternal deposition of macromolecules and pre-made cellular machineries such as ribosomes and mitochondria [?, ?, ?]. Taken together, the observations outlined in this introduction suggest that the major metabolic objective of the early embryo is to reorganize the maternally deposited macromolecules.
Figure 1.1: Total energy expenditure of embryogenesis measured by fuel depletion and heat dissipation. (A) The concentration of total glycogen and triglyceride (TAG) in embryos at the beginning and end of embryogenesis. (B) Table of change in glycogen and TAG content represented in nmoles, the amount of oxygen required for full oxidation, and the amount of heat released from full combustion. * and ** are the total oxygen consumption and total heat dissipation values measured by respirometry and calorimetry, respectively [7]. (C) The heat dissipation throughout embryogenesis in 22 °C. The values were obtained from the calorimeter setup detailed in the text.
Chapter 2

Methods and Materials

In this Chapter, I discuss the technical details of different methods and materials used throughout this thesis.

2.1 Fly stocks and genetics

2.1.1 Fly stocks and husbandry

*Drosophila* stocks used in this study were provided by the Bloomington Stock Center. Unfertilized embryos were produced by crossing st1 $\beta$Tub85DD ss1 es/TM3, Sb males to Oregon R virgin females. $\beta$Tub85DD is a male-sterile allele and was obtained from the Bloomington Stock P{neoFRT}82B P{ovoD1-18}3R/st1 $\beta$Tub85DDss1 es/TM3, Sb. Adult flies were matured in 25 C° on fresh yeast paste for at least three and at most 14 days to collect embryos. All embryos used in this study were at developmental stages prior to gastrulation, which precluded the determination of the sex of embryos. All embryos were grown on apple juice plates at 25 C°. All fly stocks were maintained by standard methods at 25 C°, and were grown on a standard cornmeal, molasses, and yeast media.
2.1.2 Plasmid construction and expression of RNRL

The coding sequence for RnrL was cloned into the transformation vector pTIGER [?] between the KpnI and EcoRI restriction sites by Gibson assembly using the primers RnrL_F tgcgttaggctgttcattggtacATGTTGAAGAACAAGTCCATG and RnrL_R tgctagcctctctgccggcctctctTTAAGAACCGCAGGACATG [?]. An alignment of fly, yeast, and mouse reveals a conserved aspartic acid at the fly position 68 (Figure 3.8). This position, referred to as D68N, was converted from aspartic acid to asparagine using the Q5 Site-directed mutagenesis kit by NEB with primers D68N_F - CCAGGAGCTGaACAACTTGGC and D68N_R GTGGTCACGCCGCAGTAC. The wild type and D68N alleles were integrated into the third chromosome using the φC31-based integration at the attP2 site estimated to be at the chromosomal position 68A4 [?]. RNRL expression was driven by the UAS promoter by crossing to MTD-Gal4.

2.2 Calorimetry

Calorimetry experiments were all performed by Professor Yatsuhisa Nagano at Osaka University. Below is the description of the calorimeter set up, similar to previous work that measured heat dissipation from Xenopus Laevis embryos [?].

The calorimeter was equipped with two ampoule measuring cylinders (LKB 2277-201), one of which held the embryo, while the other was a reference ampoule. Both ampoules were housed in an isothermal bath at 295 K. Small heat flow differences between sample and reference ampoules were measured by the output of the attached thermopiles. Eggs were loaded on a wet filter paper (Whatman #3 doused in 0.9% NaCl solution) and incubated with atmospheric gas, which was humidified through the 0.9% NaCl solution on the filter paper. The signal was fed every second into a personal computer to be accumulated for 10 s. The average of every 10 consecutive
signals and their standard deviation were recorded. For each experiment, four or five freshly fertilized CantonS embryos that had been dechorionated were used. In the four replicate experiments consisting of a total of 19 embryos, 17 hatched in approximately 24 hours.

2.3 Metabolic assays

2.3.1 Embryo collection

For metabolomics, embryos were collected at one hour intervals on apple juice plates with small amounts of yeast paste, at 25 C\textdegree. We collected embryos at three discrete time points, each representing the period before, during and after major DNA synthesis. These developmental time points were each approximately one hour long, and the developmental stage was determined in halocarbon oil. Embryos prior to the ninth division are discernible by the absence of pole cells at the posterior. Embryos undergoing the ninth to 13th divisions are discernible by the halo around the periphery and the presence of the pole cells. Finally, embryos that have completed 13 divisions are discernible by the presence of the cellularization front \[?\]. The staged embryos were dechorionated in bleach, washed thoroughly with water, transferred to sterile microcentrifuge tubes, flash frozen in liquid nitrogen, and kept on dry ice until further processing.

2.3.2 Glycogen and triglyceride measurements by biochemical assays

This section refers to the biochemical assays used to generate the data in Figure 1.1A. Embryos were collected at one hour intervals on apple juice plates with small amounts of yeast paste at room temperature. We collected embryos at two discrete time points,
0-1 hours post fertilization and 22-23 hours post fertilization. Pools of ~20 embryos were dechorionated in bleach, washed thoroughly with water, and transferred to sterile microcentrifuge tubes.

For glycogen quantification, glycogen was extracted as described by Parrou and Francoise, 1997 [?], with minor modifications. Embryos in microcentrifuge tubes were suspended in 50 μM of 0.25 M Na₂CO₃ and boiled at 95-98 C° for four hours. Then, 30 μL of 1 M acetic acid and 120 μL of 0.2 M sodium acetate was added to this solution. This solution was incubated overnight at 57 C° with 1.0 U/mL of amyloglucosidase (Sigma A7420). The next day, we used Glucose Quantification Assay kit (Sigma GAGO20) to determine the amount of glucose present in the sample. In brief, glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Then, hydrogen peroxide reacts with a probe that eventually forms a stable colored product. The intensity of the resulting signal is measured through a colorimeter.

For triglyceride quantification, we used the Triglyceride Assay kit (Abcam ab65336) where we used the protocol specified in the kit for tissue samples. In brief, triglycerides are converted to free fatty acids and glycerol, and the glycerol is oxidized into a product. This product reacts with a probe and generates fluorescence that is measured through a microplate reader.

2.3.3 Metabolite measurements by HPLC-MS

Frozen embryo samples were homogenized using a prechilled plastic pestle and mortar in -20 C° extraction solvent of 40:40:20 methanol:acetonitrile:water. Internal standards of known amount of U-15N labeled dNTPs were added to this solution (Cambridge Isotopes, MA). The samples were centrifuged at 16000g for 10 minutes in 4 C°, and the supernatant was injected into the high-pressure liquid chromatograph-mass spectrometer (HPLC-MS). The samples were analyzed by reverse-phase ion-pairing liquid chromatography coupled to an Orbitrap mass spectrometer (QExactive+, Ther-
moFisher, MA) by electrospray ionization in negative ion mode [?]. Metabolites were identified by mass-to-charge ratio and retention time match to authenticated standards. Absolute dNTP concentrations were determined by taking the ratio of signal from unlabeled metabolites to that of spiked in 15N-labeled metabolites of known concentrations. Resulting mass spectra and chromatograms were processed using Metabolomic Analysis and Visualization Engine (MAVEN) [?].

2.4 Injection experiments

2.4.1 Embryo injection and imaging

Embryos were collected at one hour intervals on apple juice plates with small amounts of yeast paste, at 25 °C. Embryos were dechorionated in bleach, washed in water, glued onto coverslips, dessicated, and immersed in halocarbon oil for injection. Injection solutions all contained 0.1 mM RhodamineB dye as a loading control with the small molecule of interest, and injections were done at least 20 minutes prior to cycle 10. The injected embryos were imaged using a 20x oil immersion objective on a Leica-SP5 laser-scanning confocal microscope.

2.4.2 Injection for metabolite measurements

For embryos that were injected and then homogenized for HPLC-MS, a different injection technique was used [?]. Embryos were collected at one hour intervals on apple juice plates with small amounts of yeast paste, at 25 °C. Embryos were taken from the plate and washed thoroughly in water. Using a brush, these embryos were lined up on a coverslip and desiccated for 10 minutes. Embryos were then covered with halocarbon oil in which they were staged and injected with the small molecule of interest. Next, the injected embryos were carefully removed from the coverslip and placed on a nilon mesh. After dabbing the oil away, the embryos were placed in sterile
microcentrifuge tubes and flash frozen in liquid nitrogen. From this point, the steps for metabolite extraction was equivalent to that above.
Chapter 3

Dynamic Control of dNTP Synthesis in Early Embryos

As reviewed previously, the protein, membrane lipid, and RNA content of fly embryos stay relatively constant over time. However, the amount of DNA increases dramatically throughout embryogenesis, following the rapid increase in number of cells. Especially in the early *Drosophila* embryo, fertilization is followed by 13 nuclear cleavage cycles that occur at a breakneck speed of 10–20 minutes per division. As a consequence, the demand for deoxynucleoside triphosphates (dNTP) in early embryos increases exponentially [?, ?]. Compared to the extensive knowledge of cell cycle regulatory factors and developmental events taking place during this time, we have limited understanding of how embryos meet the rapidly increasing demand for dNTPs. In this chapter, we will address this question using quantitative mass spectrometry, live imaging, and genetic manipulations.
3.1 Maternal stores of dNTPs in Drosophila embryos cannot form 6000 genomes needed by MZT

After fertilization, fly embryos undergo 13 synchronous nuclear divisions until the maternal to zygotic transition (MZT), a crucial event in development when zygotic transcription is initiated [??]. Nuclear division cycles prior to MZT are extremely rapid and most occur in the absence of gap phases and cell cycle checkpoint regulation [??]. In just two hours, 13 synchronous divisions produce about 6000 nuclei (less than because not all nuclei migrate to the surface during nuclear cycle nine). This creates a massive demand for building blocks of DNA, given by the product of the number of nuclei at the end of the cycles, ploidy, and the genome size [??].

How might the fly embryo satisfy this demand? To answer this question, we measured dNTP content in 0-1 hour old embryos. Embryos at this age have undergone at most eight divisions, which generate less than 5% of the 6000 nuclei that are synthesized in the first two hours. We collected pools of ~50 embryos and determined dNTP concentration through a high-pressure liquid chromatograph-mass spectrometer (HPLC-MS). We measured absolute concentration by spiking in standards of 15N isotope labeled dNTPs. The concentration values in Figure 1A were derived by assuming the embryos aqueous volume to be 7.5 nL [??]. dGTP concentration was not measured because the HPLC-MS could not differentiate signal from the isomer ATP, which is generally present in cells at much higher concentrations [??]. Our measurements of dATP, dCTP, and dTTP show that 0-1 hour old embryos contain only 30% of dATP and 50% of dC/TTP required for the synthesis of 6000 nuclei (Table 1). The concentration of dNTPs in Table 1 are convertible to nuclear equivalents as
in the following example for 73 μM of dATP.

\[ 73 \, \mu\text{M} \times 7.5 \, \text{nL} \times \text{Avogadro’s number}/(2 \times 175 \, \text{Mbp} \times \text{AT content of genome}) \]

which is equal to 1652 nuclear equivalents. This suggests that embryos contain enough dATP to undergo 10-11 synchronous divisions post fertilization (since \(2^{10} < 1652 < 2^{11}\)). Similar calculations can be made for other animals using values from literature (Table 1) [? , ?]. Xenopus oocytes contain dNTPs sufficient for 11 cycles, or half of that required by MZT, which occurs during the 12th division five hours post fertilization.

For sea urchins, in which MZT starts as early as the first cell cycle, oocytes store dNTPs sufficient for one cell cycle [?]. Thus, fly, frog, and sea urchin embryos must start producing dNTPs early on in development.

Table 3.1: Table for concentration of dNTPs in embryos in μM. Data for Drosophila are represented as mean ± two standard errors, where n=14. (x) represents the number of nuclear equivalents, the derivation for which is detailed in the text. Values for other organisms were obtained from literature [? , ? , ?].

<table>
<thead>
<tr>
<th></th>
<th>S. phage Hela cell</th>
<th>S. Purpuratus oocyte</th>
<th>X. Laevis oocyte</th>
<th>D. melanogaster embryo 0-1 hpf</th>
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<tr>
<td>dATP</td>
<td>9 (0.003)</td>
<td>11 (3)</td>
<td>13 (2087)</td>
<td>73 ± 5 (1652 ± 137)</td>
</tr>
<tr>
<td>dTTP</td>
<td>20 (0.006)</td>
<td>26 (7)</td>
<td>12 (1927)</td>
<td>116 ± 9 (2631 ± 256)</td>
</tr>
<tr>
<td>dCTP</td>
<td>13 (0.004)</td>
<td>18 (7)</td>
<td>19 (4672)</td>
<td>119 ± 8 (3559 ± 228)</td>
</tr>
<tr>
<td>dGTP</td>
<td>9 (0.003)</td>
<td>2 (1)</td>
<td>12 (2951)</td>
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3.2 RNR synthesizes dNTPs on the go

Ribonucleotide reductase (RNR) is the only known enzyme that catalyzes the rate limiting step of de novo dNTP synthesis, which is the main source of dNTPs in cycling cells [?]. Therefore, we expected early Drosophila embryos to produce dNTPs through RNR, and RNR inhibition to lead to cell cycle failure. To test this, we injected H2Av-
GFP transgenic embryos with hydroxyurea (HU), a well characterized RNR inhibitor, and monitored cell cycles by live imaging (Figure 3.1A) [?]. Most of these embryos underwent catastrophic mitoses at the 11th division, during which the nuclei were able to undergo chromosome condensation and develop to metaphase, but failed to separate during anaphase (Figure 3.1B). These failures are similar to those observed after DNA synthesis inhibition, indicative of chromosomal defects from DNA replication stress [?]. Co-injection of HU and dNTPs allowed progression through the 13 synchronous nuclear division cycles (Figure 3.1B). These results strongly suggest that RNR produces a significant portion of dNTPs required during the nuclear cleavage cycles.
Figure 3.1: Embryos synthesize dNTPs on the go through RNR. (A) Schematic of the injection experiments. Embryos are glued to a coverslip and injected with the small molecule of interest and the loading control dye (RhodamineB). For all experiments, roughly 70 pL (1% of the embryo aqueous volume) was injected. (B) The proportion of embryos that successfully completed 13 divisions after the injections. n is the number of embryos imaged after injection.
dNTPs can also be produced through the deoxynucleotide salvage pathway, which in some contexts contributes significantly to dNTP production during cell cycle [?]. Flux through the salvage pathway is controlled in large part by deoxycytidine kinase (dCK) and thymidine kinase 1 (TK1). dCK can contribute to the production of all four dNTPs by phosphorylating deoxycytidine (dC), deoxyadenosine (dA) and deoxyguanosine (dG), and TK1 contributes to dTTP synthesis by phosphorylating thymidine and deoxyuridine [?]. The substrates of these two enzymes, deoxynucleosides (dN), are produced through degradation of dNTPs. To achieve net positive synthesis of dNTPs through the salvage pathway, cells must uptake dNs from the environment, which is not possible for oviparous embryos. Sea urchin embryos can produce dNTPs for multiple rounds of divisions in the absence of RNR activity, potentially by salvaging maternally deposited dNs [?]. We estimated the maximum contribution of the salvage pathway to total dNTP production by measuring the concentrations of dNs in 0-1 hour old embryos (Figure 3.2). The concentrations of dA, dC, and dT were very low in abundance below 10 μM, or ∼300 nuclear equivalents, which can be calculated in the same way as dNTP nuclear equivalents Figure 3.2. Low dN levels in 0-1 hour old embryos imply that the salvage pathway does not contribute significantly to dNTP production, and that RNR produces most of the dNTPs during the nuclear cleavage cycles.

3.3 RNR is under allosteric control by dNTPs in the early embryo

RNR activity is regulated at multiple levels, including transcription, protein degradation, protein modification, and allosteric regulation by dNTPs. In particular, dNTP concentration governs RNR activity through a complex set of allosteric effects that are highly conserved across species from yeast to humans (Figure 3.3A) [?]. In
Figure 3.2: Embryos contain low concentrations of deoxyadenosine, deoxycytidine, and thymidine (dA, dC, dT), substrates of the dNTP salvage pathway. Each biological sample consisted of ∼50 embryos 0-1 hours old. Each biological replicate was split into two technical replicates, to one of which known concentrations of deoxynucleosides were added. The numbers denoted on the x-axis labels represent the amount of deoxynucleoside standards added in μM equivalents for the concentration in a single embryo. The y-axis denotes the total ions detected by the mass spectrometer for each metabolite. The concentration of all deoxynucleosides are significantly less than 10 μM in embryos. The concentration of dA and dC were both near the detection limit. The error bars denote two units of the standard error of the mean, where n=5.

Brief, dATP regulates overall activity by binding to the activity site of RNR, and can effectively stop dNTP production. At the same time, ATP, dATP, dGTP, and dTTP bind to the specificity site of the enzyme and dictate which NDP species is reduced. Since RNR activity is required for cell cycle progression (Figure 3.1), the cell cycle progression can be used as a reporter to probe the regulatory effects of dNTPs on RNR activity (Figure 3.3).
Figure 3.3: Embryonic RNR can be allosterically regulated by exogenously provided dNTPs. (A) Schematic for allosteric regulation of RNR, represented by dotted lines (adapted from [?]). (B) The proportion of embryos that successfully completed 13 divisions after the injections. n is the number of embryos imaged after injection.
Injection of high amounts of dATP (causing a fourfold increase of dATP concentration in the embryo) resulted in catastrophic mitoses in ways similar to those caused by HU injections, as expected by the inhibitory effect of dATP on RNR activity (Figure 3.3B) [?]. On the other hand, injection of dGTP induced cell cycle arrest at cycle 13. This may be because dGTP is known to induce dATP production by binding to the specificity site of RNR. Production of dATP can in turn suppress RNR activity, and cause a shortage of dCTP and dTTP. Accordingly, embryos injected with equal ratios of dGTP, dCTP, and dTTP completed their cell cycles normally. dTTP injection alone had no effect, possibly because dTTP injection could induce the production of dGTP and subsequently dATP, while preserving dCTP content by inhibiting dCTP deaminase [?]. On the other hand, dCTP, which is known to have no regulatory effect on RNR, had no effect, suggesting that the massive imbalance of dNTPs alone does not affect the cell cycle progression (Figure 3.3B).

Our injection experiments demonstrated how RNR activity can be regulated by exogenously provided dNTPs (Figure 3.3B). Is RNR activity inhibited by the endogenous levels of dATP? If this is indeed the case, then RNR activity should be continuously inhibited in the absence of dATP consumption. To test this prediction, we measured the dNTP concentrations over time in fertilized embryos and unfertilized embryos, the latter of which do not synthesize nuclei but are known to have various cellular processes of embryogenesis intact (Figure 3.4) [?]. In each case, we inferred RNR activity by calculating the net production of dNTP, which is the sum of total dNTP consumption and the net change in dNTP concentration. A fertilized embryo synthesizes 6000 nuclei in the first two hours. 6000 nuclear equivalents is approximately 265 μM of dATP in concentration, by the same calculations detailed previously. Since the concentration of dATP decreases from 73 μM to 38 μM, the net production of dATP is 230 μM. In unfertilized embryos, the increase in dATP over time is equal to the net production, which is almost negligible (Figure 3.4). Thus,
RNR appears to be inhibited by the endogenous levels of dATP and this inhibition is relieved as maternal stores are depleted by DNA polymerization. According to this model, the concentration of dATP in 1-2 hour old embryos that are synthesizing large amounts of DNA has to be lower than that of 0-1 hour old embryos, which is indeed the case (Figure 3.4).
Figure 3.4: Concentration profiles of dATP, dCTP, and dTTP over time in fertilized and unfertilized embryos pooled by hours post fertilization (hpf). In fertilized embryos, replication consumes dNTPs and RNR produces it. In unfertilized embryos, there is no consumption of dNTPs. Each sample consisted of 20 or more embryos, and (-) depicts the mean value. n is the number of samples. The values inserted on the bottom left corner of the panels are the average enzyme activity levels during the two hours. The activity level is derived from the mass balance equation of dNTP over the first two hours, where total RNR activity = Δ[dNTP] consumption by replication. * corresponds to p-values from two sided t-tests, where p < 0.01 :*, p < 0.001 :**, and p < 0.0001 :***.
3.4 Dynamic control of dNTP metabolism by feedback inhibition

Thus far, we have established RNR as the supplier of dNTPs during the early nuclear cleavage cycles (Figures 3.1-3.2), and showed that RNR is inhibited by dATP in naturally occurring concentrations (Figure 3.3). When does dNTP synthesis by RNR start? What are the ranges of initial dNTP and RNR concentrations in the embryo that ensure sufficient and timely synthesis of dNTPs? To explore these questions in a systematic way, we constructed a simplified mathematical model of dNTP metabolism.

Our model focuses on dATP, since dATP is the master regulator of overall RNR activity (Figure 3.5A). We did not include other components of dNTP metabolism, because each additional component introduces multiple unknown parameters, making the model difficult to manage [?]. We assumed that dATP is consumed only by DNA polymerization (C(t)), which is modelled as a sequence of exponentially increasing pulses (Figure 3.5B). The magnitude and duration of the pulses reflects the number of genomes and lengths of S phase and mitosis, both of which are known [?]. Next, we assumed that all production of dATP occurs through RNR. Although dADP is the direct product of RNR, we combined the terms for dADP and dATP in the model since NDP kinase reactions have values two orders of magnitude higher than that of RNR [?, ?]. Since the concentrations of all the substrates (NDPs) remain relatively constant during the time points of interest (Figure 3.5C), V in the production term is effectively a proxy for RNR activity, which we assume to be constant in time. Finally, the inhibition term is a simple phenomenological representation of the inhibitory effect (Hill coefficient 2) of dATP on mouse RNR observed in vitro [?]. Based on these interactions, we obtain the following equation describing dATP concentration over time, where P (product) denotes dATP concentration.
\[
\frac{dP}{dt} = V \frac{K_i^2}{p^2 + K_i^2} - C(t).
\]

The model contains only three free parameters: the initial condition \((P(0))\), the intrinsic activity of the maternally deposited enzyme \((V)\), and the strength of inhibition \((K_i)\). The values of these parameters can be constrained to a region of a three-dimensional parameter space, denoted as \(\Omega\). For each point in \(\Omega\), the corresponding simulation \(P(t)\) has to be positive, and the average of \(P(t)\) has to fall within two standard deviations of the mean of concentrations observed at the matching time points (Figure 3.4).
Figure 3.5: Model for dATP production and consumption in the early embryo. (A) Summary of the model. dATP is produced by RNR and consumed by DNA polymerization. dATP allosterically inhibits RNR. (B) dATP consumption rate is proportional to the number of nuclei, which increases exponentially in time. (C) Relative concentration (by total ions detected by the mass spectrometer) of ribonucleoside diphosphate species over time. Each biological sample consisted of >20 embryos collected at one hour intervals. Error bars denote two units of the standard error of the mean where $7 \leq n \leq 12$. 
We constructed $\Omega$ by doing a grid search for $K_i$, $V$ and $P(0)$ and found that all the feasible values of were under $\sim 30 \mu$M, which is twofold lower than the average dATP concentrations during the first two hours (Figure 3.6B). In addition, the range of allowable values reflected those measured in vitro from Escherichia coli and mouse RNR [? , ?]. Minor variations to the model, such as allowing slow accumulation of the enzyme content over time, or changing the value of the Hill coefficient of the dATP mediated inhibition, do not change our qualitative conclusions on the presence of the feedback inhibition. Thus, our model recapitulates the inhibition of RNR by dATP using only the concentration profile of dATP from fertilized embryos (Figure 3.4).
Figure 3.6: Model for dATP production recapitulates the inhibition of RNR by dATP. (A) Representative concentration profiles of dATP. In orange is the concentration profile that matches the measurements from Figure 3.4 as described in the text. The bars on the right represent mean ± two standard deviations of dATP measurements at the respective time intervals. In black are the profiles that were either too high/low in abundance to fit the data. The parameter tuples are also shown in (B) by matching shapes x, +, and o. (B) Set of feasible parameter tuples that agree with measurements in Figure 2. The color scale represents initial dATP values. Maximum V value was 1000 μM/sec, obtained by assuming the maximum concentration of RNR to be 1mM, and the maximum value to be 1/sec \([\text{?}]\). K_i values can be lower.
3.5 Perturbations to RNR activity lead to predictable changes in dNTP concentration

To test our model of dNTP metabolism, we perturbed the system in three different ways, each of which corresponds to changing one of the three parameters: the initial concentration of dATP (P(0)), the enzyme activity (V), and the strength of negative feedback (K_i).

First, we induced changes to the initial concentration of dATP (P(0)). By a general property of dynamical systems regulated by feedback inhibition, the dATP levels over time are expected to be robust to relatively large variations in initial concentrations. In fact, our model predicts that the concentration of dATP at the end of the nuclear cycles converges to the same value for a wide range of initial concentrations (Figure 3.7A). To test this prediction, we injected embryos with variable amounts of dNTPs at 0-1 hours. For each experiment, all four dNTPs were injected at equal ratios. We measured the dNTP concentration in embryos shortly after the injections, and two hours later in embryos that had undergone 13 synchronous divisions. The concentration of dNTPs after two hours converged to similar values independent of the amount injected previously (Figure 3.7B). Thus, the model reveals the impressive robustness of the dNTP supply system in pregastrular embryos.
Figure 3.7: dNTP content at gastrulation is robust to variations in initial concentrations. (A) Simulated concentration profiles of dATP, where $c = 5$ μM, $V = 1.5$ μM/sec, and dATP$(0)$ is varied from 0 to 256 μM. All the profiles converge by $t=120$ minutes. (B) Concentration of dNTPs in embryos that were injected with variable amounts of dNTPs. For all injections, all four dNTPs were injected at the same indicated concentration. The volume injected was approximately 70 pL, or 1% of the embryos aqueous volume. 0-1 hours post fertilization (hpf) embryos are those that had just been injected and had not undergone major DNA synthesis events yet. 2-3 hpf embryos have undergone 6000 nuclear synthesis events. Each dot represents a concentration measured from a pool of $\sim$15 embryos.
3.5.1 Proof for convergence in feedback inhibited systems

As a short detour, I will provide a formal proof of the convergence of feedback inhibited dynamical systems. All credit for this proof goes to Professor Eduardo Sontag at Rutgers University. Before stating the theorem, we need to define the relevant components of the dynamical system in mathematical terms.

Let \( p(t) \) be a 1-dimensional, continuous, and differentiable function in time defined by the following differential equation

\[
\frac{dp}{dt} = u(t) + f(p).
\]

Here, \( u(t) \) and \( f(p) \) are continuous and differentiable functions, where \( u(t) \) is independent of \( p \). In relation to our model for dATP concentration, \( p \) corresponds \([dATP]\), \( u(t) \) corresponds to the consumption of dATP by DNA polymerization, and \( f(p) \) corresponds to the production of dATP by RNR. To represent feedback inhibition, we will assume that \( f'(p) < 0 \) for all \( p \).

**Theorem 1.** Let \( p_1(t) \) and \( p_2(t) \) be functions defined in the same way as \( p(t) \) above, where \( p_1(0) = \pi_1 \) and \( p_2(0) = \pi_2 \). Then, there exists \( \mu \) such that

\[
|p_1(t) - p_2(t)| \leq \exp^{-\mu t}|\pi_1 - \pi_2|.
\]

First, we need the following simple lemma.

**Lemma 1.** Assume in the above system that \( \pi_1 > \pi_2 \). Then, \( p_1(t) \geq p_2(t) \) for all \( t \).

**Proof.** Assume for contradiction that there exists \( T \) such that \( p_1(T) < p_2(T) \). Since \( p_1(t) \) and \( p_2(t) \) are continuous, so is \( p_1(t) - p_2(t) \). Since \( p_1(0) - p_2(0) > 0 \) and \( p_1(T) - p_2(T) < 0 \), there exists \( 0 < T' < T \) such that \( p_1(T') = p_2(T') \). Since \( p_1 \) and \( p_2 \) are entirely identical at \( T' \), \( p_1(t) = p_2(t) \) for all \( t \geq T' \). Thus, we contradicted our assumption that \( p_1(T) < p_2(T) \).
Now we can proof Theorem 1.

**Proof.** Assume without loss of generality that $\pi_1 > \pi_2$. From Lemma 1, $p_1(t) - p_2(t) \geq 0$ for all $t$. Next, define $\mu$ as such,

$$\mu := -\max_p [f'(p)]$$

Then, we can write the following inequalities.

$$\frac{d|p_1(t) - p_2(t)|}{dt} = \frac{d(p_1(t) - p_2(t))}{dt} = \frac{dp_1}{dt} - \frac{dp_2}{dt} = u(t) + f(p_1) - (u(t) + f(p_2)) = f(p_1) - f(p_2) = \int_{p_2}^{p_1} f'(p) dp \leq \int_{p_2}^{p_1} -\mu dp \leq -\mu|p_1 - p_2|$$

Since $|p_1(t) - p_2(t)|$ decreases faster than $\exp^{-\mu t} |p_1 - p_2|$ at all $t$,

$$|p_1(t) - p_2(t)| \leq \exp^{-\mu t} |\pi_1 - \pi_2|.$$
Perhaps the simplest test for our model is to change the amount of RNR (V). As in the simulation marked by (+) in Figure 3.7, the concentrations of dNTPs are expected to increase as parameter V is increased. To implement this change, we used the Gal4-UAS system to overexpress wild type RNRL in early embryos from the maternal pool of transcripts. We found that the concentration of dNTPs approximately doubled in these embryos (Figure 3.8). Since we only overexpressed the larger subunit of RNR, whose activity requires both subunits, we cannot precisely quantify the increase in the total RNR catalytic capacity (V). Nevertheless, the significant increase in dNTP concentrations in MTD > RNRLWT embryos establishes a direct link between RNR and dNTP levels.
Figure 3.8: Genetic perturbations to RNR activity lead to predictable changes in dNTP concentration. (A) On the right is the protein sequence alignment of yeast, fly, and mouse RNRL. We introduced a point mutation at the 68th position in fly RNRL (57th position in yeast and mouse RNRL), which is located in the highly conserved dATP binding domain. On the left is the schematic of the effect of D68N on RNR activity, which has been shown in yeast and mouse to abolish dATP mediated feedback inhibition [?, ?]. (B) dNTP concentration of RNRLWT and RNRLD68N expressing embryos compared to that of Oregon R embryos. Expression was achieved by a UAS-Gal4 system, driven by a strong maternal driver (MTD-Gal4). Each sample consisted of 15 or more embryos, and (-) depicts the mean value. n is the number of samples. * corresponds to p-values from two sided t-tests, where p < 0.0001:***. 
Finally, we perturbed the system by abolishing the negative feedback by dATP, which corresponds to setting large $K_i$ values in our model. To do this, we used the Gal4-UAS system to overexpress RNRL with a point mutation from a maternal pool of transcripts. This point mutation, which changes the aspartate at the 68th position into asparagine (denoted as henceforth) is located in a highly conserved domain for dATP mediated inhibition (Figure 3.8A). Experiments in mouse and yeast have shown that the effect of dATP inhibition on RNR was completely abolished by this point mutation [?, ?]. Our model predicts RNRL$_{D68N}$ expression should increase the concentration of dNTPs much more than that of RNRL$_{WT}$ expression. Indeed, the concentration of dNTPs in MTD > RNRL$_{D68N}$ embryos was fivefold higher than that of MTD > RNRL$_{WT}$ embryos (Figure 3.8). Furthermore, metabolomic profiling of MTD > RNRL$_{D68N}$ versus MTD > RNRL$_{WT}$ embryos shows that we have indeed specifically perturbed dNTP metabolism by introducing the D68N mutation (Figure 3.9A). Interestingly, MTD > RNRL$_{D68N}$ embryos all failed to complete embryogenesis with severe anterior defects (Figure 3.9B), while the hatching rate of MTD > RNRL$_{WT}$ embryos was comparable to that of Oregon R. These results demonstrate that RNR is responsible for dNTP production, and reveals a functionally crucial role of the dATP mediated feedback inhibition in embryogenesis.
Figure 3.9: Metabolic and developmental phenotype of RNRL$^{D68N}$ expressing embryos. (A) Comparison of relative total ion counts of metabolites between MTD $>$ RNRL$^{WT}$ and MTD $>$ RNRL$^{D68N}$ embryos at 0-1 hours post fertilization. Relative concentrations are plotted in log2 scale, and the error bars represent two standard errors of the mean, where n=5. dNTP metabolism related metabolites are colored in red. x=y line is colored in orange. Abbreviations include FBP: fructose-1,6-bisphosphate and HMP: hexose-monophosphate. (B) A table of hatch rate and cuticles of Oregon R, MTD $>$ RNRL$^{WT}$, and MTD $>$ RNRL$^{D68N}$ embryos. RNRL$^{D68N}$ expression leads to particularly severe head structure defects.
3.6 Summary

Early *Drosophila* embryos face a massive metabolic challenge of providing DNA precursors for the exponentially increasing number of nuclei. We found that the maternally deposited dNTP content accounts for less than half of the nuclei present at the end of 13 nuclear cleavages, and the rest are synthesized on the go. Most of the dNTPs are synthesized through RNR, the rate limiting enzyme of de novo dNTP synthesis. Interestingly, RNR is feedback inhibited by endogenous levels of dATP and is reactivated as dATP is depleted via DNA polymerization. To understand the dynamical properties of this feedback system, we constructed a model of dNTP metabolism. In addition to providing a compact summary of our data, the model makes clear testable predictions. For instance, the levels of dNTPs should be robust to large variations in the starting amounts, and should increase dramatically in the absence of feedback inhibition. We confirmed these predictions experimentally, and in the process showed that feedback inhibition of RNR is essential for normal progression of embryogenesis.
Chapter 4

Discussion and Future Directions

4.1 *Drosophila* development as a platform to study dNTP metabolism

Cellular dNTP levels are tightly controlled. It has been long known that dNTP concentration unbalanced, too high, or too low can lead to high mutation rates and genomic instability \[^1\]. Misregulation of dNTP metabolism has been linked with multiple diseases including mitochondrial disorders, susceptibility to viral infections, and cancer \[^2\]. As the gate keeper of dNTP synthesis, RNR has a complex role in cancer development, and is an important chemotherapeutic drug target \[^3\]. However, our mechanistic understanding of the relationship between RNR regulation, mutation rates, and physiology in multicellular organisms is still nascent. There have been two previous *in vivo* studies of RNR misregulation in multicellular organisms \[^4\], \[^5\]. In one of these, the broad overexpression of the small subunit of RNR in mice surprisingly lead to increase in occurrence of lung tumors \[^6\]. However, dNTP concentrations and mutation rates were not quantified, and the reason for the tissue specific effects were unclear.
Interestingly, we also observed tissue specific effects in RNRL\textsubscript{D68N} over-expressing in embryos. Global increase of dNTP concentration lead to severe defects in the anterior part of the embryo, while the posterior and mid-body structures remained relatively normal (Figure 3.9B). In addition, overexpression of RNRL\textsubscript{D68N} driven in all tissues (by Act5C) or in follicle cells (by traffic jam) did not confer discernible morphological defects. Together, these results present complex tissue specific effects of RNR misregulation. Going forward, we can use powerful genetic tools of \textit{Drosophila} development for mechanistic studies of dNTP metabolism \textit{in vivo}.

### 4.2 Upregulation of energy metabolism in early embryos

The mature animal oocyte is a metabolically quiescent cell. Upon activation, a calcium wave traverses through the egg and initiates a diverse array of cellular activities \cite{?}. Accordingly, measurements of embryonic respiration from the past century have shown rapid increase of energy metabolism in early embryogenesis in many different species \cite{?, ?, ?, ?, ?}. However, the control mechanisms or even the metabolic fluxes that contribute to this increase in energy production are largely unknown. In the early \textit{Drosophila} embryo, the rapid increase in cellular activity is represented by the exponential increase in the number of nuclei. Along with the demand for dNTPs, the amount of energy consumed by nuclear divisions also increases dramatically during this time. To see how the embryo might be satisfying this energetic demand, we examined the changes of the embryo's metabolome during the first 2 – 3 hours of development. The relative concentration profiles presented in Figure 4.1 was generated through HPLC-MS, in conjunction with the measurements of dNTP content in Chapter 3.
While most of the metabolites remained relatively constant in time, fructose–1,6–bisphosphate (FBP) increased nearly eight fold in just two hours. FBP is the product of the phosphofructokinase, the rate limiting enzyme of glycolysis, as well as the feed forward activator of pyruvate kinase in lower glycolysis [?]. Therefore, the rapid increase in FBP levels suggests that embryos upregulate glycolysis. In addition, the TCA cycle components have a modest but significant increase over time, which may indicate increase in capacity for TCA cycle flux. Similar changes of the metabolome are also observed in unfertilized embryos, suggesting that these changes do not occur through transcriptional control (Figure 4.2).
Figure 4.1: Metabolome of fertilized embryos during the first 2-3 hours of development. (A) The relative concentration profiles of fructose–1,6–bisphosphate, malate, and α-ketoglutarate 2-3 hours post fertilization. The y-axis values are total ion counts represented as mean ± two standard errors where n=5. (B) The relative concentration profile of the metabolome of fertilized embryos, where values are depicted on a color scale representing log2 fold change with respect to the 0-1 hour post fertilization time point. The metabolites on the y-axis are organized top down in descending order of the average fold change value. For both (A) and (B), metabolite measurements were made from pools of >20 embryos at the respective developmental stages. Abbreviations include FBP: fructose–1,6–bisphosphate, HMP: hexose–monophosphate, IMP: inositol–monophosphate, and GLYC3P: glycerol–3–phosphate.
Figure 4.2: Metabolome of unfertilized embryos during the first 2-3 hours of development. (A) The relative concentration profiles of fructose–1,6–bisphosphate, malate, and α-ketoglutarate 2-3 hours post oviposition. The y-axis values are total ion counts represented as mean ± two standard errors where n=5. (B) The relative concentration profile of the metabolome of unfertilized embryos, where values are depicted on a color scale representing log2 fold change with respect to the 0-1 hour post oviposition time point. The metabolites on the y-axis are organized top down in descending order of the average fold change value. For both (A) and (B), metabolite measurements were made from pools of >20 embryos at the respective developmental stages. Abbreviations include FBP: fructose–1,6–bisphosphate, HMP: hexose–monophosphate, IMP: inositol–monophosphate, DHAP: dihydroacetone–phosphate, and GLYC3P: glycerol–3–phosphate.
In sum, the increase of FBP and TCA cycle intermediates suggest that embryos upregulate energy metabolism within 2-3 hours of fertilization. It is interesting to note that in mature oocytes, energy metabolism is repressed by the inhibition of glycolysis and the disassembly of mitochondria. The metabolic quiescence of mature oocytes is induced by the loss of Akt signal, which is turned back on during embryogenesis [?]. In addition, Akt signaling is known to increase glycolytic flux in cancer cells [?]. Therefore, Akt is one of the candidate signaling molecules that may upregulate early embryonic energy metabolism. Indeed, recent work by Kunh et al., has demonstrated that the Tor pathway, which can be controlled by Akt activity, is responsible for the upregulation of yolk catabolism in Drosophila embryos [?, ?]. In future work, we can use imaging, mass spectrometry, and genetic tools presented in this thesis, to understand the control mechanisms in energy metabolism that allow embryos to jump-start life.
Appendix A

Matlab Code for dATP model

This chapter delineates the Matlab code used for simulation of dATP concentration profile by the differential equation described in Chapter 3.

To start, the function sampler.m generates a large random set of parameters. Using dATPsim.m, we simulate the matching concentration profile for each of the generated parameter sets, and determine whether the parameter set is feasible by comparing the simulated profile to measured dATP concentrations. The differential equation by which dATP concentration is defined is detailed in dATPdiffeq.m. All three files are shown below, which also contains the details of the implementation in the comments.

A.1 sampler.m

```matlab
function [y, yp] = sampler(N)

% Function that samples parameter values to find the
% feasible space. Input N is the total number of parameters
% that will be tested.
```

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% For all the values used here and in other functions, there is a conversion between the units of the y value used in the matlab code and the units of uM used to present measurements from experiments.

% One unit of y in the code refers to the amount of dATP required for one nuclei. Therefore, to convert from uM concentrations (X) to nuclear equivalents (y), we need to perform the following operations as also detailed in the text.

\[ y = X \times 7.5 \text{ nL} \times 6.022 \times 10^{-23} / (2 \times 0.58 \times 175000000 \text{ bp}) \]

Therefore, one unit in Matlab equals 0.045 uM

% Set up the variables to be used

trials = N; %total number of parameters to be tested
T = 132; %duration of the simulation, set to 132 minutes
test_set = []; %array to store all the tested parameters
test_setY = []; %matrix of all the tested simulation profiles

% The for loop that generates the simulation profiles from randomly generated parameters, each described below. The ranges from which each parameter is sampled are set manually.
for i = 1:trials
    Ki = 10^(rand() * 3);  % strength of inhibition
    Vmax = 10^(rand() * 7);  % total enzyme capacity
    n = 2;  % hill coefficient
    slope = 0;  % production rate of RNR
    y0 = 1735 * rand();  % initial concentration of y
    param = [Ki Vmax n slope y0];

    % run the simulation
    [y, yp] = dATPsim(T, param);

    % if y at all time points are positive, save the
    % parameter values and the simulation profile
    if (double(y > =0))
        test_set = [test_set; [Ki, Vmax, y0, slope]];
        test_setY = [test_setY; y];
    end
end

% Now, parse through the obtained concentration profiles
% by comparing them to the measured dATP concentrations.
% Below, Z represents the feasible parameter set that
% matches measured data.

Z = [];
for i = 1:length(test_setMax)
    stage2 = mean(test_setY(i, 1:60));
\[ \text{stage4} = \text{mean}(\text{test set}Y(i,61:120)); \]

\[ \text{if (stage2 < 2000) \&\& (stage2 > 1240) \ldots} \]
\[ \text{\&\& (stage4 > 514) \&\& (stage4 < 1760)} \]
\[ Z = [Z, [\text{test set}(i,3) \ast 0.045 \ldots} \]
\[ \text{; test set}(i,1) \ast 4.5 \ast 10^{-2} \ldots} \]
\[ \text{; test set}(i,2) \ast 4.5 \ast 10^{-2} / 60)]; \]

end
end

\textbf{A.2 \quad dATPsim.m}

\begin{verbatim}
function [y, yp] = dATPsim(T, param)

% Function that solves the ODE to generate the
% concentration profile of dATP. Input T is the duration
% of the simulation, and param describes other
% parameters of the model.

tspan = [0 T];
P = [param];
y0 = P(5);
options = odeset('RelTol',1e-6, 'AbsTol',1e-6);
% the ODE is defined in dATPdiffeq.m
sol = ode23s(@(t,y) dATPdiffeq(t,y,P), tspan, y0, options);
x = linspace(0,T,132);
[y, yp] = deval(sol, x);
\end{verbatim}
A.3 dATPdiffeq.m

function dydt = dATPdiffeq(T,y,P)

% Function for the differential equation of y, which represents concentration of dATP in the embryo.
% Input P contains parameters Ki, Vmax, n, slope, and y0.

% Times for cell cycle events
% For the 9 cycles that take place in the middle of the embryo, we assume that each division takes 9 minutes, with 3 minutes allotted for mitosis.
% The S phase and mitosis timings below come from Papoulas, 2010.
% cycle 10 = 6.5 + 3 (S phase: 6.5 min, mitosis: 3 min)
% cycle 11 = 7.4 + 3.3
% cycle 12 = 7.8 + 4
% cycle 13 = 13.5 + 6.1

Ki = P(1);
Vmax = P(2);
n = P(3);
slope = P(4);

% The number of nuclei increase exponentially from cycle
% to cycle. C represents the consumption term.

if (T<81) %prior to cycle 9
  cycle = floor(T/9);
  C = 2^cycle*sin(pi*(T-9*cycle)/6)*(6>(T-9*cycle))/3.8197;
else if (81<=T)&&(T< 87.5) %cycle 10
  C = 2^9*sin(pi*(T-81)/6.5)/4.14;
else if (90.5<=T)&&(T< 97.9) %cycle 11
  C = 2^10*sin(pi*(T-90.5)/7.4)/4.711;
else if (101.2<=T)&&(T< 109) %cycle 12
  C = 2^11*sin(pi*(T-101.2)/7.8)/4.9656;
else if (113<=T)&&(T< 126.5) %cycle 13
  C = 2^12*sin(pi*(T-113)/13.5)/8.5944;
else
  C = 0;
end

% C is multiplied by 3/4 since the total number of nuclei
% at the end of the cycles is closer to 6000 than 8000.
% The production term is simply Vmax times the product
% inhibition term with hill coefficient n (here set to 2)
dydt = -C*0.75+(1+slopex*T)*Vmax*1/(1+(y/(Ki))ˆn);
end
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