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Regulation of post-embryonic development in Caenorhabditis elegans

Deciphering the impact of CDK-9 on CTD Serine-2phosphorylation

Stubbe, Francois-Xavier

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REGULATION OF POST-EMBRYONIC DEVELOPMENT IN *CAENORHABDITIS ELEGANS*: DECIPHERING THE IMPACT OF CDK-9 ON CTD SERINE-2 PHOSPHORYLATION

Mémoire présenté pour l'obtention

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François-Xavier STUBBE

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Université de Namur
FACULTE DES SCIENCES
Secrétariat du Département de Biologie
Rue de Bruxelles 61 - 5000 NAMUR
Téléphone: + 32(0)81.72.44.18 - Téléfax: + 32(0)81.72.44.20
E-mail: joelle.jonet@unamur.be - http://www.unamur.be

Regulation of Post-embryonic Development in *Caenorhabditis elegans*: Deciphering the Impact of CDK-9 on CTD Serine-2 Phosphorylation

STUBBE François-Xavier

Abstract

Transcription of protein coding genes is carried out by the RNA polymerase II (Pol II). It is now clearly established that Pol II itself is subject to many modifications that can profoundly influence how factors required for transcription and RNA processing are recruited. The largest subunit of Pol II harbors a tail like C-terminal domain (CTD) composed of repeats of the consensus heptapeptide sequence $Y_1S_2P_3T_4S_5P_6S_7$. It has been proposed that many different potential combinations of CTD modifications makes up a code that orchestrate the complex sequential recruitments of factors involved in transcription and RNA processing. One of the most abundant CTD modification is the phosphorylation of the CTD-Ser2, a mark classically associated with transcription elongation. The positive elongation complex CDK9/Cyclin T(P-TEFb) has been shown to phosphorylate CTD-Ser2 and to be broadly essential for expression of early embryonic genes.

In the model organism *Caenorhabditis elegans*, the RNAi knock down of *cdk-9* mRNA induces an early embryonic arrest mimicking an RNAi knock down of *ama-1*, the biggest Pol II subunit. We have constructed a *cdk-9* analogue sensitive (*cdk-9as*) strain seeking to investigate the role of CDK-9 during development. We showed that the inhibition of CDK-9as with the bulky ATP analogue 3MB-PP1 does not perfectly phenocopy RNAi knock down but induces a reduction in brood size and partial embryonic lethality. It has been proposed that CDK-12, but not CDK-9, is the major CTD-Ser2 kinase in *C. elegans*. Using our newly constructed *cdk-9as* mutant strain, we investigated the relative contribution of CDK-9 in the phosphorylation of CTD-Ser2. Surprisingly, we did not detect a drop in the phosphorylation level upon CDK-9as inhibition. CDK-12as inhibition causes a severe decrease in CTD-Ser2P but detectable signal is still present. We constructed a mutant strain containing both *cdk-9as* and *cdk-12as* and showed that simultaneous kinase inhibition at high concentration causes a more severe drop in CTD-Ser2 phosphorylation level than CDK-12as inhibition alone. We postulate that the CTD kinase activity of CDK-9 and CDK-12 may be partially redundant. When CDK-9as is inhibited, CDK-12 alone can compensate for the loss in CTD-Ser2 phosphorylation. Taken together our results show that CDK-9 dependent CTD-Ser2 phosphorylation is required for completion of embryogenesis but the CDK-9 itself is dispensable to maintain proper level of CTD-Ser2 phosphorylation.

Mémoire de master 120 en biochimie et biologie moléculaire et cellulaire

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Supervisor: Damien Hermand

Université de Namur
FACULTE DES SCIENCES
Secrétariat du Département de Biologie
Rue de Bruxelles 61 - 5000 NAMUR
Téléphone: + 32(0)81.72.44.18 - Téléfax: + 32(0)81.72.44.20
E-mail: joelle.jonet@unamur.be - http://www.unamur.be

Régulation du développement post-embryonnaire chez *Caenorhabditis elegans* : Décrypter l'impact de CDK-9 sur la phosphorylation de la sérine-2 du CTD

STUBBE François-Xavier

Résumé

La transcription des gènes codant pour des protéines est réalisée par l'ARN polymérase II (Pol II). Pol II possède une extension C-terminale (CTD) composée d'une répétition de l'heptatapeptide $Y_1S_2P_3T_4S_5P_6S_7$ qui peut être abondamment modifié. Ces modifications influencent le recrutement de facteurs impliqués dans la transcription et le métabolisme des ARN. Une des modifications les plus abondantes, la phosphorylation de la sérine 2 du CTD (CTD-Ser2), est classiquement associée à la phase élongative de la transcription. Le facteur positif d'élongation CDK9/Cycline T (P-TEFb) est connu pour phosphoryler CTD-Ser2 et pour être requis pour l'expression des gènes embryonnaires.

Chez *Caenorhabditis elegans*, la déplétion par ARN interférence (RNAi) de *cdk-9* cause un arrêt développemental précoce identique à la déplétion de *ama-1*, la plus grosse sous-unité de Pol II. Nous avons construit un mutant *cdk-9* analogue sensible (*cdk-9as*) dans le but d'étudier le rôle de CDK-9 dans le développement. L'inhibition de CDK-9as par le 3MB-PP1, un analogue de l'ATP, ne phénocopie pas parfaitement le RNAi mais provoque une augmentation de la mortalité embryonnaire ainsi qu'une réduction de la taille de la descendance. De façon surprenante, l'inhibition de CDK-9as n'a pas causé de réduction du niveau de CTD-Ser2 phosphorylée alors que CDK-9 est décrit comme une kinase de CTD-Ser2. Au contraire, l'inhibition de CDK-12as, une autre kinase de CTD-Ser2, cause une importante réduction de CTD-Ser2 phosphorylée. Nous avons construit une souche possédant à la fois l'allèle *cdk-9as* et *cdk-12as*, permettant ainsi l'inhibition simultanée des deux kinases, et avons montré qu'à haute concentration le niveau de CTD-Ser2 phosphorylée est encore plus réduit que suite à la seule inhibition de CDK-12as. Nous postulons que la fonction kinase du CTD de CDK-9 et de CDK-12 puisse être partiellement redondante. Quand CDK-9as est inhibé, CDK-12 peut assurer la phosphorylation de la CTD-Ser2 par elle-même. Ensemble, ces données suggèrent que CDK-9 est requis pour le bon déroulement de l'embryogenèse mais pas pour maintenir le niveau de CTD-Ser2 phosphorylée.

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“Science is not only a disciple of reason but, also, one of romance and passion”

Stephen Hawking

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I. INTRODUCTION

A. From DNA to protein: a central dogma

How the information contained in DNA (deoxyribonucleic acid) can encode the blueprint of complex organisms is one of the long-standing questions of Biology. Over the last decades, numerous studies have revealed various aspects of gene expression. The first step is transcription, the process by which DNA is copied to make an RNA (ribonucleic acid) molecule. This process is carried out by a group of multi-subunit enzymes called RNA Polymerases (RNAP), which are found in all three domains of life: eubacteria, archaea and eukaryota (for review: (Burton, 2014)). In turns, RNAs have two different potential fate, depending on their type they will either have an intrinsic biological function or they will be translated by ribosomes into proteins. Unlike prokaryotes, there is a physical separation between those processes in eukaryotes: transcription is carried in nucleus while translation occurs in the cytoplasm. This concept of directional flow of genetic information, from DNA to RNA, to make proteins is known as the "central dogma of molecular biology" (Crick, 1958).

B. Transcription

1. The different DNA dependent RNA polymerases

Transcription evolved from a single catalytic subunit polymerase to a multi-subunit complex found in all three kingdoms of life: *archea*, *prokaryota* and *eukaryota*. In contrast to eubacteria and archaea that use a single RNAP to carry transcription, eukaryota possess at least three: RNAP I (Pol I), II (Pol II) and III (Pol III). They are macromolecular complexes of 589, 514 and 693 KDa and they are respectively composed of 14, 12 and 17 subunits. Of those subunits, 5 are shared among the polymerases (Cramer et al., 2008).

Having a greater number of RNAP allows for more specialization in gene regulation. Indeed, each RNAP is responsible for the transcription of a defined set of sequences. Pol I is involved in the transcription of the large ribosomal (rRNA) precursor except the 5S rRNA (Goodfellow & Zomerdijk, 2013). Pol I is by far the most abundant polymerase since rRNA accounts for 80% of total transcribed RNAs. Pol II transcribes coding sequences into mRNA that will be translated by ribosomes into proteins, as well as several non-coding RNAs (ncRNA). Over the last decade, ncRNAs have gained lots of attention in the scientific community since it has become clear that they are not junk but that they, or their transcription, are in charge for a wide range of functions such as transcriptional regulation or heterochromatin formation. Pol III is responsible for the transcription of short non-coding RNA, including 5S RNA, and all the transfer RNAs (tRNAs) (for review:(Cramer et al., 2008)). Two additional RNA polymerases, RNAP IV and RNAP V, are specifically found in plants where they transcribe noncoding RNAs (Egloff & Murphy, 2008).

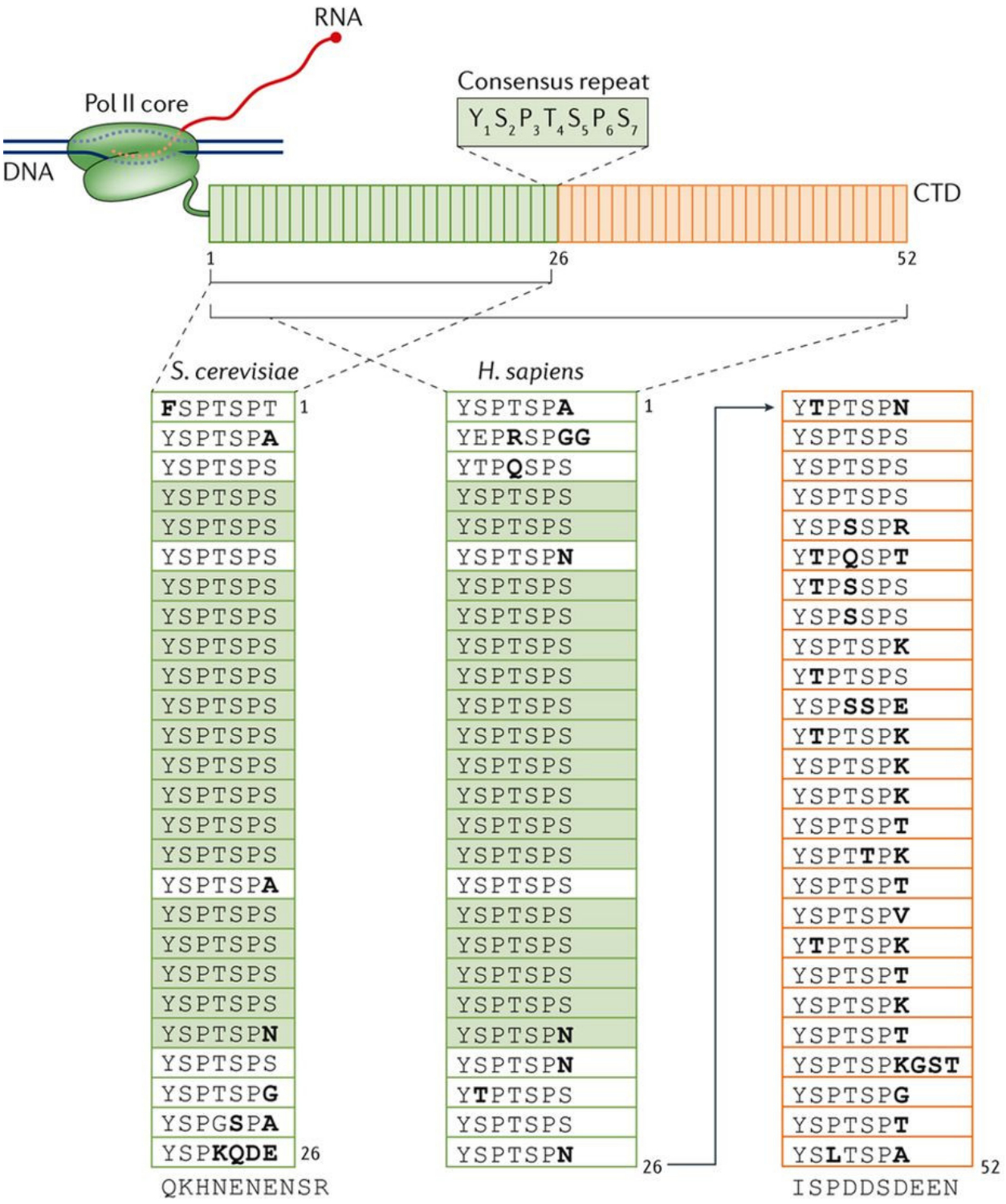


Figure 1: RNA polymerase II CTD. Comparison of *S. cerevisiae* and *H. sapiens*. The degenarated amino acids are in bold (Harlen & Chruchman, 2017)

2. RNA Polymerase II transcription

2.1. The complex story of the Pol II C-terminal domain (CTD)

Pol II is by far the most studied polymerase as it transcribes protein coding genes. It is a large complex composed of 12 subunits, named Rpb1 to Rpb12 by decreasing order of molecular weight, in yeast. Pol II is unique among all polymerases in that the subunit Rpb1, known as AMA-1 in *Caenorhabditis elegans*, harbors a flexible, tail-like extension, C-terminal domain (CTD). The CTD is composed of tandem heptad repeats with the consensus sequence tyrosine-serine-proline-threonine-serine-proline-serine (Y1S2P3T4S5P6S7), which is well-conserved across all eukaryotes (Figure 1). The number of repetitions roughly correlates with the complexity of the organism and ranges from 26 in budding yeast to 52 in humans with 42 in *C. elegans* (Nonet et al., 1987) .

Studies performed on *Drosophila*, yeast and mammalian cells have shown that the CTD plays a key role since its deletion is lethal in such organisms (Corden, 2013). However, *in vitro* studies have shown that it is dispensable for catalysis during transcription (Kim & Dahmus, 1989). It has also been shown that truncating the CTD down to 10 repeats in yeast does not impact viability, thus suggesting that some repeats are functionally redundant (Nonet et al., 1987). This is also true in mammals where lethality is observed if more than 23 repeats are removed (Chapman et al., 2005). Altogether, those studies highlight that the CTD is not necessary for the catalytic function of the polymerase but is required for proper response to regulatory events and for the synchronization of transcription with co-transcriptional processes such as RNA processing and chromatin remodeling.

It is now believed that the CTD acts as a regulatory platform offering a diversity of binding surfaces to a plethora of factors required throughout the transcription cycle. Rather than carrying every factor during the whole cycle, the CTD interacts dynamically with each factor at the appropriate time thanks to a combination of post-transcriptional modifications and conformational changes within the CTD. This ballet of specific modifications and subsequent recruitments evokes a code, referred as the “CTD-code”, that specifies the position of Pol II in the transcription cycle (Buratowski, 2003).

Elegant biochemical and genetic studies have revealed that each residue of the consensus heptapeptide can be modified. Every repeat can be phosphorylated on its 3 serines (CTD-Ser2, CTD-Ser5, CTD-Ser7), its tyrosine (CTD-Tyr1) and its threonine (CTD-Thr4). The two prolines (CTD-Pro3, CTD-Pro6) can be found in either *cis* or *trans* configuration. The CTD can also be O-GlcNAcylated on CTD-Ser2, CTD-Ser5 and CTD-Thr4 (Ranuncolo et al., 2012). Non-consensus repeat can also be modified. For instance, some repeats contain arginine and lysine (often replacing CTD-Ser7) that can be methylated or acetylated (Voss et al., 2015). Such possibilities of modifications have for long misled biologists into thinking of a very complex CTD-code. However, recent mass spectrometry analysis has shown that CTD-Ser2P and CTD-Ser5P are much more abundant, by a factor of 100-fold, than any other residue phosphorylation (Suh et al, 2016).

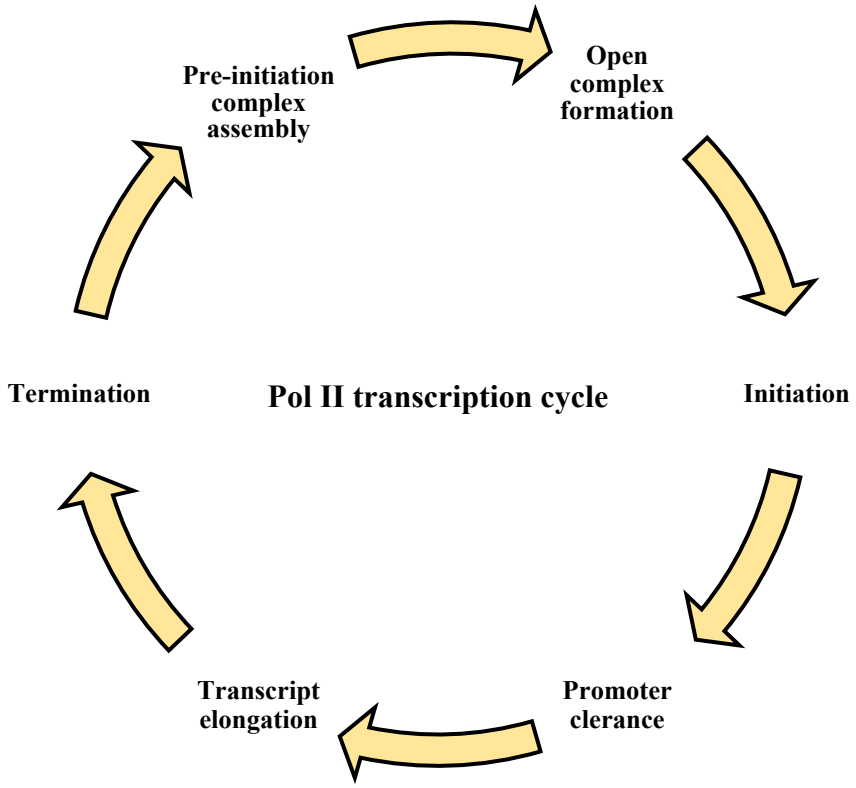


Figure 2: Schematic view of the transcription cycle.

2.2. Pol II transcription cycle

As for the cell cycle, gene transcription by Pol II is cyclic: once a gene has been transcribed, Pol II can re-initiate transcription. This cycle can be divided into distinct steps: pre-initiation complex (PIC) assembly, open complex formation, initiation, promoter clearance, elongation and termination (Figure 2) (Sveistrup, 2004).

Transcription starts with the assembly of general transcription factors (TFIIB, TFIID, TFIIE, TFIIH), forming the PIC, at a specific promoter region along the DNA. There are different elements promoting transcription such as TATA, CAAT or GC box, Initiator sequence or Proximal Sequence. The complex TFIID binds to the TATA box, 25 bp upstream the TSS, which allows the recruitment of the rest of the PIC. The holoenzyme TFIIH is organized into two functionally distinct subcomplexes: the core module and the CDK-activating kinase (CAK) module. The core module is an ATP-dependent 3'to 5' helicase, which opens the dsRNA (promoter melting) (Grünberg & Hahn, 2013). This allows the Mediator protein complex to arise, carrying Pol II by interacting with its unphosphorylated CTD. This is the open complex formation (Kim et al., 2000). At this stage of transcription, Pol II is still bound to the mediator complex and starts transcribing short aborted RNA products (Luse, 2013). Phosphorylation of TFIIH by the Mediator associated kinase kinase CDK8 (Cyclin dependent kinase) and the subsequent phosphorylation of the polymerase evicts it from the PIC, thus leading to elongation.

However, on many genes, Pol II pauses after transcribing about 20 to 120 nucleotides downstream to the TSS. This is due to the binding of NELF (negative transcription elongation factors) and DSIF (DRB-Sensitivity Inducing-Factor Spt4 and Spt5) (Marshall et al., 2003). The importance of understanding promoter-proximal pausing has long been recognized, not only because of the importance of stress response pathways but also because important developmental genes such as MYC and transcription of the human papilloma virus (HIV) were long known to be regulated at this step (Lu et al., 2015). Genome wide studies in murine, human, and *Drosophila* cells have revealed that such promoter proximal pausing is a widespread mechanism that regulates the rate of gene transcription notably by helping to proper capping (Nechaev et al., 2010). Pol II release requires P-TEFb (the positive elongation factor), a heterodimer comprising CDK9 and its regulatory subunit Cyclin T (T1 or T2). Upon its activation by CDK7, P-TEFb phosphorylates NELF (absent in most lower eukaryotes including *C. elegans*) and DSIF on the C-terminal region (CTR) of its subunit Spt5. Those phosphorylations cause NELF to dissociate from chromatin while DSIF switches from being a pausing factor to a positive elongation factor, thus driving Pol II to productive elongation (Yamaguchi et al., 2013). During productive elongation, the chromatin state must be modified to allow Pol II to move forward as well as to prevent cryptic intragenic transcription (for review: (Talbert & Henikoff, 2017)). This is notably achieved by the FACT complex, which tags along Pol II, and can dislodge H2A-H2B histone dimer from nucleosomes. This process is known as 'nucleosome breathing', it makes the DNA wrapped around the nucleosome more accessible and facilitates the passage of Pol II (Hondele et al., 2013).

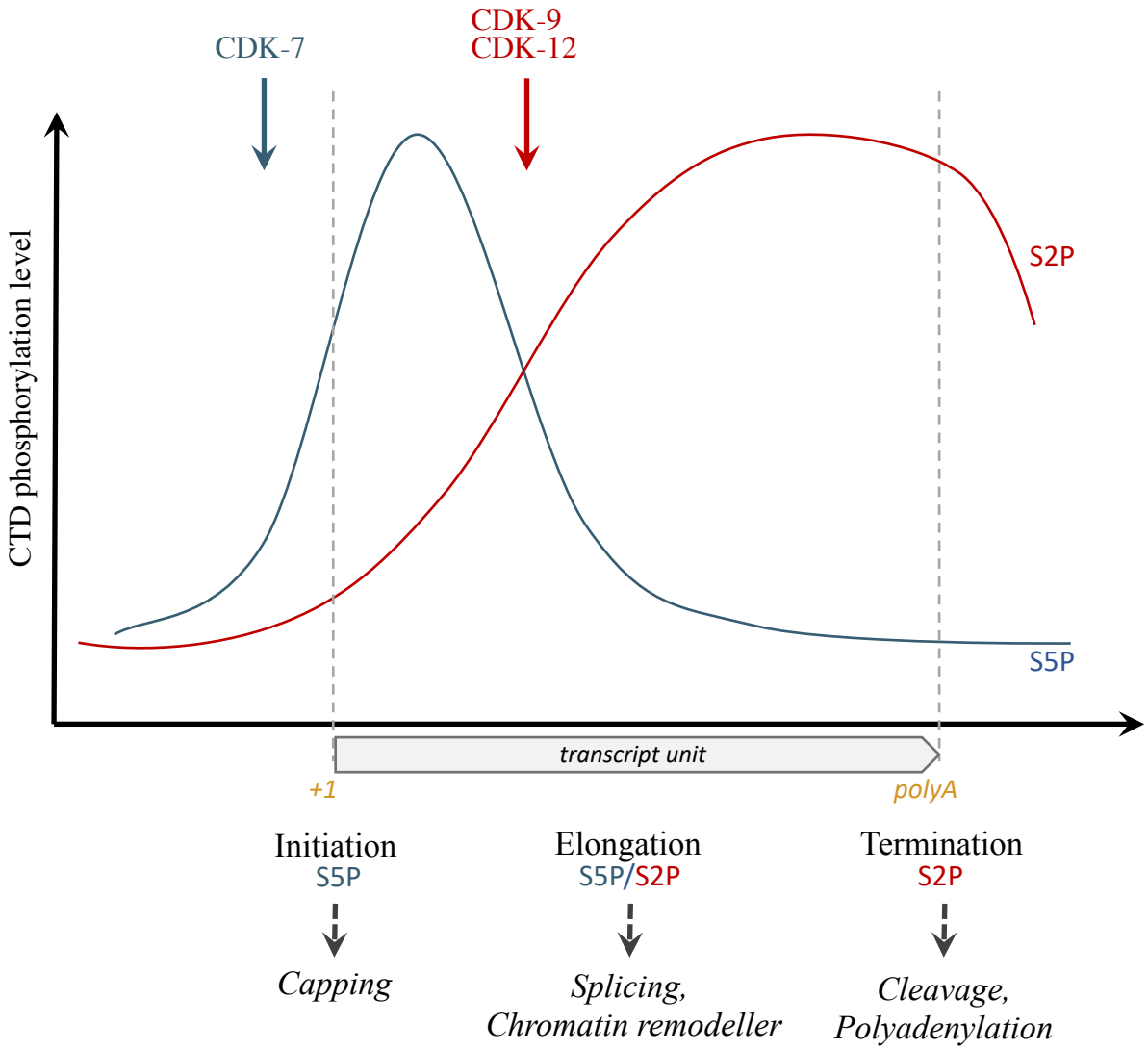


Figure 3: CTD-Ser2 and CTD-Ser5 phosphorylation along the transcription unit. CTD-Ser2P is represented in brown and CTD-Ser5P is in blue.

Towards the 3' end, Pol II passes through signals inducing a transition from transcription to termination. In this final this step, the RNA is cleaved, poly-adenylated and exported to the cytoplasm while the remaining moiety still associated with the polymerase is degraded by the exoribonuclease XRN2/Rat1, which is proposed to lead the termination. In addition to the end of RNA polymerization, Pol II is released from DNA and thus completes a round of transcription. Chromatin immunoprecipitation (ChIP) experiments have shown that Pol II accumulates downstream of the TTS (Transcription Termination Site, namely the last nucleotide found in the mRNA before the poly A tail). This is thought to facilitate the recruitment of factors required in mRNA 3' end formation and termination. However, until recently, the mechanistic nature of the transition between elongation remained unclear. Earlier this year, ChIP and sequencing analyses showed that unphosphorylated Spt5 also accumulate downstream of the TSS (Parua et al., 2018). The same team identified Dis2, a CDK9 substrate, as a phosphatase of CKD9-dependent phosphorylation sites on Spt5. Those results suggest a model of transcriptional exit: the dephosphorylation of Spt5 by Dis2 reverses elongation-rate enhancement (Parua et al., 2018), thus slowing down the polymerases which can then be captured by the 'torpedo' exoribonuclease XRN2/Rat1(Sansó et al., 2016).

2.3. Pol II transcription cycle, a matter of modified CTD

(a) CTD-Ser2 and CTD-Ser5 cycle consensus

ChIP experiments have shown that the phosphorylation status of the CTD changes in a predictable pattern while RNA pol II moves along the transcription unit (Buratowski, 2003). Pol II is recruited to the promoter with an unphosphorylated CTD. Right after initiation, the CTD is heavily phosphorylated on CTD-Ser5. With productive elongation and Pol II progression along the gene body, CTD-Ser5 is dephosphorylated while the level of CTD-Ser2 phosphorylation rises. This cycle of phosphorylation allows the co-transcriptional RNA maturation to occur by helping the capping, the splicing and the poly-adenylation of the RNA (Figure 3).

(b) The phosphorylation cycle of CTD-S2 and CTD-S5

Cyclin-Dependent kinases (CDKs) are part of a family of serine/threonine kinases first discovered as regulators of the cell cycle in the fission yeast (Beach, Durkacz & Nurse 1982). As their name suggest, CDK's activity depends on a separate subunit called cyclin. The level of CDK remains stable while the level of cyclins fluctuates and periodically activate CDKs. There is another kind of CDKs for which the abundance of the cyclin partner does not vary during the cell cycle. Those CDKs are involved in the control of gene transcription. As such, CDKs can be divided into two functionally distinct groups: the cell-cycle related CDKs and the transcriptional CDKs.

One CDK, **CDK7**, has been proposed to act both as a cell cycle regulator and as a transcriptional regulator. Indeed, it was first discovered in *Xenopus* as part of a biochemical activity called CDK activating kinase (CAK) that phosphorylates the T-loop of virtually all CDKs. This phosphorylation is required for CDKs complete activation (Fesquet et al., 1993). CDK7 is also a component of the general transcription factor TFIIH, along with cyclin H, and is known to phosphorylate the CTD-Ser5 and the CTD-Ser7.

Table 1: The RNA polymerase II CTD CDKs/Cyclins ortholog couples in various species. The complex they belong to is also indicated: TFIIH (Transcription factor II H), P-TEFb (Positive Transcription Elongation Factor b) and CTDK (C-Terminal Domain Kinase)

	TFIIH	P-TEFb	CTDK
S. cerevisiae	Kin28/Ccl1	Bur1/Bur2	Ctk1/Ctk2
S. pombe	Mcs6/Mcs2	Cdk9/Pch1	Lsk1/Lsc1
Metazoa	CDK7/Cyclin H	CDK9/Cyclin T	CDK12/Cyclin K

The kinase module **CDK8** of the Mediator complex also phosphorylates CTD-Ser5. This modification is known to occur early in the transcription cycle, reaching a peak at the transcription start site (TSS) where it recruits capping enzymes (Mayer et al, 2012). The negative charges added by phosphorylation destabilize the interaction between the mediator and the CTD, which evicts the polymerase from the PIC, thus leading to elongation. Soon after the CTD discovery, it has been shown that a mutant where all CTD-Ser5 have been replaced by an alanine[1] (CTD-S5A) is lethal. However, fusing the mammalian capping enzyme Mce1 to a CTD S5A is sufficient to restore viability. This reveals that the only essential function of the CTD-Ser5P is to recruit the RNA capping machinery as other functions are dispensable for viability (Schwer & Schuman, 2011).

Soon after initiation, the level of CTD-Ser5P steadily decreases while CTD-Ser2 phosphorylation, the other most abundant modification, increases until reaching a peak near the 3' end. This transition is insured by the phosphatase RPAP2 (Rtr1 in yeast). The rise of CTD-Ser2 phosphorylation is likely insured by **CDK12** and its partner, cyclin K. As mentioned in the paragraph above, CTD-Ser5P is required to properly recruit the capping machinery. As such, one can expect that CTD-Ser2P might be required for another RNA maturation process. It has long been known that a phosphorylated CTD strongly enhance splicing *in vitro* (Hirose et al, 1999) and that several components of the spliceosome bind the CTD (David et al., 2011). The use of CTD-S2A (Every CTD-Ser2 is replaced by an alanine) mutant in human cells has linked splicing and S2P as the mutant does not properly recruit the spliceosome. However, in yeast, a CTD-S2A mutant is not lethal while deletion of the spliceosome is (Gu, Eick & Bensaude, 2013).

After termination, CTD-Ser2 is dephosphorylated by Fcp1 phosphatase, allowing recycling of Pol II complexes for re-initiation (Cho et al., 2001). At this point, the remaining level of CTD-Ser5P is removed by the phosphatase Ssu72 (Krishnamurthy et al., 2004). In an unphosphorylated state, Pol II is released from DNA and can enter a new cycle of transcription (Hsin & Manley, 2012).

In short, thanks to its dynamic plasticity generated by differential phosphorylation at multiple sites, the CTD acts as a regulatory platform offering a diversity of binding surfaces required by many proteins to dock on Pol II. The CTD plays a key role in tethering factors that process the nascent transcript and modify chromatin template to the elongating Pol II (Meinhart et al., 2005).

(c) The CTD-Ser2 kinases CDK9 and CDK12

As already mentioned, the transition of Pol II from pausing to productive elongation requires the kinase activity of the P-TEFb complex, formed of CDK9 and its cyclin T. There are two isoforms of the cyclin T, T1 and T2. Even if the degree to which specific genes depend on one or another isoform is unknown, it has been shown that HIV's transactivator of transcription (TAT) binds P-TEFb through interacting specifically with cyclin T1 (Cho et al., 2007).

P-TEFb is part of several macromolecular complexes regulating its activity. When there is no transcription, P-TEFb is sequestered and inhibited by the 7SK-RNA thus repressing transcription elongation. In contrary, The SEC and BDR4 are active forms of P-TEFb. They both stimulate the kinase activity of CDK9 that can then phosphorylate its targets, which include CTD-Ser2. Although both complexes can phosphorylate CTD-Ser2, little is known

[1] The alanine mutation prevents phosphorylation

about the relative contribution of each complex. Interestingly, it has recently been reported that chemical inhibition of BDR4 causes severe effect on CTD-Ser2 phosphorylation and transcription elongation while not impacting CDK9 genomic occupancy. This suggests that BRD4 is not required to recruit P-TEFb but rather stimulate it (Winter et al, 2017). Furthermore, BDR4 can phosphorylate CTD-Ser2 *in vitro* thus suggesting that it can stimulate transcription elongation on its own (Hill et al., 2015).

It has long been assumed that CDK9 was the main CTD-Ser2 kinase since it is the homologue of the budding yeast Bur1. Bur1 is a kinase that contributes to phosphorylation on the CTD-Ser2 mark at the 5' ends of genes (Qiu et al., 2009). However, it has been shown in vitro that CDK9 phosphorylates essentially CTD-Ser5 when the CTD is primed with phosphorylated CTD-Ser7 (Czudnochowski et al., 2012). Furthermore, CDK12, the closest metazoan homolog of the yeast Ctk1, in complex with cyclin K, has been shown to be the major CTD-Ser2 kinase in yeast, Drosophila and human cells. (Bartkowiak et al., 2010). In contrast to CDK9, which locates to the 5' end of the gene, CDK12 occupancy increases toward the 3' end of the gene and may account for the high Ser2 phosphorylation in this region (Bartkowiak et al., 2010). It was then proposed that CTD-Ser2P in metazoan matches the elongation process in the budding yeast: Cdk9 phosphorylates Pol II CTD and DSIF (and NELF when present), and these events precede and are required for Cdk12-mediated phosphorylation of CTD-Ser2 on elongating Pol II (Bartkowiak and Greenleaf, 2011).

C. The model organism *Caenorhabditis elegans*

1. A nematode taking over in genetic labs

In June of 1963, Sydney Brenner wrote in a letter to the Laboratory of Molecular Biology (LMB), Cambridge, that "nearly all the classical problems of molecular biology have either been solved or will be in the next decade", the future of molecular biology lies in the extension of research to other areas of biology, notably development. To answer the fundamental developmental question of "How genes might specify the complex structures found in higher organism" (Brenner, 1974), he became interested in how neurons form and how they wire in the right way to create functional nervous systems. He chose to take a model organism approach and so his first order of business was to determine what model would be suitable for such studies. After careful considerations, he settled on *Caenorhabditis elegans* (*C. elegans*) and introduced the worm into genetics labs in the sixties. It quickly became apparent that the nematode *C. elegans* is a great all-around model organism for studying essentially all aspects of development.

The nematode *Caenorhabditis elegans* (*Caeno*, recent; *rhabditis*, rod; *elegans*, nice) was initially described and named *Rhabditis elegans* by Maupas in 1900. It's only in 1948 that the potential of *rhabditis* species for genetic research was pointed out by Dougherty and Calhoun (1948). They saw in the self-fertilizing and bacteria feeding *C. briggsae* strain, described by Margaret Brigg, a perfect genetic system to reduce the variability of genetic recombination (Ferris & Hieb, 2015). Two strains of *C. elegans* have historical importance. One strain, Bergerac, was collected from the soil in France by Victor Nigon of the university of Lyon.The other one, Bristol, was isolated from mushroom compost near Bristol by Staniland in 1956 (Ferris and Hieb, 2015). There are profound differences between those two strains, which justify that virtually all *C. elegans* genetics has been done with the Bristol strain. Firstly, the

Bergerac strain was found to exhibit a high spontaneous mutation frequency due to the transposition of the Tc1 transposon which is present in high copy number. Multiple crossing with the low copy number Bristol strain allowed geneticists to generate some of the strains used for transposon tagging. Secondly, Bergerac males are infertile and hermaphrodites are heat sensitive. They become sterile if cultivated at temperatures above 18°C while Bristol hermaphrodites can be cultured up to 25°C.

Sydney Brenner requested the Bristol strain from Ellsworth Dougherty in 1964 and derived a line that he referred in his notebook as N2. The notation N2 stands for Brenner's second attempt to establish a line of *C. elegans*. The first attempt, referred as N1, being his unsuccessful trial to isolate a *C. elegans* from his own garden. The N2 strain was defined as the *wild type* reference in 1965 and Brenner was awarded the noble prize in 2002 for establishing and using the nematode *C. elegans* as an experimental model system.

2. *C. elegans* biology

2.1. General description

The nematode *C. elegans* belong to the phylum Nematoda (round worms) which is a group containing over 20 000 species found all over the world. Nematodes are very diverse and have adapted to many different life-styles. *C. elegans* are small, growing to 1 mm in length and 50 µM in width, free-living, non-pathogenic, non-parasitic, nematodes. In the wild, they live in the soil where they feed on bacteria growing on rotten vegetable matter. In the lab, *C. elegans* can easily be maintained on agar plate or liquid culture with *Escherichia coli* as a food source. It can also be grown axenically in liquid media and large number can be grown in mass culture. It has a lifespan of approximately 2 to 3 weeks and every individual will give birth to about 250 worms in a few days.

C. elegans is a simple organism, both anatomically and genetically. The adult essentially comprises a tube, the exterior cuticle along with body wall muscles and a nervous system, containing a smaller tube, the digestive system. The two concentric tubes are separated by a fluid-filled pseudocoelomic space which contains the reproductive system. Most of the volume of the animal is taken up by the reproductive system. Of the 959 somatic nuclei in the hermaphrodite (1031 in males), 302 are neurons. Neural structures include a battery of sense organs in the head which mediate responses to taste, smell and touch. *C. elegans* does not have eyes but it possesses photoreceptor enabling it to some response to light. The worm has been extensively studied for the behavior it exhibits and for its capacity of rudimentary learning. Movement is allowed by 81 muscle cells organized into 4 longitudinal bans of muscles paired sub-dorsally and sub-ventrally. Alternative flexing and relaxation generate dorsal-ventral waves propelling the animal forward (Chapell & Wharton, 1986).

Despite its apparent simplicity, *C. elegans* shares many essential molecular signaling pathways, genes and biological characteristic with more complex organisms such as humans. Thanks to its completely transparent body, biologists have been able to track, follow and map every cell until completion of the cell lineage thus creating a complete "wiring diagram" of the cell contacts in the animal (Chapell & Wharton, 1986). This model organism is then particularly interesting to study embryogenesis, morphogenesis, development, nerve function, behavior and aging and how they are determined by genes. Thus *C. elegans* provides the researcher with the ideal compromise between complexity and tractability.

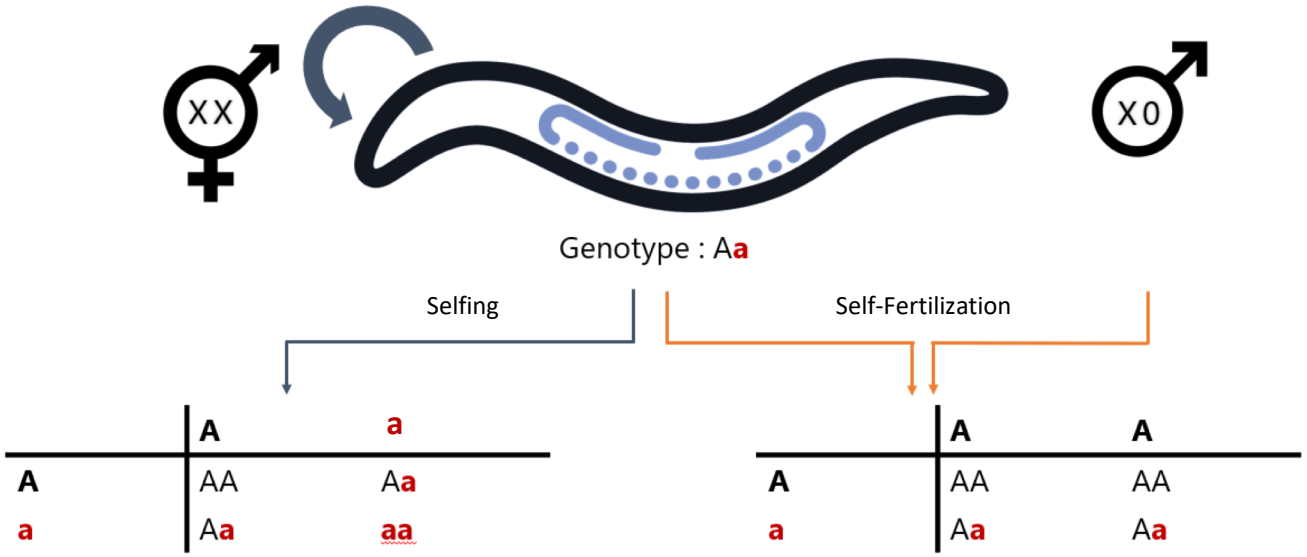


Figure 4: *C. elegans* reproduction at 20°C. Worms have two sexes, the self-fertilizing hermaphrodites (XX) and males (XO). The hermaphrodite germline produces both oocytes and sperm. Via self-fertilisation (or selfing) and adult produces offsprings following Mendel's rules. In the lab, this characteristic is very handy since a single hermaphrodite is enough to maintain a stock population. Occasionally, the X chromosome fail to separate properly in the hermaphrodite germline resulting in male progeny with only one X chromosome. Cross-fertilization between a male and a hermaphrodite will result in more or less 50% males in the progeny. Using this cross-fertilization, alleles can be outcrossed of *C. elegans* genome.

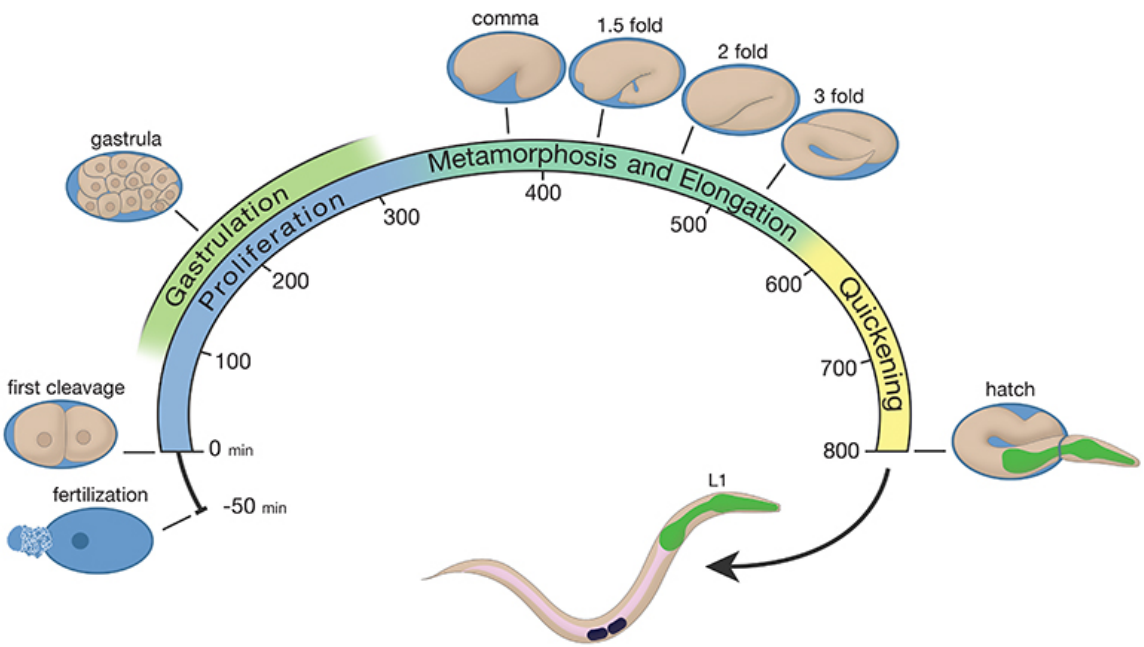


Figure 5: *C. elegans* embryonic development. *C. elegans* embryonic development last about 800 minutes and is divided into well-defined stages: Proliferation, Gastrulation, Elongation and quickening. From WormAtlas.

2.2. Reproduction

C. elegans is an androdioecious nematode with both hermaphrodites (XX) and males (XO). Hermaphrodites are self-fertilizing, they first develop a testis producing sperm, which is stocked in a spermatheca. Then, the gonad switches off to make oocytes, which are fertilized by aforementioned sperm.

In the lab, males arise spontaneously at low frequency (male frequency < 0.002) which corresponds to the frequency of spontaneous nondisjunction of the X chromosome. Such events lead to the production of an aneuploid sperm or egg that will result in an XO male. Since hermaphrodites cannot cross with one another, any outcrossing that does occur must do so via males (Figure 4). This mating system is advantageous for geneticists. In the absence of males, any new mutation that arises has a good chance of becoming fixed because each animal is the result of a crossover with itself thus promoting homozygosity. An isogenic lineage can thus be derived from a single hermaphrodite.

A population purely hermaphroditic will have a higher growth rate than a population that is a mixture of males and hermaphrodites. This means that a mutation that occurs will be selected against unless males confer some other advantage that is linked to their mode of reproduction. However, the fact that *C. elegans* can reproduce exclusive by self-fertilization, even if males can be maintained, is a question that still puzzle biologists. Nowadays, it is commonly accepted that males are maintained because they mitigate inbreeding depression. However, it raises the question of how selfing is maintained within populations that have the possibility to fully reproduce by outcrossing?

2.3. Life cycle

The worm is conceived as a single cell, which undergoes a complex process of development (Figure 5), starting with embryonic cleavage, proceeding through morphogenesis and growth to the adult. Right after mature oocytes fertilization by the sperm contained in the spermatheca, the new zygote develops a very resistant chitinous cuticle. Eggs are held into the parental worms for about 3 hours after what they are laid and continue to develop *extra utero*. When laid, embryos are still in the proliferation phase of embryogenesis. During this phase, cells undergo stereotyped cell divisions and they are all similar in term of cytoplasmic structures. However, some cells start moving away from their sister cell in a process known as “global cell sorting”. Other cells undergo immediate apoptosis upon mitosis while the sister cell keeps proliferating. At the 30-cells stage, gastrulation starts the formation of the 3 germ layers (endoderm, ectoderm, mesoderm). By global cell sorting, functional cell groups arise in the embryo (Bischoff & Schnabel, 2006). At this point starts morphogenesis, which overlaps with the end of gastrulation, during which cells become specialized and tissue are formed. Once the developing tissues are wormed shaped, they become folded in the eggshell. This mark the transition to the elongation phase, during which the embryo will develop from the one-fold stage to the 600 cells three-fold stage. Altogether, embryogenesis last about 14 and is concluded by *C. elegans* hatching (Figure 6) (Chisholm, 2000).

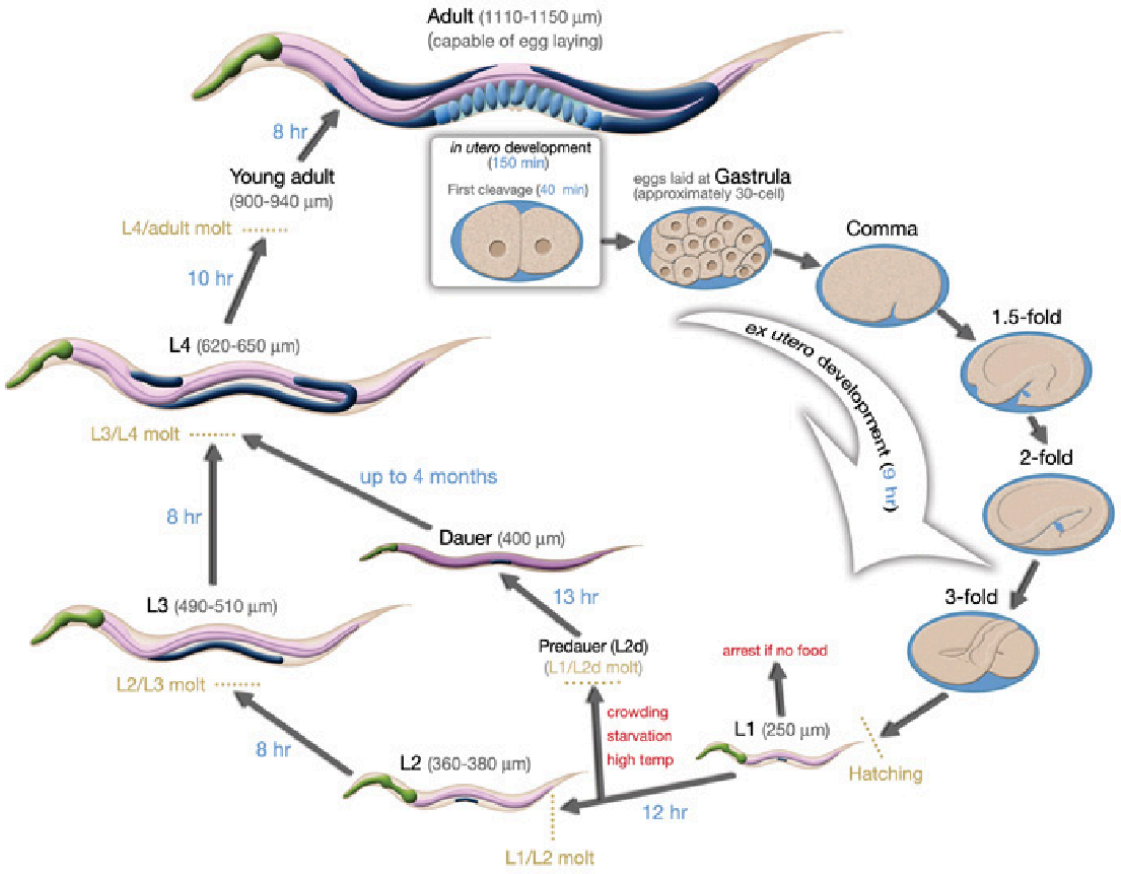


Figure 6: *C. elegans* life cycle at 20°C. *C. elegans* life cycle is about 3 days long and divided in distinct developmental stages: the embryonic stages, the larval stages (L1 to L4) and the fertile adult stage. If larvae hatch in absence of food, they can enter developmental arrest called L1 arrest. If nutrients are lacking, developing larvae can enter an alternative L3 stage called dauer stage. When the conditions are right, dauers can resume development. From WormAtlas.

Following hatching, post-embryonic development starts (Figure 6). Newly hatched nematodes called larval stage 1 worms or "L1 worms" grow to adulthood through 3 additional stages called L2, L3 and L4, in about 50 hours. Each of those larval stages is marked by a molt. A new cuticle is synthesized under the old one, the pharynx briefly stops pumping (period called lethargus) and the old skin is shed. The L4 molt is the entry point to adulthood as gonadogenesis is completed and the vulva opens. The now sexually mature worm can mate and reproduce by self-fertilization. Unmated hermaphrodites lay fertilized oocytes, embryos, during 4 days. Once all the sperm stocked in the spermatheca is used, they start laying unfertilized oocytes for a day or so.

The life cycle described above occurs in un-stressful conditions. When the environment becomes harsh (limited food supply, heat stress or overcrowded population), *C. elegans* can enter an alternative L3 stage called the dauer stage (Cassada & Rusell, 1975). Dauer worms are morphologically distinct from larvae normally developing. They are thinner, they possess a specialize cuticle much more resistant to hard conditions such as acids, detergents or anesthetics. The dauer stage is a non-feeding stage, there is an internal plug on the oral orifice and their pharynx do not pump (Vowels & Thomas, 1992). However, dauer nematodes are motile and can adopt a special food searching behavior called nictation. They stand on their tails and wave their heads in the air. This might enable the worms to stick to a moving insect and use it as a transport to a novel food source (Lee et al, 2011). Once food has been found or that the external conditions are back to normal, worms exit the dauer stage and resume their development.

In *C. elegans*, there is another alternative stage called the L1 arrest. Contrary to the dauer stage, there is no morphological change in the arrested L1 larvae. Worms undergo such arrest if they hatch in the absence of food. This is due to the disruption of an insulin-like pathway leading to the accumulation of an active form of the transcription factor DAF-16/FOXO. DAF-16/FOXO transcriptionally inhibits *daf-12* and *dbl-1*, two genes required for fed larvae development (Hibshman et al., 2017). Simply put, external nutrients are detected by chemosensory neurons, which induce the production of Insulin-Like peptides (ILPs). ILPs act as a trigger that activates the Insulin-like Receptor (InsR) DAF-2. Activated InsR/DAF-2 antagonize DAF-16 and promotes post-embryonic development. If no nutrients are available, DAF-2 is not activated and cannot alleviate the repression of DAF-16 and development is put on hold (for review: (Baugh, 2013)). Arrested larvae slow down their metabolism and increase their stress resistance. The L1 arrested stage can be maintained up to 4 weeks (Roux et al., 2016).

2.4. Tissue specificity of CTD-Ser2 phosphorylation in *C. elegans*

Until recently, little was known about the interplay and specific action of CDK-9 and CDK-12. In human cells and *Drosophila,* it has been shown that CDK9 primes CDK12 CTD-Ser2 phosphorylation in somatic cells. RNAi-based data from Kelly's lab states that CTD-Ser2 phosphorylation is independent of CDK-9 in the germline where only CDK-12 is required, but that in the somatic tissues both kinases are needed (Bowman et al., 2013). When they performed RNAi against CDK-12, immunofluorescence showed a complete loss of signal in the Z2 and Z3 primordial cells (germline precursors) as well as a reduced level of CTD-Ser2P in other cells.

Interestingly, even if CDK-9 inhibition does not affect CTD-Ser2 level in the germline it seems to be essential for proper germline development. Bowman and co-workers showed that the absence of CDK-9 in germ cells caused dramatic sterility. Only about 1/10 of the normal brood size was produced. The produced oocytes showed normal level of CTD-Ser2P thus further supporting that CTD-Ser2 phosphorylation in the germline does not require CDK-9 but solely CDK-12 (Bowman et al., 2013).

All together, these results suggest that, as it is the case in yeast, CTD-Ser2P in *C. elegans* germline is mainly carried on by CDK-12 but no CDK-9. Even if CDK-9 does not phosphorylate CTD-Ser2 in the germline, it is important for its proper development thus suggesting that it has another crucial role to play in this tissue (Bowman & Kelly, 2014). However, neither CDK-12 nor CTD-Ser2P are required since the germline develops correctly in the absence either of those (Bowman et al., 2013).

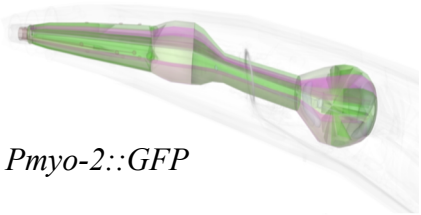
II. Objectives of the work

Ongoing work in our lab revealed that chemical inhibition of CDK-12 in the nematode *Caenorhabditis elegans* causes an early developmental arrest at the L1 stage, a decrease in detectable mRNA of specific groups of genes and a global decrease of CTD-Ser2P level. In a process called trans-splicing, specific to *C. elegans* and a few other organisms, such as trypanosomes (Sutton & Boothroyd, 1886), around 70% of *C. elegans* mRNAs are capped at their 5' end with a SL (splice leader) RNA (Allen et al., 2011). There are 2 types of SL RNAs: the SL1 RNA is specific to promoter proximal genes (single gene and first gene in operon) while the SL2 RNA is used at downstream operon genes (position 2 and over) (Allen et al., 2011). RNA-seq performed on worms arrested in L1 after CDK-12as inhibition has shown that the steady-state level of SL2 trans-spliced mRNAs was decreased. These observations led to a model in which CDK-12-dependent CTD-Ser2P would be needed to dock proteins involved in SL2 trans-splicing. Without docking of those proteins, the SL2 moiety is not added efficiently, which produces unprotected mRNA quickly degraded by exonucleases. Quite unexpectedly, these data also support that embryogenesis can occur in the absence of detectable CTD-Ser2P.

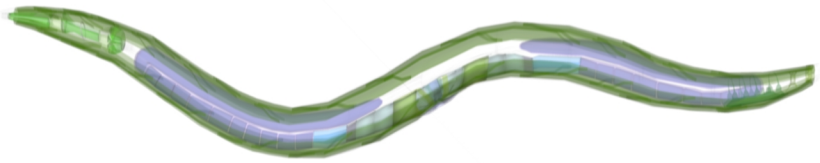
Contrarily to Bowman and co-workers, we did not observe tissue specificity upon CDK-12 chemical inhibition. Quantitative western blots performed in our lab also indicate that low level of CTD-Ser2P is still present when CDK-12 is inhibited. These data open the possibility that CDK-9 might be responsible for the remaining signal observed. Currently, there is no conditional CDK-9 mutant available in any multicellular organism.

The principal objective of this work is to construct and characterize a strain of *C. elegans* having a conditional, analogue-sensitive endogenous CDK-9. In a second time, a strain containing both the homozygous CDK-9 and CDK-12 analogue-sensitive alleles will be constructed. With those tools, we aim to analyze the relative contribution of both kinases to the phosphorylation of CTD-Ser2 and to better understand its biological function in the development of a multi-cellular organism.

A.

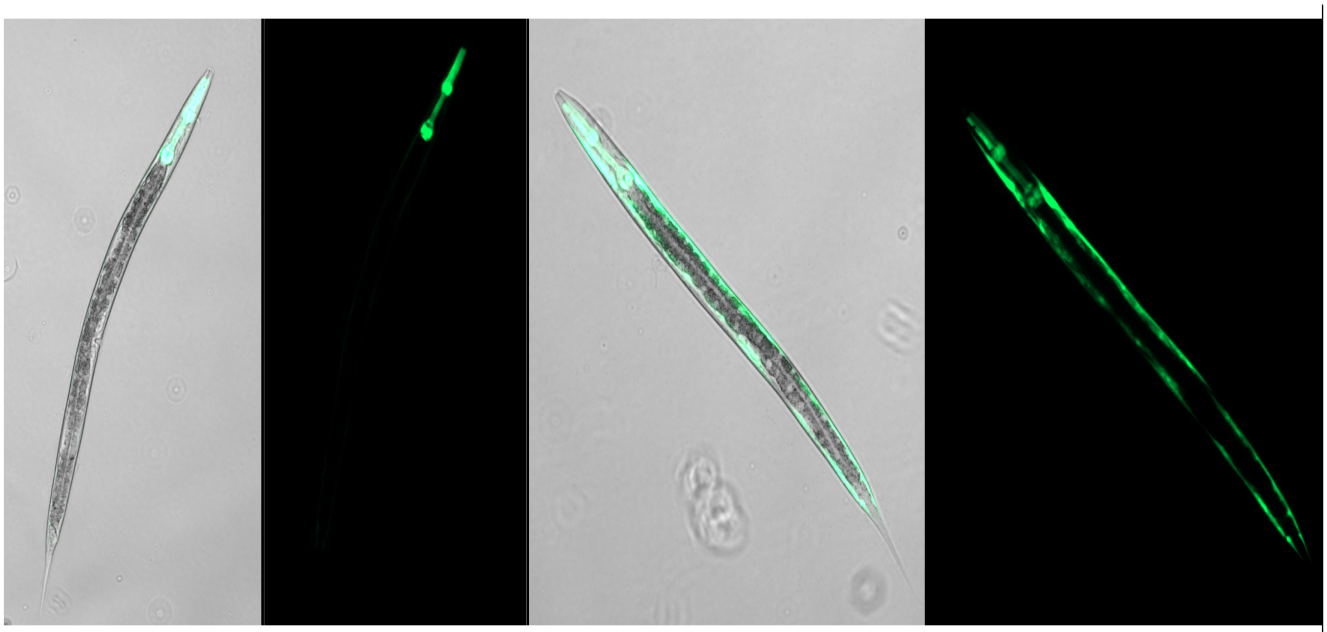


Pmyo-2::GFP



Pmyo-3::GFP

B.



Pmyo-2::GFP *Pmyo-3::GFP*

Figure 7: Microinjection training. (**A**) Expression of either *Pmyo-2::GFP* and *Pmyo-3::GFP* are shown in green : GFP under *myo-3* promoter is expressed in muscle tissues while GFP under *myo-2* promoter is expressed in pharyngeal tissue (Wormbase). (**B**) Fluorescent micrographies of worms transformed with either plasmid or both.

III. Results

A. Strain Construction

The first aim of this work is the construction of a *C. elegans* mutant strain. Mutants are powerful tools for geneticists because mutations can interrupt cellular processes, often holding the key to understanding gene function. Also, it can be very handy to be able to overexpress a specific gene, to tag a protein and so on. To answer the need of *C. elegans* transgenesis, several methods have been developed over the years. Transgenesis is commonly performed by microinjecting DNA directly into the distal part of the gonad (Mello et al., 1991). In this part of the gonad, germ cells are not yet individualized, and nuclei share a core of cytoplasm. Such features allow very efficient targeting of the germline and, eventually, DNA injected there can be delivered to many germ cells at once thus increasing the amount of putative transformants in the progeny. Having no micro-injection experience in our lab, we first had to develop the technique (See Material & Methods).

1. Micro-Injection of *Pmyo-2::GFP* and *Pmyo-3::GFP*

To create a transgenic *C. elegans* strain, micro-injection must be performed into the gonad of the worm. Because each gonad arm is a syncytium of nuclei that will eventually be surrounded by membranes, the injected DNA can enter the premeiotic nuclei. If the body cavity is injected, there will be no transformant in the progeny. As such, we needed a training step to make sure we could successfully inject into the gonad. We used a mixed of plasmids, *Pmyo-2::GFP* and *Pmyo-3::GFP* , available in our lab, that are commonly used as a co-marker of injection. Both *myo-2* and *myo-3* encodes isotypes of muscle type specific myosin. While *myo-2* is expressed in pharyngeal tissue, *myo-3* is expressed in muscle tissue (Figure 7A) (Ardizzi & Epstein., 1997).

Two injected worms were pooled on one plate. After 4 days of incubation at 20°C, the progeny was checked with a fluorescence microscope for GFP signal. If GFP signal was detected, the plate was marked as “positive” (Figure 7B). Out of 20 plates (40 injected worms), we could not detect any positive plates. We raised the plasmid concentration and succeeded in obtaining transformants worms. On average, 43% of our plates were positives. Such data is consistent with literature and made us conclude that our injection technique is effective.

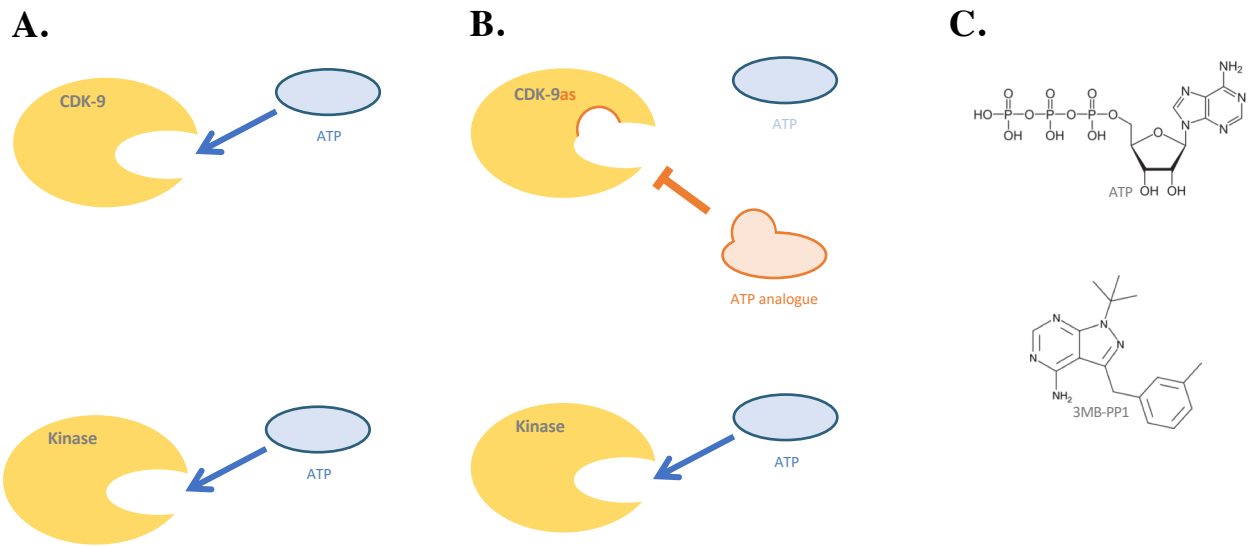


Figure 8: The Shokat analog sensitive mutant. The shokat analog-sensitive (*-as*) mutation allows to specifically sensitize a kinase to a synthetic inhibitor. (**A**) Kinases not possessing the *as* mutation can bind ATP. (**B**) The kinase-as has a normal kinase activity but can be specifically inhibited by a bulky ATP analogue. (**C**) The structure of ATP and the bulky ATP analogue 3MB-PP1.

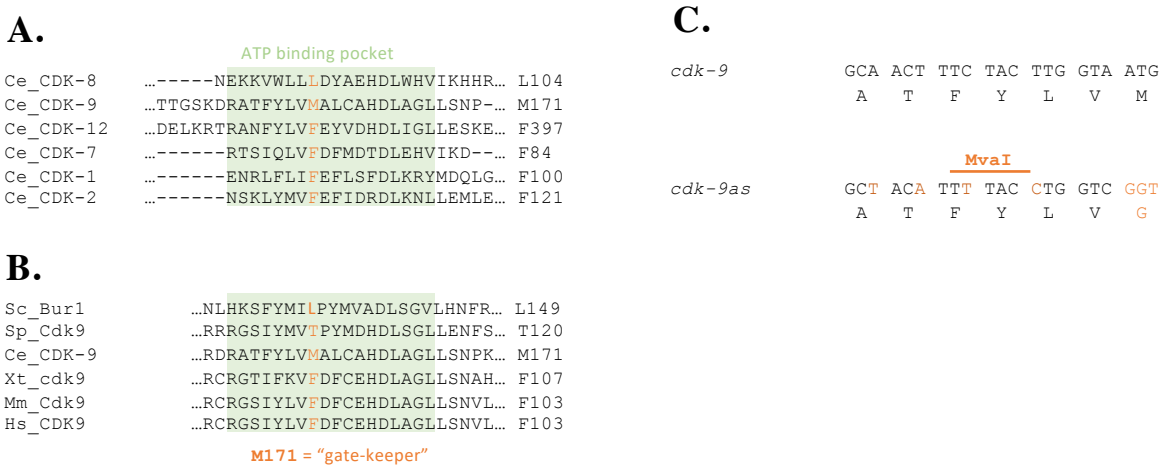


Figure 9: Identification and mutation of the CDK-9 gatekeeper residue. (**A**) Protein sequence alignment of *C. elegans* (Ce) cyclins CDK-1, CDK-2, CDK-7, CDK-8, CDK-9 and CDK-12. For each sequence, the well conserved gatekeeper residue is highlighted in orange. (**B**) Protein sequence algniment of *C. elegans* CDK-9 and its orthologs in different organisms : Bur1 in *S. cerevisiae* (Sc), Cdk9 in *S. pombe* (Sp), cdk9 in *X. tropicalis* (Xt), Cdk9 is *M. musculus* (Mm) and CDK-9 in *H. sapiens* (Hs). The conserved gatekeeper residue is highlighted in orange. Alignment has been performed using clustal Omega (**C**) DNA sequence alignment of *cdk-9* showing the mutations mutations to create the *cdk-9as* mutant. The ATG methionine codon is replaced by GGT glycine codon. Two silent mutations are introduced to create a MvaI restriction site. To make sure the crRNA doesn't match with the modified locus even if the PAM sequence has been removed, a silent mutation is introduced in the GCA alanine 165 codon and the ACA threonine 166 codon.

2. Construction of a *cdk-9as* mutant strain

2.1. The Shokat analog-sensitive mutant: a genetic-chemical way to specifically inhibit a given kinase

Protein kinases form a very large family, with 438 members identified in *C. elegans* (Manning, 2005). They have many diverse roles since about 30% of all the proteins can have their activity modulated by phosphorylation (Cohen, 2000).

Due to the high level of homology between their catalytic domain, it is unconceivable to imagine a library of kinase specific inhibitors. Nevertheless, inhibitors exist and are continuously being developed as they yield potential in cancer therapy (Morales & Giordano, 2016). However, most of them inhibit multiple kinases simultaneously. To get around the need of such inhibitors, Kevan Shokat and his co-workers developed a method to inhibit individual kinase by sensitizing them to a specific inhibitor (Alaimo et al., 2001). The method consists in the introduction of a single mutation converting a conserved non-essential bulky residue (methionine, leucine, phenylalanine, threonine, etc.), referred to as the gatekeeper, into a smaller residue (glycine, alanine) thus creating an enlarged catalytic pocket. Such engineered kinases are called "analog-sensitive kinase" (*as*-kinase) (Figure 8A, B) (Bishop et al., 2001).

Shokat derived from the general inhibitor PP1 a few analogues (1NM-PP1, 3MB-PP1, …) able to specifically bind those pockets but not WT pockets due to steric clashes with the gatekeeper (Zhang et al., 2013). PP1 derivates are bulky ATP analogues inhibiting as-kinase by competing with the binding of ATP (Figure 8B). Those analogues do not possess phosphates that can be transferred to the target protein and, by such, they inhibit kinase activity (Figure 8C).

Quantitative comparison of WT and -as kinases revealed that the -as mutation alters neither the substrate specificity nor the phosphorylation specificity (Elphick et al., 2009). Those -as kinases are powerful tools to study the role of the kinase *in vivo*. Furthermore, such kinases can be used to identify the direct targets of a specific kinase. Indeed, ATP where the adenine ring has been enlarged (A*TP) can be used as substrate for the as-kinase (Koch & Hauf, 2010). This modified ATP cannot bind WT kinases but can bind as-kinases. By marking the ATP γ-phosphate radioactively (Shah & Shokat, 2002) or chemically (Allen et al., 2005), specific targets of the as-kinases can be labelled and thus identified.

2.1. Identification of CDK-9 gatekeeper residue

As described above, in order to sensitize CDK-9 to a bulky ATP derivate, the large gatekeeper residue has to be mutated. Since kinases have a high degree of homology and we already knew the gatekeeper residue of CDK-12, a sequence alignment of multiple kinases identified the corresponding gatekeeper of CDK-9. Indeed, we aligned CDK-9 and CDK-12 along with other *C. elegans* CDKs as well as CDK-9 orthologs in other organisms and found that the methionine 171 (M171) was most likely to be CDK-9 gatekeeper residue (Figure 9A).

The genetic manipulation consists in the mutation of the ATG methionine codon into a GGT glycine codon. We will also introduce a silent mutation near the M171G to create a MvaI restriction site (Figure 9B). This second mutation allows screening for as-mutant by PCR-Restriction assay and therefore creates an easy to follow molecular scar.

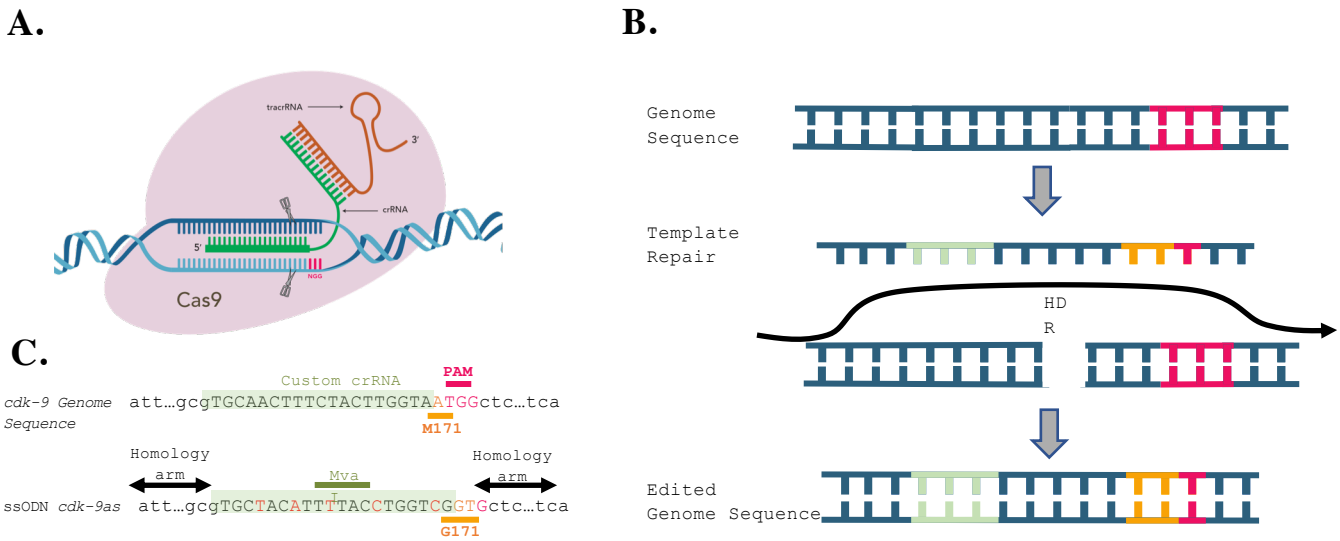


Figure 10: A CRISPR-Cas9 based strain construction. (**A**) Schematic representation of the CRISPR-Cas9 system. The target sequence (complementary to the crRNA) is followed by a protospacer adjacent motif (PAM, pink) which differs depending on Cas9 origin (-NGG *in S. thermophilus*) and is needed for Cas9 to grab onto DNA. Then, Cas9 unzips DNA double helix and the crRNA matched the target sequence. (Adapted from Integrated DNA Technologies' website). (**B**) Upon Cas9 cleavage, DNA lesion can be repaired by a mechanism called homology directed repair (HDR) which uses the injected ssODN as template to guide the repair**.** (**C**) Genomic *cdk-9* sequence and the custom crRNA targeting *cdk-9*'s gatekeeper and its neighboring nucleotides (green box). The template repair used to edit the genomic sequence is a single stranded oligodeoxynucleotide containing the desired mutations (M171G, MvaI restriction site and silent mutations) and flanked by 35 nucleotides long homology arms (ssODN *cdk-9as*).

2.2. A CRISPR based approach

Contrary to the yeast, DNA integration by homologous recombination is very inefficient in nematodes. Microinjected DNA forms extrachromosomal arrays which are not stably inherited and do not segregate in a mendelian fashion. The recent development of CRISPR (Cluster Regularly Interspaced Short Palindromic Repeats)-Cas9 (CRISPR associated protein 9) system offers very efficient and precise genome editing tools as the presence of a double strand breaks (DSB) strongly stimulates homologous recombination. Indeed, the CRISPR-Cas9 system can be guided to a specific locus, thanks to a sgRNA, where Cas9 makes two nicks, one on each DNA strand, 3 nucleotides upstream relative to a protospacer adjacent motif (PAM). The sgRNA is composed of two RNAs: the CRISPR RNA (crRNA) that guides Cas9 to the desired genomic region and the trans-activating RNA (tracrRNA), which links the crRNA and Cas9 to form the ribonucleotidic (RNP) complex (Figure 10A)(Deltcheva et al., 2011). The RNP first binds to the PAM before looking for a match to the guide sequence. This way, even sequences that perfectly match are not recognized if they do not contain a PAM.

Introducing the M171G mutation required to create the *cdk-9as* strain, we first had to design a sgRNA targeting *cdk-9* (*cdk-9* sgRNA). Such sgRNAs can be designed by hand or using available software(s). The sgRNAs used in this work have been hand-designed and checked with IDT custom sgRNA design tool. There are different factors to take into consideration while designing sgRNAs. Those are activity, specificity and proximity to the desired modification. Ideally, the guide sequence should be unique in the genome, to minimize the chance of generating off-target mutations. We designed two sgRNAs that both score at 99, which indicates a very good specificity. The essential difference between the two designed sgRNAs is the PAM they recognize. The first sgRNA guides Cas9 to introduce a DSB 2 nucleotides before the ATG methionine codon of interest while the second introduces a DSB 8 nucleotides upstream. Considering the higher degree of proximity achieved by the first sgRNA, we decided to use it and, if failure, to try the second.

Once a DSB has been introduced, it is repaired by endogenous cellular pathways, including the homology dependent repair (HDR) pathway (Figure 10B). HDR is a precise repair mechanism that uses a template repair. It has been shown that single-stranded oligodeoxynucleotide containing the desired mutation and flanked by at least 35 nucleotides homologous to the cleavage site will be incorporated by gene conversion (Paix et al., 2014). As such, we constructed a *cdk-9as* ssODN (single strand oligonucleotide) containing the M171G mutation as well as additional mutations required to create the MvaI restriction site. We also introduced a silent mutation in order to remove the PAM sequence (Figure 10C). Indeed, even if the tracrRNA will not match a sequence containing more than 3 mismatches, compared to its own sequence, we decided to play it safe. By removing the PAM, we assure that Cas9 will not cut an already repaired *cdk-9* locus.

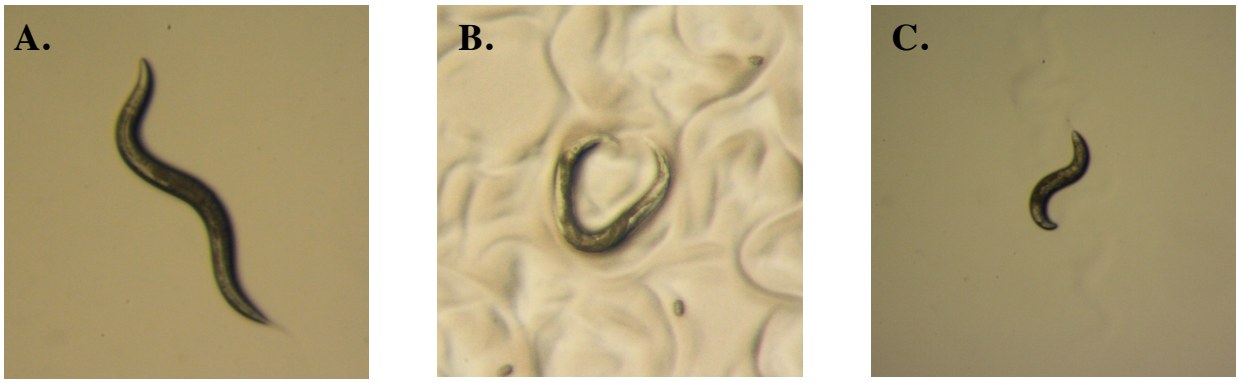


Figure 11: Phenotypes of the co-injection marker *dpy-10* phenotypes. (**A**) WT worms (*dpy-10 +/+)*. (**B**) Roller worm (*dpy-10 cn64/+)*. Because of their helically twisted body, those worms turn in circles, leaving a very particular pattern in the bacteria lawn, very easily screened by eye. (**C**) Dumpy worm (*dpy-10 cn64/cn64*).

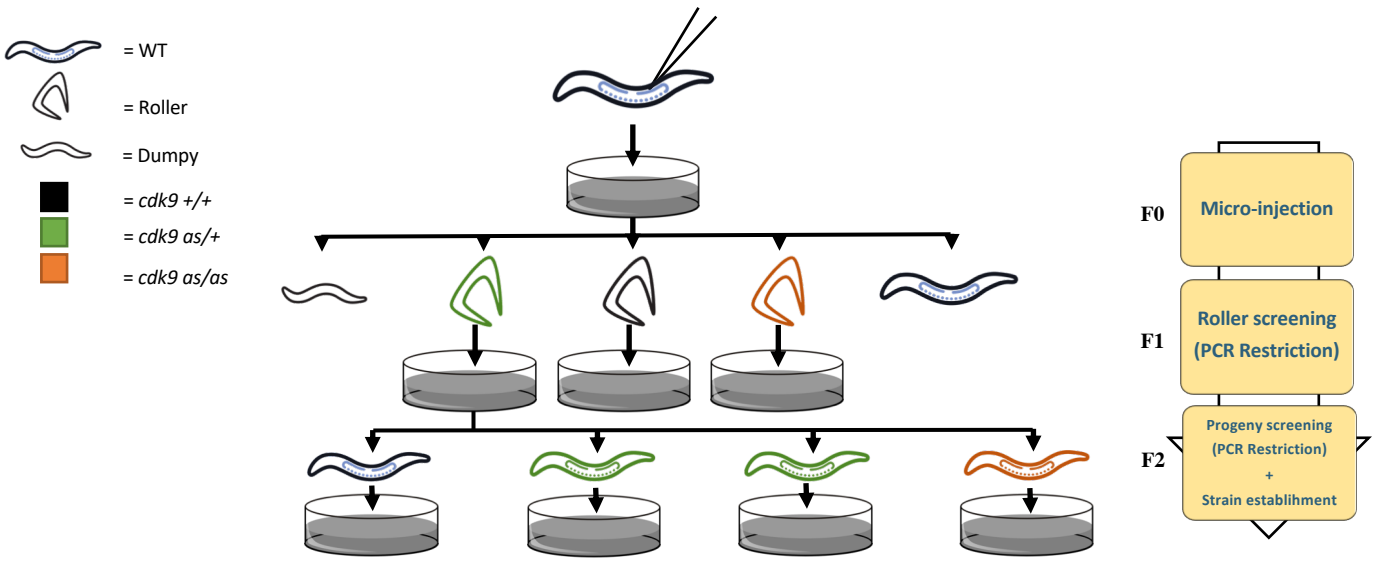


Figure 12: *cdk-9as* screening strategy. Two injected worms (F0) are pooled on an NGM plate seeded with OP50. Their progeny (F1) is visually screened for roller worms which are isolated on individual plates. Once embryos have been laid, the parental worm (F1) is checked for the desired mutation by PCR restriction assay. If the worm is *cdk-9 as/as*, all its progeny is also *cdk-9 as/as*. A worm having lost the roller phenotype can then be conserved for strain establishment. If the F1 worm is *cdk-9 as/+*, its progeny (F2) is isolated on individual plates and checked for *cdk-9 as/as*. This step is repeated until finding an as/as worm which is then used for strain establishment.

2.1. The co-CRISPR strategy, looking for roller worms

It is unfeasible to directly PCR-check the injected worms F1 progeny (first generation) as it would require singling out hundreds of worms, allowing them to lay eggs, and then PCR to identify animals positive for the *as*-mutation. If the *as*-mutation of interest does not produce an obvious phenotype in absence of inhibitor, the only obvious and easy option is to use a co-marker that could easily be screened by eye (Figure 11A, B, C). Such method is called co-CRISPR, two loci are edited simultaneously: the desired locus and an unlinked marker producing a visible phenotype. We decided to use the well-established co-CRISPR marker *dpy-10* previously described (Paix et al., 2014). The *dpy-10* co-CRISPR strategy consists in converting the WT *dpy-10* locus into the *cn64* allele upon cutting by Cas9. A missense mutation in the *cn64* allele causes a dominant roller phenotype (Figure 11B) when heterozygous (*cn64/+*) but a dumpy phenotype (Figure 11C) when homozygous (*cn64/cn64*). Statistically, it is likely that if the worm is roller then it is also positive for the *as*-mutation we aim to introduce, making roller worms a criterion of selection (Arribere et al., 2014). Since *C. elegans* is a self-hermaphrodite, worms carrying the *as*-mutation but having lost the *dpy-10* marker can be found in a roller F1 progeny. Such worms can then be isolated and used to derive homozygous mutant strain (Figure 12).

Using the micro-injection technique, we injected worms with a mix containing the protein Cas9 in complex with *cdk-9* sgRNA, the *cdk-9as* ssODN as well as the *dpy-10* sgRNA and the *dpy-10* ssODN We visually screened the F1 progeny for roller worms and identified 6 positive plates containing at least one roller out of 10 plates of injected worms. From those plates, 30 rollers were singled out.

2.2. Screening by PCR-Restriction Assay

Taking advantage of the MvaI restriction site created along with the *as*-mutation, we designed a PCR restriction assay (Figure 13). The assay consists in the amplification of the *as*-mutation and the surrounding sequences. The amplicon is then digested by MvaI and screening is performed by restriction fragment length polymorphism.

We designed a first set of primers to assess if the 30 singled out roller worms carried the *as*-mutation. We identified 14 heterozygous mutants (*cdk-9 as/+*). Unfortunately, we could not identify any homozygous mutants (*cdk-9 as/as*). Due to a non-specific band, very close to the diagnostic band we were looking for in the restricted WT, we could not be 100% sure we effectively had worms carrying the *as*-mutation. We designed a new set of primers, getting rid of the ambiguity, enabling us to confirm our candidates. The heterozygous candidates were found in the progeny of 5 of the initial plates, thus rising our CRISPR-Cas9 efficiency for point mutations up to 50%.

We chose 2 confirmed rollers and screened 8 worms (total 16 worms) in their progeny. We found 14 *cdk-9 as/+* but, unfortunately, no *cdk-9 as/as* worms. We kept 5 of those candidates and screened their progeny. For reasons still unclear, the second set of primers stopped working, forcing us to try yet another set. Using this new set, we identified 5 *cdk-9 as/as* candidates.

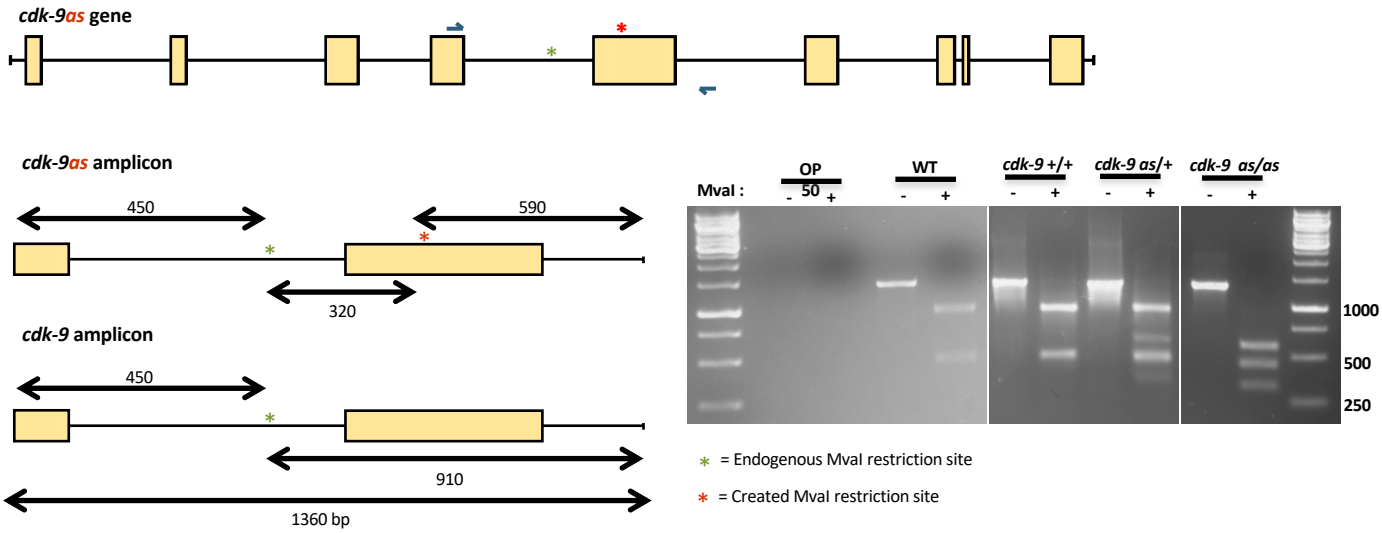


Figure 13: PCR restriction assay by fragment length polymorphism. Screening for as-mutation is done by PCR restriction assay since ssODN *cdk-9as* also adds a MvaI restriction site. PCR amplification gives an amplicon of length 1350 bp. After restriction with MvaI, the *cdk-9* genetic state (+/+, *as*/+, *as*/*as*) can be assessed by fragment length polymorphism: (1) *cdk-9* +/+ has 2 bands (450 bp, 910 bp), (2) *cdk-9 as*/*as* has 3 bands (320 bp, 450 bp, 590 bp) and (3) *cdk-9 as*/+ has 4 bands (320 bp, 450 bp, 590 bp, 910 bp).

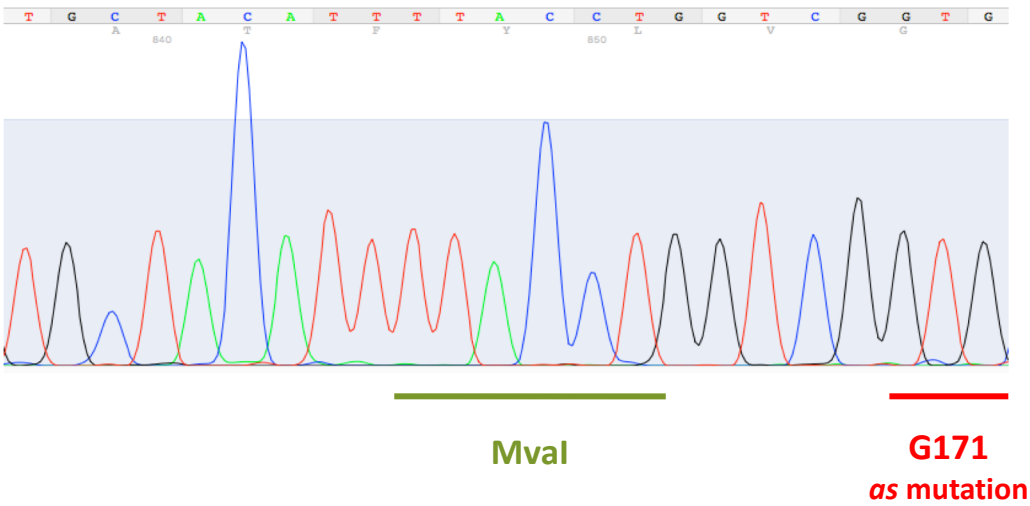


Figure 14: Confirmation of *cdk-9as* by PCR sequencing

2.3. Strain Confirmation by PCR-Sequencing

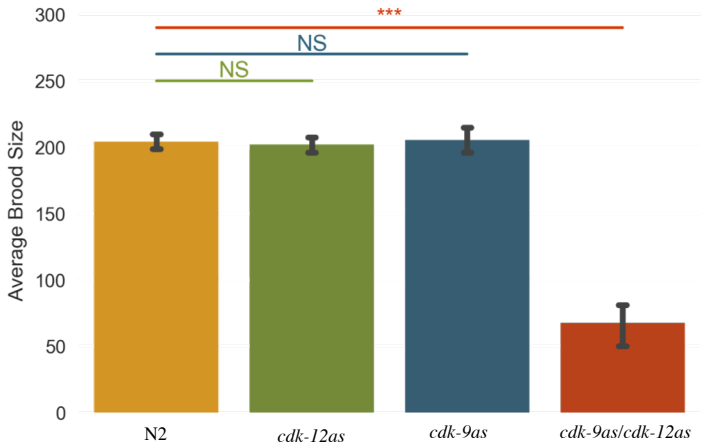
We sequenced 2 candidates from 5 worms identified in the PCR-Restriction Assay. The resulting chromatograms had evenly-spaced crisp peaks, with a low baseline noise (Figure 14). As there was no ambiguity, the desired mutations in the repaired region were confirmed.

3. Construction of a *cdk-9as/cdk-12as* mutant strain

There are two putative CTD-Ser2 kinases: CDK-9 and CDK-12. Our lab already generated a *cdk-12as* mutant strain. Since we successfully created a *cdk-9as* strain, we decided to create a double mutant to study what would happen to CTD-Ser2 phosphorylation in absence of both kinases. The procedure is fairly similar to the *cdk-9as* strain construction. Indeed, the same mix containing Cas9 in complex with *cdk-9* sgRNA, the *cdk-9as* ssODN as well as the *dpy-10* sgRNA and the *dpy-10* ssODN is injected into worm gonads. The sole difference is that instead of injecting N2 worms, *cdk-12as* worms were injected.

Using the same PCR-restriction assay, we found 6 candidates out of 9 screened worms From those, we derived 3 homozygous mutant worms, 2 were confirmed by PCR-sequencing.

A.



B.

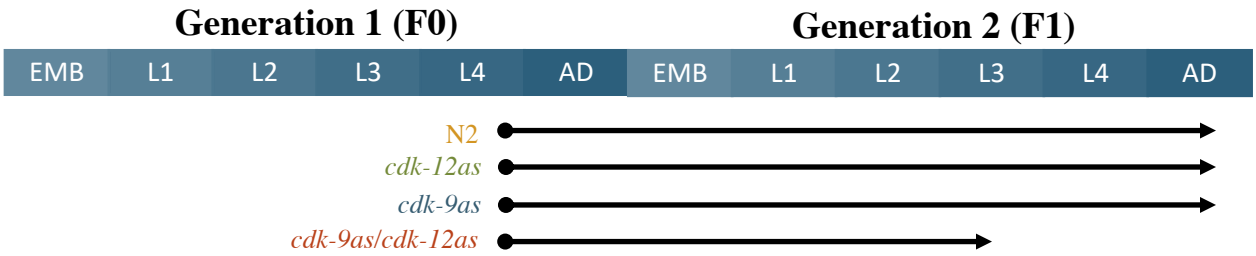


Figure 15: Single CDK-*as* mutants develop like N2 in absence of the inhibitor.
(**A**) Measure of the brood size of *N2*, *cdk-12as*, *cdk-9as* and *cdk-9as/cdk-12as* worms at 20°C. N = 5 (**B**) *Single* CDK-*as* mutants do not have growth delay compared to N2. Observation of the progeny 3 days after it has been laid.

B. Single CTD kinase mutants develop like *wild type* worms in absence of the inhibitor

Characterizing the phenotype of the *cdk-9as* strain as well as the *cdk-9as/cdk-12as* strain in the absence of the specific ATP analog 3MB-PP1 is essential prior the any experiment. It is important to assess the phenotype of a strain in normal conditions and compare it to the N2 WT reference as any differences would impair observations caused by CDK inhibition.

1. Self-brood size

The first phenotype to be assessed was brood size. Average brood size provides a useful metric concerning the overall fitness of a strain, as many developmental abnormalities directly or indirectly affect the fertility of the worms. *C. elegans* can be culture at either 20°C or 25°C. However, in our lab, we only culture it at 20°C. As such, the brood size was assessed at 20°C. The single CDK mutants (*cdk-9as* and *cdk-12as*) showed no statistical reduction in average brood size compared to the N2 strain (Figure 15A). However, the double mutant (*cdk-9as/cdk-12as*) showed a strongly reduced average brood size compared to the *wild type* N2 strain (Figure 15A).

2. Assessing growth delay

In addition to the brood size experiment, we assessed the growth rate for each strain. Concretely, L4 worms were put on a plate and allowed to lay eggs. Three days later, the developmental stage of the F1 progeny was observed. The F1 progeny of the WT N2 strain was adult and no embryos were already laid (Figure 15B). Similarly, the F1 progeny of *cdk-9as* and *cdk-12as* had also reached adulthood (Figure 15B). However, we cannot exclude that a little growth delay exists in those strains compared to the WT since the experiment does not have the resolution allowing the detection of subtle growth delays. Our experiment gives an order of magnitude: single *cdk-as* mutant strains have a growth rate very similar to the WT and, they can thus be cultivated in similar conditions. On the other hand, the *cdk-9as/cdk-12as* strain had not reached adulthood but were still mostly L3 worms (Figure 15B). It took the double mutant strain about a day longer than the WT to reach adulthood.

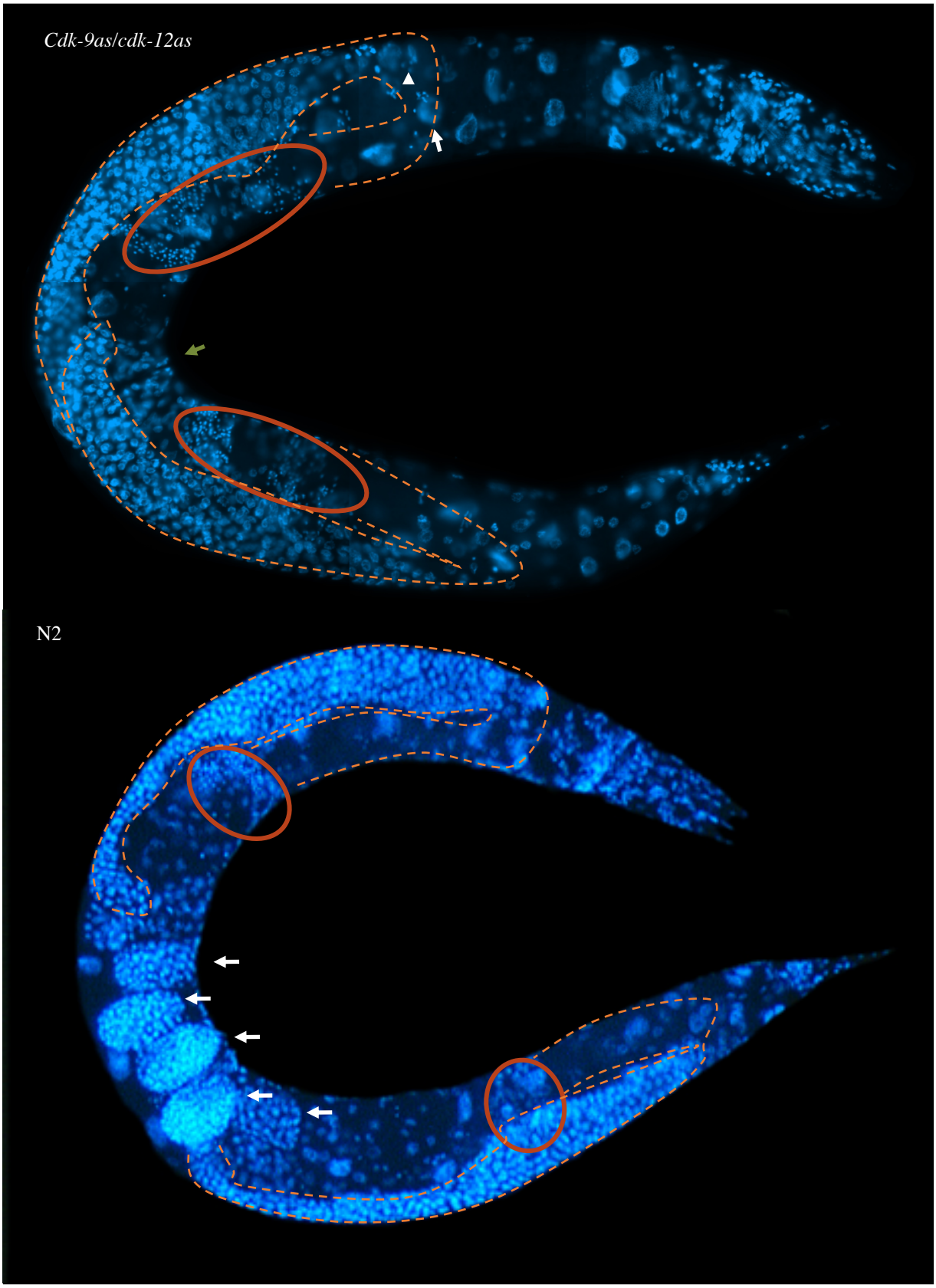


Figure 16: DAPI staining of a young adult *cdk-9as/cdk-12as*. Abnormally shaped gonad (doted line), extended spermatheca (circled). Worms are capable of producing oocytes (withe arrows) and the vulva (green arrow) is visible and open. As comparison, an N2 adult worm DAPI stained is also presented (http://www.signalingcellcyclegroup.wustl.edu/)

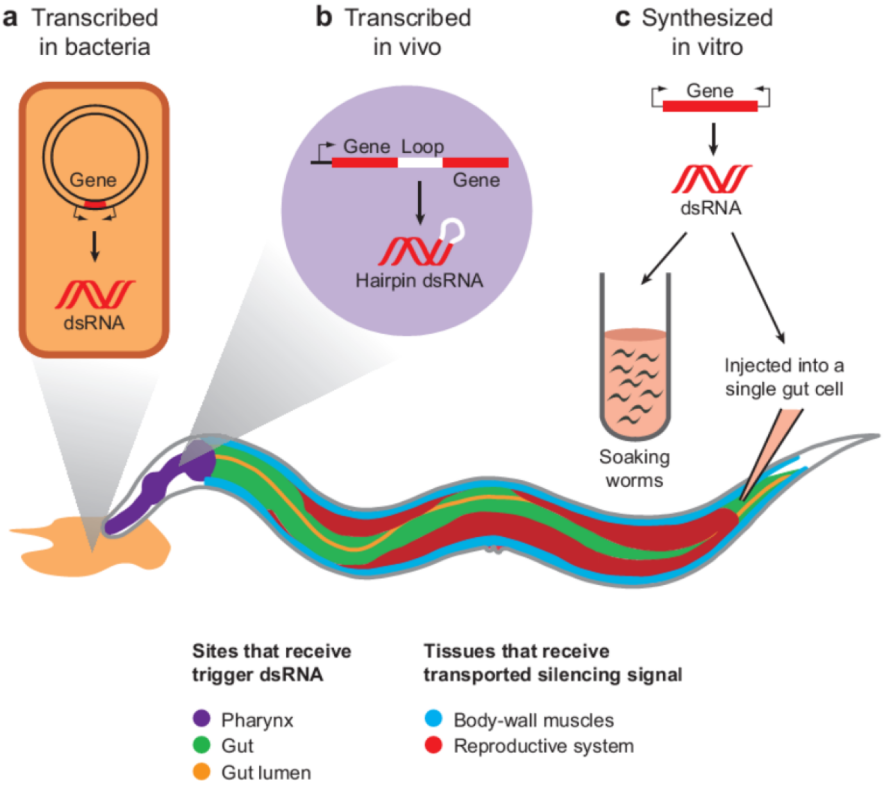
3. Microscopic characterization

In order to characterize the phenotype of the young adults obtained, a microscopic characterization by DIC and DAPI staining was performed (Figure 16). We did not detect obvious morphological defects in *cdk-9as* and *cdk-12as* compared to WT N2 worms, at least at the level of expertise of the laboratory.

Contrary to *cdk-9as* and *cdk-12as*, the double mutant strain presents obvious morphological abnormalities. We observed that gonad morphology and function is impaired. Indeed, adult worms possess gonads that are much bigger than normal with aberrant structures. Nevertheless, those gonads seem to be capable of producing oocytes. However, we observed worms in which only one gonad seemed to be working, as one side of the uterus was empty while the other one contained embryos. Furthermore, a few worms were found with no gonad at all. Unfortunately, such worms could not be DAPI stained or observed under the microscope as their frequency in the population is low. We also observed enlarged and diffuse spermatheca compared to the *wild type*. In general, in the *cdk-9as/cdk-12as* strain, gametogenesis appears strongly affected.

Taken together, the reduced brood size, the growth delay and the synthetic effects make the *cdk-9as/cdk-12as* strain inadequate for further experiments requiring more material than the observation of just a few worms. The following experiments will thus essentially focus on the *cdk-9as* strain.

A.



B.

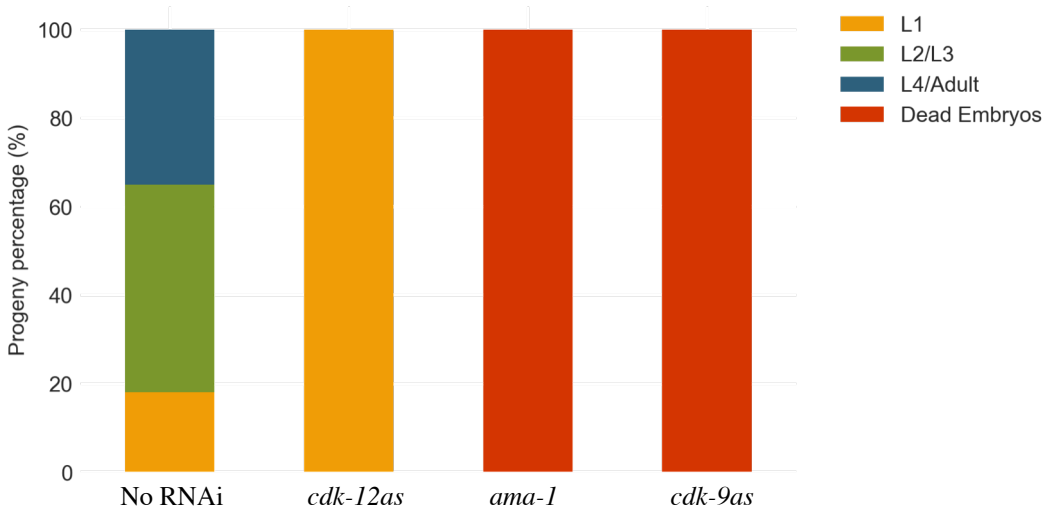


Figure 17: Knockdown of *cdk-9* mRNA phenocopies knockdown of *ama-1* mRNA. (**A**) Different methods to trigger RNAi in *C. elegans* (Jose & Hunter, 2007). (**B**) Quantification of the F1 progeny of N2 worms upon RNAi by micro-injection. The first 24H progeny was discarded (injected worm is moved to a new plate).

C. Confirming literature: RNAi by microinjection

In the nineties, Fire and Mello discovered the RNA interference (RNAi) pathway in *C. elegans* from the serendipity that injecting double stranded RNA induces dramatic downregulation of the corresponding gene expression, both in the animal and its germ line (Fire et al., 1998). Briefly, the RNAi mechanism consists in the cleavage of the dsRNA by the Dicer endonuclease into 21-23 nucleotides long primary small-interfering RNAs (siRNAs). These siRNAs associate with RISC (RNA-induced silencing complex) and guide it to the target mRNAs, which is then degraded.

RNAi can be triggered by 4 different ways in worms: micro-injecting dsRNA produced by *in vitro* transcription, soaking worms with dsRNA, feeding with bacteria expressing dsRNA, using a transgene expressing an hairpin dsRNA (Figure 17A) (Jose & Hunter, 2007). RNAi by injection is very labor intensive compared to the other methods. However, it gives reliable gene inhibition from worm to worm. Since we have experience with microinjection, we decided to perform RNAi by micro-injecting *in vitro* transcribed dsRNA directly into N2 young adults. In contrary to transgenesis, the injection does not necessarily have to be performed in the gonad as equivalent down-regulation effects can be obtained irrespective to the injection site.

1. Knockdown of *cdk-12* induces an L1 arrest

As *cdk-12* is used as control in our experiments, we performed RNAi to knock down its expression. Indeed, previous experiments in our lab have shown that either a loss-of-function mutant, or RNAi by soaking and feeding as well as the inhibition of CDK-12as all results in a penetrant L1 arrest. However, RNAi by injection of young adults had never been attempted. We performed the RNAi knockdown by injection in order to confirm the L1 arrest inhibition. A “No RNAi” control was also done by micro-injecting young adults with the T7 reaction buffer (no dsRNA) (Figure 17B).

The *cdk-12* (micro-injection RNAi) embryos arrested their development at the L1 stage (Figure 17B), which agrees with previous results from the lab. CDK-12 is dispensable for the embryonic development and is required at the post-embryonic level.

2. *cdk-9* knockdown phenocopies *ama-1* knockdown

CDK-9 is known to be required for efficient transcription. As a benchmark for a general transcription defect, we performed an *ama-1* RNAi knockdown therefore targeting the largest subunit of Pol II, AMA-1. Indeed, transcription is known to be dispensable until around the 100 cells stage. The proteins and mRNAs maternally inherited are sufficient to cover the needs for early embryonic development. As expected upon *ama-1* knockdown, we observed 100% embryonic lethality (Figure 17B).

Similarly, *cdk-9* RNAi knockdown induces a 100% embryonic lethality (Figure 17B). Embryos were not collected and observed by microscopy. Therefore, we cannot assess if embryos arrested at the 100 cells stage. Nevertheless, visual assessment shows that embryos lack differentiation signs. CDK-9 (RNAi) embryos do not reach the elongation stages of embryonic development (fold stages). Taken together, those results confirm that transcription is essential for embryonic development and that the knockdown of Cdk-9 mimmicks the knockdown of Pol II, in sharp contrast to the knockdown of *cdk-12*.

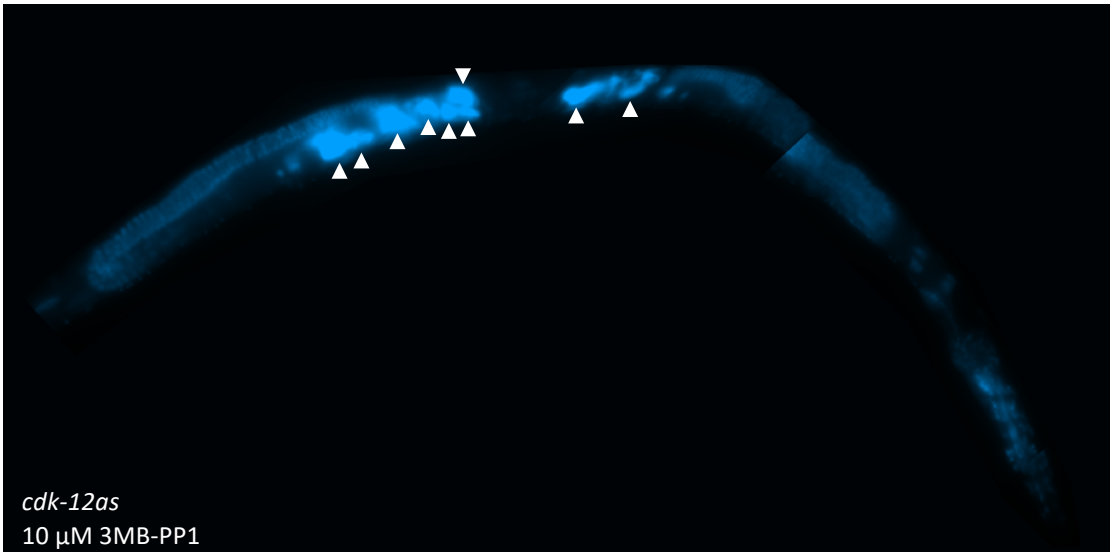


Figure 18: Strong CDK-12as inhibition induces an Emo phenoype. L4 *cdk-12as* worms were cultivated on plate in presence of high concentration (10 μM) of the inhibitor 3MB-PP1 and DAPI stained 24H later. Those worms presented endoreplication in gonad, called Emo phenotype (white arrow), and were sterile.

D. Physiologic characterization of analogue sensitive CDKs inhibition

A main advantage of the chemical genetics approach to inhibit kinase activity is the possibility to add the inhibitor at any given stage, which allows for time specific inhibition thus opening up the study of kinases beyond embryonic development context. Up to this day, nearly all the published *as*-mutants were constructed in yeast or mammalian cell lines. The only study reported to use the method in multicellular organism showed that the inhibition of the neuronal kinase SAD-1 was effective in every *C. elegans* neurons. Previous work in our lab has adapted Zhang et al.'s protocol for CDK-12as. It simply consists of culturing worms in a liquid media containing the ATP analogue inhibitor. We decided to use the ATP analog 3MB-PP1 as it seems to have an enhanced efficiency compared to the 1NA-PP1 and the 1NM-PP1 found to be the most effective inhibitors for as kinases (Zhang et al., 2013). Embryos are technically challenging to observe and characterize in liquid media due to their small size. Because of those limitations, a protocol of inhibition on plate was developed. The new method simply consists of adding the inhibitor directly into the NGM mix poured into petri dish. Practically, L4 worms were cultured on plates containing the inhibitor and the development of the F1 progeny was observed.

1. The inhibition on plate of CDK-12as induces a L1 arrest

The *cdk-12as* strain is well characterized in our lab as it has been a subject of investigation since 2014. As indicated above, the inactivation of CDK-12 by any mean results in a penetrant L1 arrest. However, CDK-12as inhibition has never been attempted on solid media. In order to confirm the phenotype and validate CDK-12as as a control for CDK-9as inhibition, we cultivated *cdk-12as* worms on plates containing increasing concentration of the 3MB-PP1 inhibitor (1 µM, 5µM and 10 µM). Three days later, the phenotype of the F1 progeny was assessed. A low concentration of 3MB-PP1 (1µM) is enough to induce a penetrant L1 arrest. In the *wild type*, the inhibitor has no effect as the F1 progeny develops correctly even in presence of the inhibitor. At higher concentrations (5 and 10 µM), the *cdk-12as* F0 becomes sterile. No embryos were observed. DAPI staining revealed that worms presented endo-replication, known as Emo phenotype (Figure 18), in the gonad.

Taken together, those results indicate that N2 and *cdk-12as* are both good controls. N2 is a negative control not affected by the inhibitor and *cdk-12as* is a positive control, inhibited to similar extents on plate or in liquid media. Importantly, the data indicate that inhibition on plate does result in phenotypes identical to those observed by other methods, establishing the validity of the protocol.

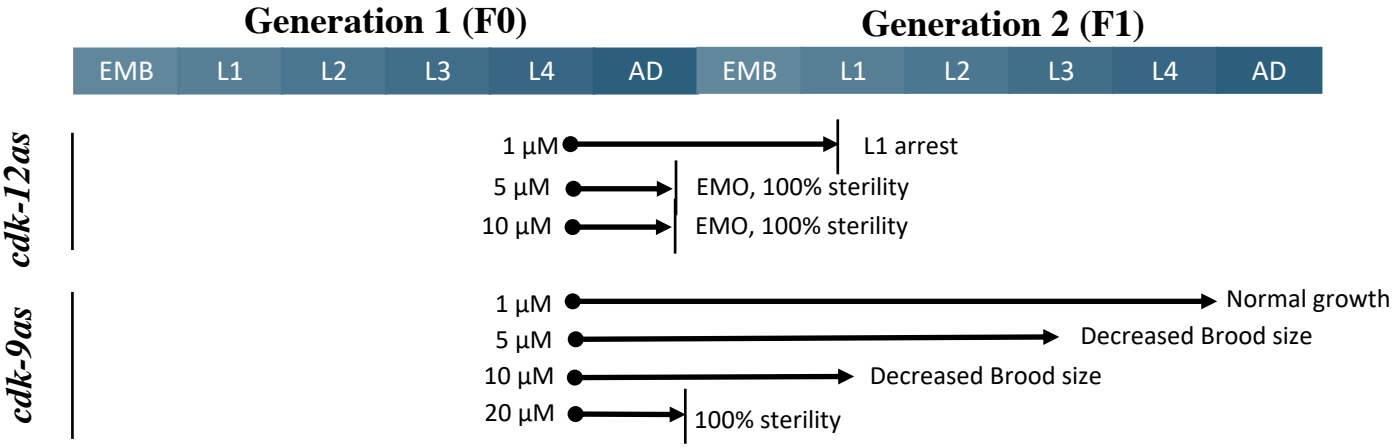


Figure 19: Summary of single *cdk-as* mutants descriptive phenotypes upon inhibition on plate. L4 worms were put on plate and the F1 progeny was observed 3 days later.

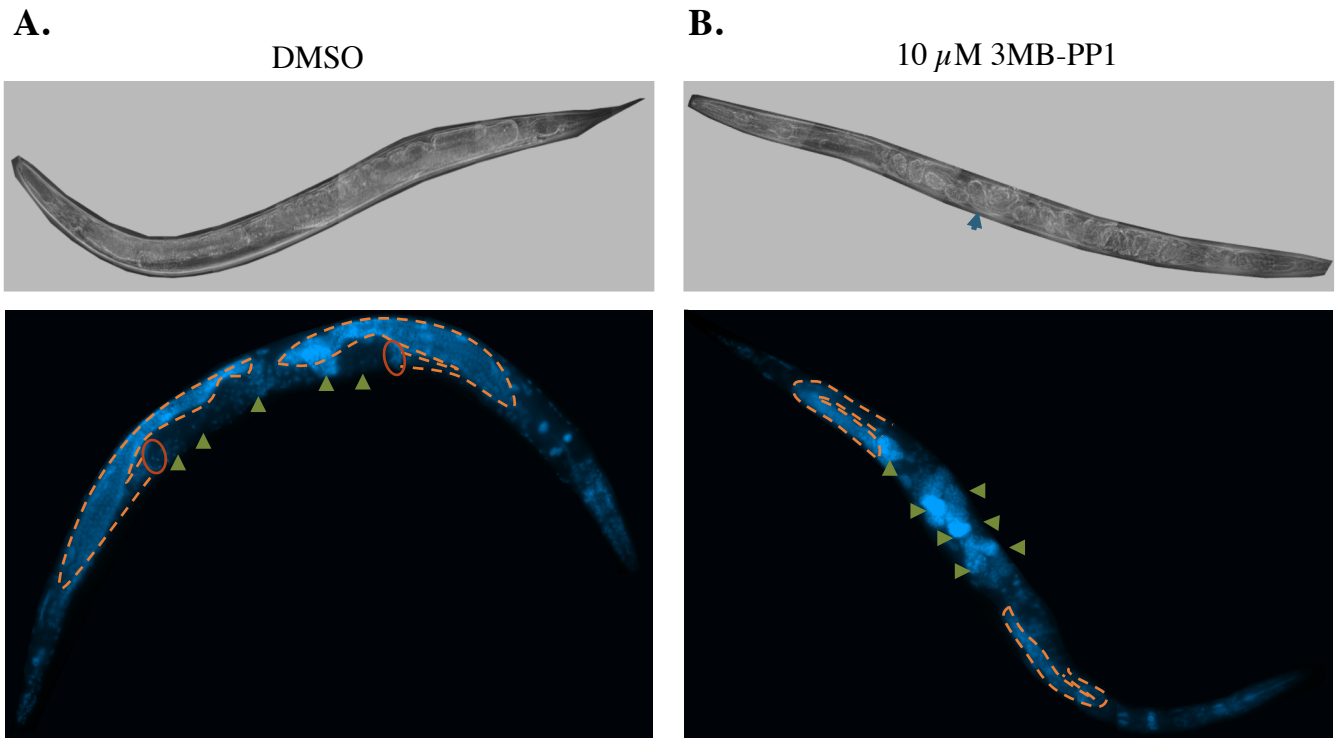


Figure 20: The inhibitor leads to morphological defects in *cdk-9as*. Top images are DIC and bottom image are DAPI staining. (**A**) L4 *cdk-9as* cultivated for 24H in presence of DMSO. The gonad is surrounded in doted line and the spermatheca is circled in full line. Embryos are shown with green arrowsheads. There are no morphological defects. (**B**) L4 CDK-9as cultured for 24 H in presence of 3MB-PP1 (10 μM). The gonad is surrounded in doted lined and embryos are shown using green arrowheads. The spermatheca is not visible on the image. The gonads are shorter compared to non-inhibited *cdk-9as* worms. There are more embryos than should normally be present in a worm (about 10) and some of them should have already been laid as they are composed of more than 30 cells. On the top panel, hatched embryos are present in the worm (blue arrow).

2. Descriptive characterization of CDK-9as inhibition

2.1. Inhibiting L4 worms induces growth delay, sterility and egg laying defect

Earlier in this work, the knockdown of *cdk-9* was performed by RNAi on young adults, which resulted in fully penetrant embryonic lethality. This data is coherent with the literature. Indeed, it has been shown that depletion of *cdk-9* mRNA or simultaneous depletion of the two cyclin orthologs (*cit-1.1* and *cit-1.2*) by RNAi caused an early developmental arrest at +/- 100 cells (Shim et al., 2002). However, the inhibition of CDK-9as at doses that affect CDK-12-as both on plate and liquid media did not induce complete embryonic lethality. Indeed, at the lowest tested concentration (1µM 3MB-PP1), which is enough to induce a L1 arrest in *cdk-12as* worms, no effect on the *cdk-9as* strain were observed with no noticeable reduction in brood size compared to the control DMSO condition (Figure 19). However, the observed brood size was affected by increasing concentration (5 µM and 10 µM) of 3MB-PP1 (Figure 19). Furthermore, in the highest tested concentration (20 µM), no F1 progeny could be observed (Figure 19).

In addition to average brood size reduction, CDK-9as inhibition causes growth delay of the F1 progeny. As for brood size, the lowest tested condition does not induce growth delay. However, such delay is observed at 5 and 10 µM of 3MB-PP1. After 3 days on inhibitory plates, worms were still L2/L3 if inhibited with 5 µM while there were still L1 if inhibited with 10 µM (Figure 19). As such, the penetrance of growth delay also increases with increasing concentration of the inhibitor.

In order to assess if worms developed correctly with the inhibitor, DAPI staining was performed on adult worms (Figure 20A, B). No defects were observed at the 1 µM concentration. However, increasing the concentration seemed to have a severe effect on the worm capacity to lay eggs. At 5 µM, embryos that should already have been laid as they had more than 30 cells were present, in the parental worms. This was even more obvious when 10 µM of inhibitor was used: there were hatched larvae inside some of the parental worms (Figure 20B). On both conditions (5 µM, 10 µM) embryos were misplaced as if the gonad and uterus lacked a well-defined structure (Figure 20B). In the highest tested concentration condition (10 µM), some gonads were reduced in size compare to the WT. In brief, DAPI staining and DIC imaging show that embryos accumulate in the parental worms longer, sometimes reaching the point of hatching while still inside the parental uterus.

In summary, the inhibition of L4 worms induces sterility, egg laying defects, embryonic lethality and growth delay with the highest concentration tested inducing complete sterility.

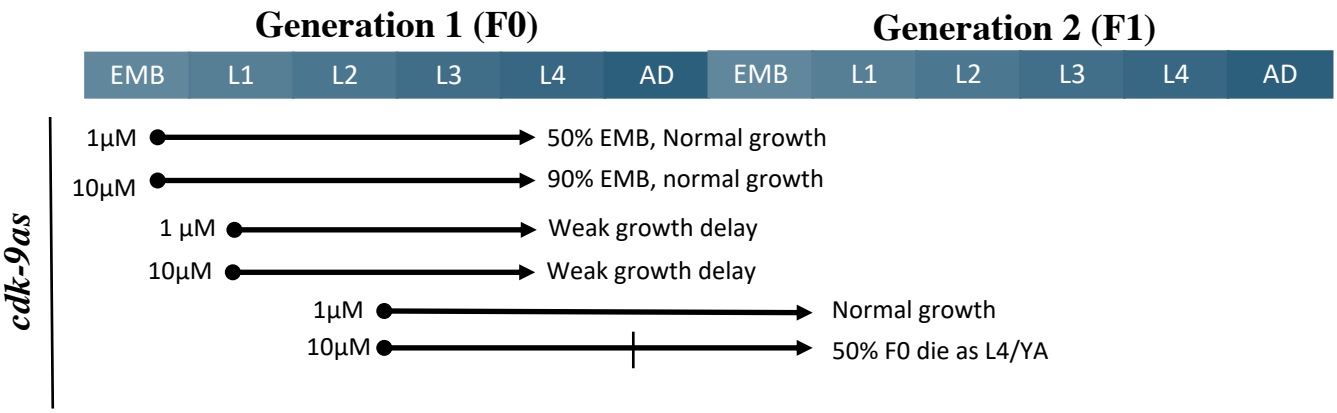


Figure 21: Summary of single CDK-9as inhibition at different developmental stages. L4 worms were cultured in liquid media supplemented with 3MB-PP1 and the F1 progeny was observed 3 day later.

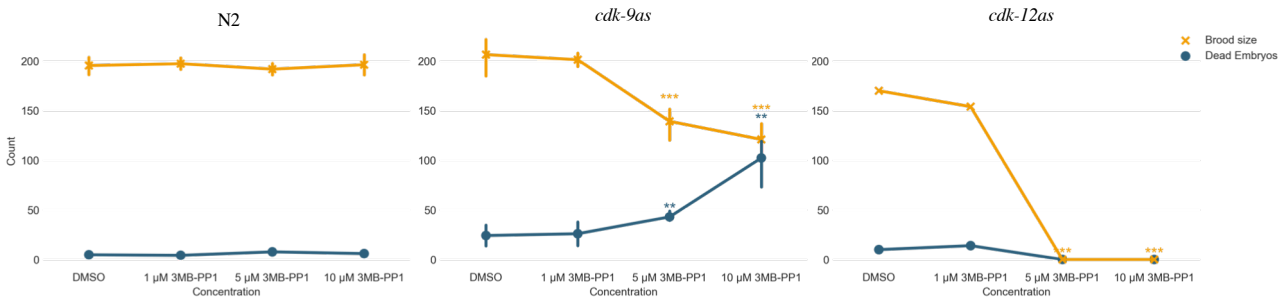


Figure 22: CDK-9as inhibition induces sterility and embryonic mortality. For each tested strain (*N2*, *cdk-9as*, *cdk-12as*), 5 L4 worms were isolated on plate containing DMSO or 3MB-PP1 (1μM, 5μM, 10 μM) for 4 days. The first 24H progeny is discarded (the parental is moved to a new plate) in order to copy the RNAi progeny observation procedure. Brood size comprises all the larvaes and embryos (dead or alive) present on the plate. The dead embryo count is the difference between the embryo count after 3 days and the embryo count after 5 days.

2.1. Inhibiting other *C. elegans* developmental stages

In order to better define the developmental time frame where CDK-9 is of importance, the inhibition was also performed on other *C. elegans* developmental stages (Embryo, L1, L1/L3, L4). The desired stage is cultured in liquid, supplemented with 3MB-PP1 (1 or 10 µM), and characterize 3 days later. The experiment has been performed by Fanélie Bauer.

The inhibition of post-bleaching embryos induces partial embryonic lethality. On 1 µM inhibitory plate, 50% of the inhibited embryos have died. At 10 µM, 90% have died. Embryos that hatch become worms that develop similarly to the N2 WT strain (Figure 21). When synchronized (starved) L1s were inhibited, no difference was observed between the 1 µM and 10 µM conditions (Figure 21). In both conditions, worms had a slight growth delay but reached adulthood (Figure 21). However, when L2/L3 were inhibited, the conditions similarity did not hold true. At 1 µM, the observed F1 progeny was smaller than on a non-inhibitory plate. This is the reflect of either sterility, mortality or both. At 10 µM, 50% of the parental worms died at the L4/Young adult stage (Figure 21). Altogether, those data suggest that the inhibition of CDK-9as causes defects in embryogenesis and/or late gonadogenesis.

2.1. CDK-9as inhibition is reversible

The inhibition of CDK-9as is reversible. Indeed, when the F1 progeny of inhibited worms was transferred to a fresh plate not containing inhibitor, it grew to adulthood and was fertile.

3. CDK-9as inhibition induces partial embryonic mortality and sterility

In order to quantify the proportion of embryonic lethality in the progeny, we performed an inhibitory experiment copying the RNAi protocol. On day one, a young adult is isolated on an inhibitory plate. After 24H, the worm is placed on a new inhibitory plate and allowed to lay eggs for 2 days. *cdk-9as* was tested in parallel to the control strains, N2 and *cdk-12as* at 3 different concentrations of inhibitor. As expected, the inhibitor does not interfere with the WT strain brood size or survival rate of the laid progeny (Figure 22). Similarly, CDK-12as inhibition at high (5 µM, 10 µM) 3MB-PP1 induces complete sterility (Figure 22).

CDK-9as induces a very highly significant reduction in brood size at 5 and 10 µM but not at 1 µM. Along with the brood size reduction, an increase in embryonic mortality is observed. At 5 µM, 31.2% of the embryos laid are dead while at 10 µM it goes up to 83.7% (Figure 22). Furthermore, the observed embryos lacked differentiation signs. Even if CDK-9as inhibition does not 100% phenocopy *cdk-9* RNAi knockdown, it nevertheless induces a similar phenotype: an early arrest during embryonic development.

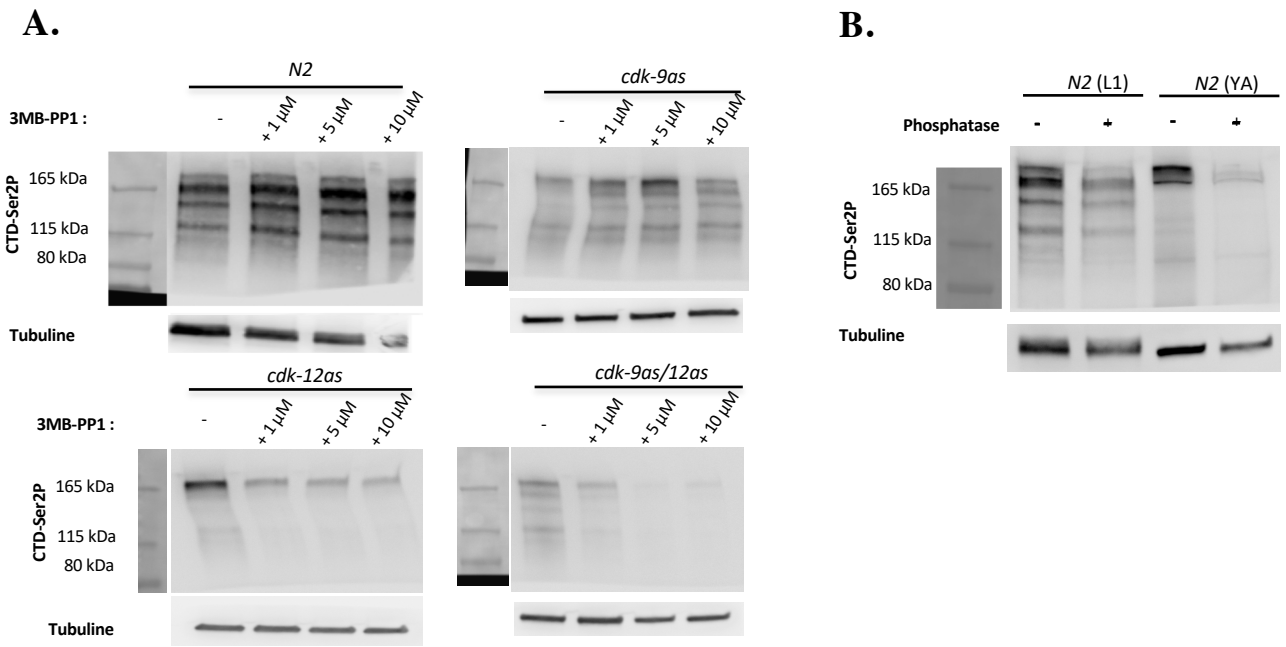


Figure 23: The inhibition of CDK-9as in L1 worms does not reduce CTD-Ser2 phosphorylation level. (**A**) Protein extracts of about 100 000 synchronized starved L1 worms that were supplemented for 30 minutes with DMSO or 3MB-PP1. The level of what was probed by western blotting using the 3E10 antibody. (**B**) Protein extracts from about 100 000 L1 worms (N2) and 500 young adults (N2) were treated or not by a lambda phosphatase and probed with the 3E10 antibody. A global reduction of signal is observed in extracts treated with phosphatase. This means that every observed band correspond to phosphorylated serine.

E. 3MB-PP1 inhibition: impact on CTD-Ser2 phosphorylation

The second aim of this work is the study of the impact of CDK-9 on CTD-Ser2P. As described in the introduction, it is currently believed that CDK-9 is partly responsible for CTD-Ser2P. Having a *cdk-9as C. elegans* strain encompassing a conditional is a powerful tool as it allows us to inhibit the kinase at a given time thus allowing the study of phosphorylation CDK-9 dependent.

1. CDK-9as inhibition does not reduce CTD-Ser2P level in L1 worms

Previous work in our lab has already characterized the role of CDK-12as inhibition in the development of the worm, specifically showing a L1 arrest. CDK-12 is required to drive development from the L1 stage to post-L1 development, thus we investigated the CTD-Ser2 phosphorylation level of inhibited L1 worms. We found that at low (1 µM) concentration of 3MB-PP1, the inhibition of CDK-12as results in a severe decrease of CTD-Ser2P. However, increasing 3MB-PP1 concentration does not seem to further reduce the level of CTD-Ser2P (Figure 23A).

We started our investigation of CDK-9 dependent CTD-Ser2 phosphorylation on synchronized L1 larvae. We found that inhibiting CDK-9as does not induce a drop in CTD-Ser2P at any tested 3MB-PP1 concentration (Figure 23A). Using the *cdk-9as/cdk-12as* strain, we simultaneously inhibited CDK-12as and CDK-9as. We found that low (1 µM) 3MB-PP1 concentration induces a decrease in CTD-Ser2P similar to CDK-12as inhibition in the same conditions (Figure 23A). Interestingly, increasing 3MB-PP1 concentration to 5 µM further reduces CTD-Ser2P in the *cdk-9/cdk-12as* to an almost non-detectable level (Figure 23A). The same decrease is observed at 10 µM (Figure 23A). As our western blots often show multiples bands, we questioned the specificity of the phospho-CTD-Ser2 antibody. We treated WT total protein extract from L1 and young adult worms, with a lambda phosphatase and observed a general decrease of the signal after treatment (Figure 23B). Therefore, it seems likely that the multiple bands are degradation products of the phosphorylated polymerase rather than bands corresponding to the unphosphorylated form.

Altogether, those data provide a first indication that CDK-9 is not the major CTD-Ser2 kinase and is dispensable to maintain proper level of CTD-Ser2P. We postulate that CDK-12 and CDK-9 may have partially redundant CTD-Ser2 kinase activity with CDK-12 alone compensating for the decrease of CTD-Ser2 phosphorylation upon CDK-9as inhibition.

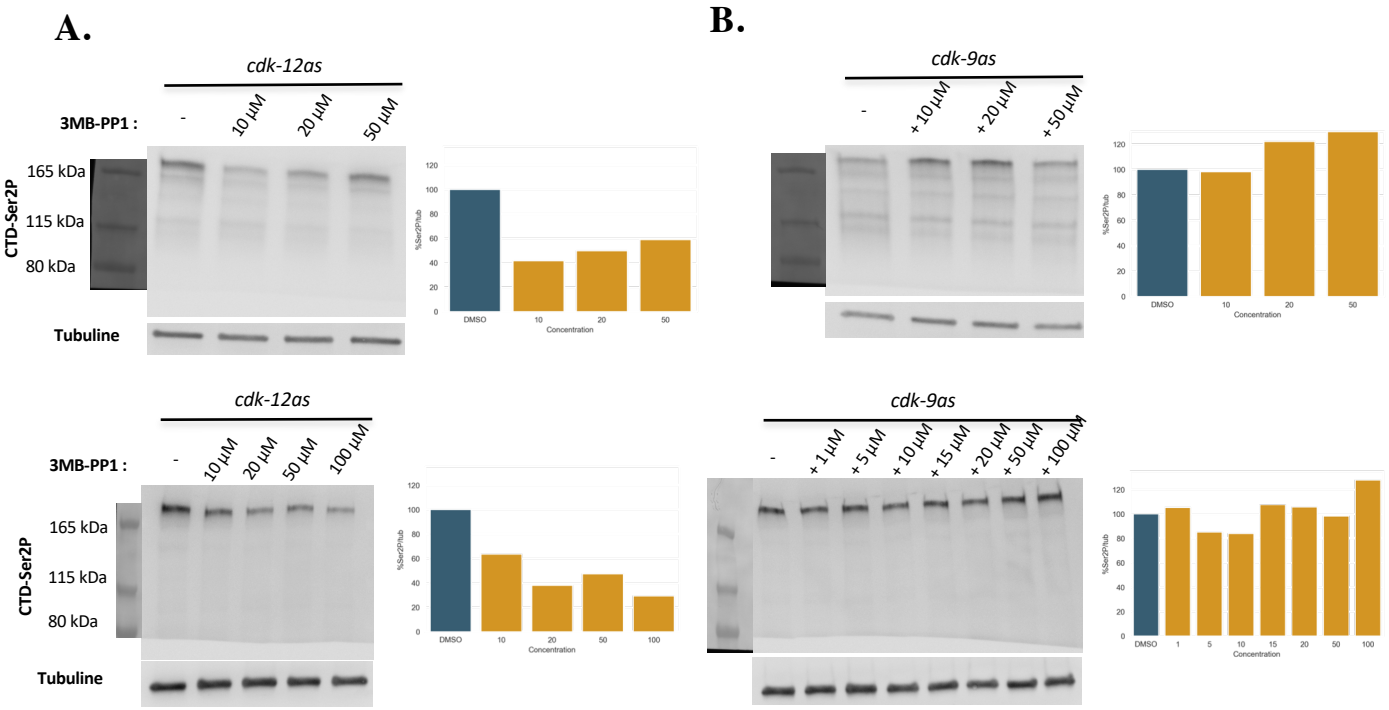


Figure 24: The inhibition of CDK-9as in adult worms does not reduce CTD-Ser2 phosphorylation level. Protein extracts of about 500 young adult worms that were supplemented for 30 minutes with DMSO or 3MB-PP1 was performed. The level of CTD-Ser2P (10 μg of the total protein extract charged on gel) was probed by western blotting using the 3E10 antibody. For each western the CTD-Ser2P signal was quantifiied. The two-bottom western blot have been performed by Fanélie Bauer.

2. CDK-9as inhibition does not reduce CTD-Ser2P in young adults

Since CDK-9as inhibition in L1 worms does not reduce CTD-Ser2P, we extended our investigation of CTD-Ser2P in young adults. We found that at low (1 µM) concentration of 3MB-PP1, the inhibition of CDK-12as in young adults does not induce a drop in CTD-Ser2P (Figure 24A). However, higher concentration (10 µM, 20 µM, 50 µM, 100 µM) induces a drop in CTD-Ser2P (Figure 24A) similar to the inhibition of CDK-12as in L1 worms with low (1 µM) 3MB-PP1 concentration (Figure 23A).

Because the inhibition of CDK-12as requires higher concentration of inhibitor in young adults (Figure 24A) than in L1s, the inhibition of CDK-9as in young adults was also performed using a wider range of inhibitor concentration (Figure 24B). The experiment was performed twice. In line with the inhibition of CDK-9as in L1s, no drop in CTD-Ser2 phosphorylation level can be observed upon CDK-9as in young adults (Figure 24B). The phosphorylation level stays quite steady at the different tested concentrations, with maybe a slight increase (Figure 24B). This provides a second indication that CDK-9 is not the major CTD-Ser2 kinase and is dispensable to maintain proper level of CTD-Ser2P.

The inhibition of CDK-9as in both L1 and young adults suggests that CDK-9as is not responsible for CTD-Ser2P. However, on plate inhibition of CDK-9as induces defects in embryogenesis and/or late gonadogenesis. Taken together, those data suggest that the observed phenotype is not linked to CTD-Ser2P but rather to another CDK-9 target.

IV. Discussion & Perspectives

A. The Shokat analogue sensitive mutant strain, a tool to study kinases *in vivo*

Studying kinases *in vivo* is a difficult task as it is technically very challenging to specifically inhibit a given kinase. Indeed, due to the high degree of homology between their catalytic domain, there are no highly specific inhibitors available. Furthermore, even if RNAi is possible in *C. elegans*, it lacks reproducibility (RNAi by soaking or feeding) and is technically labor intensive (RNAi by microinjection). The Shokat analogue sensitive mutant stands out of these RNAi limitations. This technique offers the possibility to specifically inhibit a given kinase by converting its non-essential bulky gatekeeper residue into a smaller one. This sensitizes the modified kinase to synthetic bulky ATP derivates. Using the recently developed CRISPR editing system, we successfully created and confirmed the first multicellular CDK-9as mutant strain in a *wild type* and *cdk-12as* background. Thanks to the high efficiency of the CRISPR/Cas9, the generation of such *C. elegans as*-mutant strain is relatively easy and fast. This opens up the possibility to apply the technique to other CTD kinases but also to more specific kinases.

The main advantage is that the *as*-mutation has little effects in absence of the inhibitor. Both single CDK *as*-mutant strains have a brood size similar to the WT in absence of the inhibitor. However, we only assessed brood size at 20°C and cannot exclude the possibility that the mutation is temperature sensitive. To answer this concern, average brood size at 25°C and 15°C should be assessed. In addition, the *cdk-9as* strain has a slightly increased basal embryonic lethality on DMSO than the WT N2 strain.

Contrarily to single CDK *as*-mutants, in absence of the inhibitor, the *cdk-9as/cdk-12as* strain has many developmental defects such as an extended spermatheca, a high degree of sterility and a significant growth delay. As such, even if the possibility of inhibiting simultaneously both CDK-9as and CDK-12as is interesting, the severe synthetic effects of the *cdk-9as/cdk-12as* strain in absence of the inhibitor makes it a useless tool.

B. What is the function of CDK-9 in the model organism *C. elegans*?

In the nineties, it has been reported that embryonic germ cells are transcriptionally repressed. No newly transcribed mRNA could be detected in embryonic germ cells. It is generally admitted that this silencing of the embryonic germ cells ensures that they do not differentiate in somatic tissues at a developmental stage where differentiation signals are very abundant, which would deprive the organism of a progeny. By contrast, at the 4-cells stage, newly transcribed mRNA could already be detected in somatic blastomeres (Seydoux et al., 1996). It was reported that PIE-1, a germline specific factor, was responsible for the difference of mRNA transcription between the soma and the germline. Embryos immunostaining showed that there is no CTD-Ser2P in germ line blastomeres but that CTD-Ser2P is obvious in somatic cells of embryos older than 8 cells. However, in embryos where *pie-1* had been deleted, CTD-Ser2P could also be detected in germ line cells. This indicated that PIE-1 represses Pol II CTD phosphorylation which, in turns, blocks mRNA transcription (Seydoux and Dunn, 1997). Importantly, the causal relationship between a block in CTD-Ser2P and the inhibition of transcription is not established and it is equally possible that a transcriptional arrest at early elongation results in lower CTD-Ser2P simply because the polymerase does not reach the 3'-end of the transcription unit where CTD-Ser2P massively occurs.

Before the characterization of CDK-12, CDK-9 had been identified as the CTD-Ser2 kinase in *C. elegans* and as essential for embryonic development (Shim et al., 2002). It was shown that RNAi knockdown of *cdk-9* mRNA phenocopies *ama-1* RNAi knockdown as both lead to an early developmental arrest. Furthermore, *cdk-9* RNAi knock down induced a loss of detectable CTD-Ser2P and a reduced level of some mRNAs (ex : *hsp-70*, *c-myc*, …) (Shim et al., 2002). More recently, Kelly and coworkers reported that upon *cdk-9as* RNAi knock down, the CTD-Ser2P signal does not entirely vanish in embryos. The two germ line precursors cells (Z2 and Z3) still showed CTD-Ser2P signal. Kelly's team proposed a model in which CDK-9 is required to prime CDK-12 phosphorylation in the somatic cells but to be dispensable for CTD-Ser2 phosphorylation in the germ line (Bowman et al., 2013).

Results generated in our lab, partly fit this model and suggests that CDK-12 dependent CTD-Ser2 phosphorylation is required at the L1 stage to drive the transcription of SL2 trans-spliced genes. This suggests that CTD-Ser2 phosphorylation is not required for global transcription but is rather required for specific gene expression in response to external cues. Recent semi-quantitative western blot performed in our lab showed that the level of CTD-Ser2P is greatly reduced but still detectable when CDK-12as is inhibited. This master project was proposed in order to assess whether the remaining level of CTD-Ser2 phosphorylation, upon CDK-12as inhibition, is due to the action of CDK-9.

1. Is CDK-9 required for proper embryogenesis?

RNAi knockdown of *cdk-9* mRNA and *ama-1* mRNA both result in very penetrant early embryonic lethality. This indicates that CDK-9 is required for transcription, which is essential for embryogenesis from the 8 cells stage. In the contrary, *cdk-12* RNAi knockdown does not affect embryo development as the F1 progeny of injected worms undergoes a L1 arrest rather than embryonic lethality. Thus, our RNAi data confirm Blackwell and Kelly's data, CDK-9, but not CDK-12, is required for completion of embryonic development (Shim et al., 2002) (Bowman et al., 2013).

The inhibition of L4 *cdk-9as* worms does not perfectly copy RNAi against *cdk-9* as complete embryonic lethality is not observed. Indeed, increasing 3MB-PP1 concentration reduces the brood size but increases the proportion of dead embryos in it. The observed phenotypes are dose dependent. Microscopic characterization also showed that *cdk-9as* inhibited L4 worms had trouble laying eggs as old embryos tend to accumulate in the uterus, sometimes even hatching inside the body of the parent. Those observations suggest that at low (1 µM) inhibitor concentration, there is enough CDK-9 activity to produce and lay embryos and for embryonic transcription to properly occur. Conversely, lower CDK-9 activity induces defect in embryo production, embryo laying and embryonic transcriptional failure causing lethality. An RNA-seq performed on inhibited *cdk-9as* young adults should allow to better understand the functional role of CDK-9 at the transcriptional level: is CDK-9 required for proper expression of germ line development related genes?

The hypothesis of residual CDK-9 activity is supported by the two distinct phenotypes observed upon CDK-12as inhibition. Low level of inhibition (1 µM) is not enough to induce the Emo phenotype observed at higher concentration (5 µM, 10 µM) but is nevertheless enough to induce the L1 arrest. Such dose dependent defects are not unusual with as-mutants. Indeed, using an analogue-sensitive mutant, Coudreuse and Nurse showed a different level in Cdc2 kinase activity was required to go through the different transitions of the cell cycle (Coudreuse & Nurse, 2010). They observed that a higher dose of inhibitor was required to arrest fission yeast at the G1-S than at the G2-M transition and hypothesized that a much lower kinase activity is required to initiate DNA replication than mitosis. To bypass this residual activity issue, one could create an "irreversibly sensitized" (is) mutant that can be specifically and permanently inhibited by an ATP analog bearing a thiol-reactive chloromethylketone (CMK). To do so, in addition to the gatekeeper modification, a reactive cysteine must be added in the ATP binding pocket. The reaction between the CMK and the cysteine covalently binds the ATP analogue to the kinase thus definitely inactivating it.

2. Does CDK-9 phosphorylates Pol II CTD-Ser2?

Since some CTD-Ser2P resists to CDK-12as inhibition, we could have expected CDK-9as to be responsible for it. Surprisingly, we did not detect any drop in CTD-Ser2P in either L1 or young adult inhibited *cdk-9as* nematodes. This observation is inconsistent with literature as it implies that CDK-9 is not an exclusive *in vivo* CTD-Ser2 kinase. Instead of observing the expected decrease, our results might even suggest a slight rise of CTD-Ser2 phosphorylation level upon CDK-9as inhibition. That rise was more of a tendency in our experiments than a reproducible trend. Our experiments reproducibility will be increased by measuring time spent by the worm developing in presence of food rather than assessing the age of the worm visually. We next tried to assess the amount of CTD-Ser2P detectable by immunostaining of embryos derived from inhibited parents (data not shown). We did not detect any difference in CTD-Ser2 levels but there could be a technical reason for this negative result. Indeed, the secondary antibody used was ten times too concentrated, possibly leading to detection of background. These preliminary experiments must be repeated using the right antibodies concentrations. It should also be performed in parallel by injecting RNAi against *cdk-9* in parent worms to assess the level of CTD-Ser2P in their progeny.

As the inhibition of CDK-9as alone does not induce a drop in CTD-Ser2P level but that the inhibition of CDK-12as does, we assessed the CTD-Ser2 phosphorylation level upon inhibition of both kinases at once. Using the *cdk-9as/cdk-12as* mutant strain, we simultaneously inhibited both kinases at low (1 µM) inhibitor concentration and observed a drop in CTD-Ser2P similar to the CDK-12as inhibition. Upon simultaneous inhibition at higher (5 µM, 10 µM) concentration, the CTD-Ser2P was further reduced to nearly undetectable level. The experiment has only been performed once and should be repeated to confirm the observed two steps decrease.

Taken together, our results suggest that the CTD kinase function of CDK-9 and CDK-12 are partially redundant but that CDK-12 alone can compensate for CDK-9as inhibition in terms of CTD-Ser2P. We postulate that CTD-Ser2 phosphorylation by CDK-9 is dispensable. Rather, CDK-9 inhibition causes severe phosphorylation defect of a target that has an essential biological function. Our hypothesis is backed up by a chemical genetic screen, performed in human cells, that identified the torpedo exoribonuclease XRN2 as a CDK9 substrate (Sansó et al., 2016). Indeed, they found that the inhibition of CDK9 does not reduce CTD-Ser2 phosphorylation level but impairs recruitment and activation of XRN2 thus causing termination defects (Sansó et al., 2016). The identification and study of CDK-9 substrates in *C. elegans* should allow to better understand CDK-9 importance in important biological processes.

3. Is CDK-9 dependent Pol II CDT-Ser2 phosphorylation tissue specific?

Previous work in our lab showed that CDK-12as inhibition with low concentration (1 μM) causes a severe decrease of CTD-Ser2 phosphorylation in L1 worms. Higher concentration (5 μM and 10 μM) decreases CTD-Ser2P to similar level that low concentration but induces the Emo phenotype. This may indicate that another CDK-12 substrate could be important at these developmental stages and / or that higher doses of inhibitor are required to inhibit CDK-12as at these stages, maybe because the diffusion of the inhibitor is less efficient as worms grow up. In any cases, high CDK-12 activity is likely required to resume development from L1 arrested worms as 1 μM is sufficient to block it.

We do not currently possess molecular data ruling the question if CDK-12 and CDK-9 phosphorylation are tissue specific. In 2010, Deal and Hennikof proposed the INTACT (Isolation of Nuclei Tagged in Specific Cell Types) method which uses affinity purification of tagged nuclei in specific cell types (Deal & Hennikof, 2010). The method was adapted to specifically tag and purify *C. elegans* muscle nucleus (Steiner et al., 2012). Essentially, the nuclear pore complex NPP-9 is fused to a tagging cassette containing BLRP (Binding Ligase Recognition Peptide) and placed under the muscle specific promoter *myo-3*. The BLRP is a substrate of BirA (*E.coli* biotin ligase) that can be expressed under the control of the *his-72* promoter. Co-expressing BirA with the NPP-9 tagged protein allows in vivo biotinylation and thus, upon total nuclear extraction, the purification of muscle specific nucleus (Steiner et al., 2012). Adapting this method to extract germline nuclei, by placing the NPP-9 fused construct under a germline specific promoter, would allow us to decipher the potential CDK-9 and CDK-12 CTD-Ser2 phosphorylation tissue specificity.

4. Why RNAi and as-inhibition do not agree?

The difference in CTD-Ser2P observed in between RNAi experiments (Shim et al., 2002) and CDK-9as inhibition could suggest that the action of CDK-9 on CTD-Ser2P is not direct but passes through one or more partners. Indeed, when inhibiting a CDK-9as the protein interactions are conserved. However, when RNAi knockdown is performed, there is a decrease in the protein quantity present in cells. This could mean that CDK-9 interacts with a CTD kinase whose activity is regulated by CDK-9 dependent phosphorylation. When CDK-9as is inhibited, CDK-9 regulation is alleviated and the interacting CTD kinase over phosphorylates CTD-Ser2P. However, when RNAi knock down of *cdk-9* mRNA is performed, the CTD kinase is not carried to the polymerase, thanks to loss of CDK-9 interaction. As such, it cannot phosphorylate the CTD-Ser2 thus causing a decrease in the phosphorylation level.

There is currently no published study reporting the interaction of CDK-9 and another kinase in any other organisms. The development of a specific CDK-9 antibody would allow the purification of CDK-9 and its interactants by co-immunoprecipitation. Using mass spectrometry, CDK-9 interactome could be determined which would allow a better understand its function in the transcription cycle.

5. Is CDK-9as kinase activity down upon inhibition?

In this work, we did not detect a decrease in CTD-Ser2P upon CDK-9as inhibition. However, Blackwell and coworkers showed that RNAi against *cdk-9* induces a severe decrease of CTD-Ser2P (shim et al., 2002). As this contradicts our data, a reasonable concern is that CDK-9as inhibition is partial. There is no doubt that the inhibitor is effective as a penetrant phenotype (embryonic lethality) is observed upon inhibition at higher dose that have no effect on the *wild type*. However, we currently have no specific marker of CDK-9 activity, as there are no available phospho-antibodies recognizing any CDK-9 target. It is now well established that released of Pol II from pausing to productive elongation requires SPT-5 C-terminal-region phosphorylation by CDK-9 in other organisms. Therefore, an antibody should be developed and used to quantify SPT-5 phosphorylation level upon CDK-9as inhibition.

To sum up, inhibiting CDK-9as at 10 μM inhibitor concentration partially recapitulates the embryonic lethality observed when CDK-9 is downregulated by RNAi without recapitulating a decreased in CTD-Ser2P, somehow uncoupling these two phenotypes.

V. Conclusion

In this memory thesis, we investigated the function of CDK-9 in the development of the model organism *Caenorhabditis elegans*. Using the recent CRISPR-Cas9 approach we successfully constructed and confirmed a *C. elegans* strain having a conditional, analogue-sensitive endogenous CDK-9. We showed that inhibiting CDK-9as does not perfectly phenocopies RNAi against *cdk-9* but rather leads to dose-dependent phenotypes of sterility and embryonic lethality. We then investigated the contribution of CDK-9 to the phosphorylation of CTD-Ser2. We did not detect a drop of CTD-Ser2P by western blot nor immunoblotting, which suggests that CDK-9 may not be a genuine *in vivo* CTD-Ser2 kinase. We constructed and confirmed a *C. elegans* strain containing both the *cdk-9as* and *cdk-12as* alleles and showed a further decrease of CTD-Ser2P in this strain. A drop of CTD-Ser2P similar to the sole inhibition of CDK-12as was observed but at high concentration a more severe drop was observed in the double mutant strain. We postulated that the CTD kinase activity of CDK-9 and CDK-12 is partially redundant. When CDK-9as is inhibited, CDK-12 alone can compensate for the loss in CTD-Ser2 phosphorylation.

In short, this work showed that CDK-9 kinase activity is required for proper embryogenesis but that CDK-9 itself is dispensable to maintain physiological level of CTD-Ser2 phosphorylation.

VI. Supplementary data

Table S1: Sequences used in this study

cdk-9as crRNA 1 (	Forward (5' → 3'):AATACAGGCCGAATGGTTGC Reverse (5' → 3'):AATTTCGGGCGGAAATCTGG
cdk-9as sequencing	TCGGCAAGTTTGAGTATTCC
T7	Forward & Reverse (5' → 3'): TAATACGACTCACTATAGGG
cdk-9as crRNA	TGCAACTTTCTACTTGGTAA
cdk-9as ssODN	attcaggtactggaacgacgggatccaaggatcgTGCtACaTTtTACcTGGTcGGTG ctctttgtgcacacgatttggctggtctcttatca
dpy-10 crRNA	GCUACCAUAGGCACCACGAG
dpy-10 ssODN	CACTTGAACTTCAATACGGCAAGATGAGAATGACTGGAAACCGTACCG CA

VII. Material & Methods

1. *Caenorhabditis elegans* growth conditions

All the strains of *C. elegans* used in this study are derived from the Bristol N2 strain. Worms were maintained at 20°C on Nematode Growth Media (NGM) agar plates as described in wormbook.

2. Microinjection

Micro-injection is performed as follow. We first transfer a young adult from a culture plate to an agarose pad, previously covered in halocarbon oil, using an eyelash mounted on a tip.

Note on the agarose pad: We found that agarose 2% works the best, 1% not being sticky enough.

Note on choosing the worm: Using adult worms that are not too young nor too old is a crucial part of the process. Indeed, injection of L4 worms will fail since the gonad has not switched from a sperm producing gonad to an oocyte producing gonad. In the contrary, old worms are harder to inject because their gonad is filled with oocytes. Furthermore, since those worms might have already laid lots of eggs, the progeny size potentially having the desired mutation is much smaller. We found that injecting young adults containing a single row of embryos (about 10 embryos) works the best. If less embryos are present in the tract, the gonad is collapsed on itself and harder to see and properly inject.

Once the desired worm is picked, it is attached to the pad by gently pushing it from tail to head. After immobilization, we place the worm on a microscope stage and the loaded needle (Eppendord, FemtoTip2) is mounted onto the micromanipulator (Eppendorf, FemtoJet2). After immersion in the oil, the needle is placed close to the worm and the microscope is set to 40X magnification. The needle's opening is checked by applying pressure from the peristaltic pump outside the worm.

Note on opening the needle: If enough liquid is spilled out, the actual injection is performed. If no liquid is ejected out of the needle, it is either clogged or not open enough. A possible solution is to break the needle tip open. To do so, we place a broken cover slip on a glass slide with a drop of oil. The slide is put under the microscope and bring in focus. Using the micromanipulator, the needle tip is placed at the same level than the broken cover slip. The needle is gently tap against the cover slip. This should be enough to break the tip. Once the tip is sufficiently open, proceed to injection.

To actually inject the worm, it is first placed close to the needle. Then, the needle is carefully driven into the syncytial space of the gonad and flow pressure is applied. Once the injection is done, the needle is quickly but gently removed from the worm's body and the procedure is repeated to inject the second gonad arm. A good sign of proper injection is a flow around the bend of the gonad. If scalloping is seen at the site of injection, it means the body cavity was injected. Following injection, the worm is transferred onto a fresh NGM plate and washed in recovery buffer.

3. Pmyo-2::GFP and Pmyo-3::GFP mix preparation for microinjection

The preparation of the mix is a straight forward process: 1 µL of *Pmyo-2::GFP* plasmid (20 ng/µL, pDH665) is mixed with 1 µL of *Pmyo-3::GFP* plasmid (20 ng/µL, pDH666) and 3 µL of water are added. The mix is vortexed and loaded on a needle (Eppendorf Femtotips II).

4. CRISPR mix preparation for microinjection

Preparation of the injection mix is a two step process. During the pre-incubation step, tracrRNA (resuspended to 360 µM in TRIS pH 7.5), *dpy-10* crRNA and target gene crRNA (both resuspended to 200 µM in 5 mM TRIS pH 7.5) are mixed together. Water is added for a final concentration of respectively 44 µM, 8 µM, 36 µM and 1X. The mix is incubated for 5 min at 95°C and then cooled down to room temperature. After incubation, *dpy-10* ssODN (resuspended to 16 µM in water), target gene ssODN (resuspended to 2 µg/µL) and Cas9 protein (25 µg/µL) are added to the mix. Water is added for a final concentration of respectively 0.5 µM, 6 µM and 1.67 µg/µL. The prepared mix is spun at 15800g for 5 minutes and the supernatant is loaded on a needle (Eppendorf Femtotips II). This method is adapted from (Paix et al., 2015).

5. Genomic DNA Isolation

Lysis is performed on a single worm. Worms are dipped into 10 µL of colorless goTAQ reaction buffer 1X (Promega Corporation, M7921) supplemented with 0.1 µL of proteinase K (20 mg/mL) (Roche Diagnostics, 03 115 828 001). After 4 snap freezing cycles, worms in their lysis buffer are incubated 60 min at 65°C followed by 10 min at 94°C in order to inactivate proteinase K.

6. Single Worm PCR

After Genomic DNA isolation, amplification of *cdk-9* fragments was done by PCR using 2 µL of primers (10 µM), 5 µL of Green buffer 5X, 0.25 µL of dNTP (20 mM), 0.125 µL GoTAQ polymerase (GoTaq® G2, Promega Corporation M7841) and 12.7 µL of H2O. Specific annealing temperature and extension time will be specified for each PCR. Primers were designed using ApE.

7. cdk-9 Restriction Assay

Detection of the point mutation introduced in *cdk-9as* is diagnosed by restricting *cdk-9as* amplicon with the restriction enzyme MvaI (ThermoFisher, ER0551). The mix containing 5 µL of Amplicon, 2 µL of MvaI Buffer 5x, 1 µL of MvaI and 12 µL of water is incubated for 60 min at 37°C. After incubation, a gel electrophoresis is performed on the whole mix as a diagnostic tool.

8. Brood size analysis

Five worms of each genotype were transferred to individual plates at the L4 stage and moved 24H later to a new petri dish. Two days after the adult had been removed from a plate, the F1 progeny was counted on both plates. The brood size of a given hermaphrodite was therefore the addition of the two counted plates. The statistical comparison between *wild type* and mutant strains was determined using a one-tailed paired t-test.

9. DAPI staining

DAPI staining is performed on ethanol fixed specimens. First, the worms are washed in M9 and resuspended in cold EtOH. Worms are left at least 10 minutes on ice then washed three times with PBS. Worms are pelleted and mounted on slide with Fluoroshield + DAPI(Sigma F6057).

10. Drug assay – liquid media

The mix is composed of 2 µL of 1-(1,1-dimethylethyl)-3-[(3-methylphenyl)methyl]-1H-pyrazolo[3,4-d]pyrimidin-4-amine (3MB-PP1), diluted in DMSO at either 0 µM (control), 1 µM, 10 µM or 20 µM, as well as 5 µL of concentrated *E.coli* (strain HB101) and 993 µL of S minimal media (0.1M NaCl, 50mM KH2PO4 pH 6, 5 µg/mL cholesterol, 3 mM MgSO4, 4 mM CaCl2, 1 M Potassium citrate). After 5 days, each condition (3MB-PP1 concentrations) is visually assayed for phenotypes.

11. Drug assay – solid media

To make inhibitory plates, 3MB-PP1 is diluted to the desired concentration into standard NGM mix. The inhibitor is previously diluted in DMSO so that, for every condition, 1 µL is added to 1 mL of NGM. The mix is then poured (4.5 mL) into small plates (35 mm diameter). The plate is let to dry for at least a night and is then seeded with *E. coli* (strain OP50). Once the bacteria have grown, the plate is ready to use. Plates are kept for 1 to 2 weeks.

12. In vitro transcription & RNAi

KOD PCR (Novagen 71086) using T7 primer is used to amplify the insert from a clone in a double T7 feeding vector. The reaction is performed in 50 µL with standard Novagen protocol using 10 ng of DNA. The PCR reaction should yield about 200 ng/ µL. In Vitro transcription is performed following theT7 RiboMAX™ Express Large Scale RNA Production System (Promega 1300). In vitro transcription is performed with unpurified PCR product. Once done, the transcription product is diluted 4x with ultra-pure water (Sigma, 900682-1L). This protocol follows procedure described on wormbook. The transcription reaction is loaded on a needle (Eppendorf Femtotips II). Injected worms are singled out on an NGM plate. The first 24 hours progeny is discarded and the remaining progeny phenotype is visually assessed.

13. Quantifying embryonic lethality & sterility

5 L4 worms are singled out on an inhibitory plate and allowed to grow and lay eggs for 24 hours after what they are transferred to another plate. 3 days later, the brood size (all visible embryos and larvae) are counted. Embryos that had not hatched 24 hours later are considered dead. The statistical comparison between an inhibitory condition and the DMSO condition was determined using a one-tailed paired t-test.

14. Protein isolation and Western Blot

Worms (+/- 100 000 L1 or +/- 500 young adults by condition) inhibited for 30 minutes with the desired 3MB-PP1 concentration were collected, washed once in M9 and frozen. Thawed worms were resuspended in 4x Laemmli Buffer (200mM Tris pH8.5, 8% SDS, 40% glycerol) then boiled for 5 minutes before being thoroughly vortexed and sonicated for 10 cycles (30 sec ON, 30sec OFF) on a Diagenode Bioruptor Sonicator. Protein concentration were analyzed by Pierce (ThermoFisher 22660). Equal amounts of protein were run on Protean Biorad TGX gel 4-15% at 150V for 45 minutes. Proteins were transferred to a nitrocellulose membrane using the biorad Trans-blot Turbo transfer system, set up for standard molecular weight protein (1.0A, up to 25V). The membrane was then blocked with milk (70166–500G Sigma) for at least one hour. Primary antibodies were incubated overnight at 4°C without shaking: anti-rat-CTD-S2P 3E10 1/1000 (04-1571 Merck Millipore), anti-mouse-AMA-1 1/1000 (38520002 Novus Biologicals) and anti-mouse-Tubuline 1/2000 (T5168 Sigma). The membrane was washed 3 times in PBS-T then incubated for 1hr with secondary antibodies 1/2000. Membranes were revealed by Western Lightning Plus-ECL (BioRad, 1705061).

15. Phosphatase treatment

The phosphatase treatment is performed using a Lambda protein Phosphatase (New England BioLabs, P0753S). on total protein extracts of N2 worms. The mix containing 10 µg of proteins, 5 µL of 10X NEBuffer for protein Metallo Phosphatases, 5 µL of 10 mM $MnCl_2$, 1 µL of Lambada Protein Phosphatase and water for a total volume of 50 µL. The mix is incubated for 30 minutes at 37°C and directly loaded on gel for western blotting.

16. Immunostaining

Immunofluorescence analyses on methanol/formaldehyde-fixed specimens was performed as follow. The desired worms were put directly on the poly-lysine coated slides and frozen in M9+NaN3 between the slide and a coverslip. For embryo immunostaining, the adult worms are cut in half using a 22G needle. The slides are left at -80°C for the weekend then freeze-cracked and immediately drenched for 20 minutes in MeOH. Then, 100µL formaldehyde 3.7% (Hepes pH6.9 0.08 M, MgSO4 1.6 mM, EGTA 0.8 mM, formaldehyde 3.7%, in PBS 1X) is added onto the slides and incubated at room temperature for 30 minutes. The slides are then washed 2 times in PBS and once in PBS-T (PBS 1X, Triton 0.05%). The primary antibodies are incubated overnight in humid chambers. The antibody used for L1 immunostaining: anti-CTD-S2P 3E10 1/1000 (04-1571 Merck Millipore) and anti-AMA-1 1/2000 (38520002 Novus Biologicals). The antibody used for embryo immunostaining: anti-CTD-S2P 3E10 1/1000 (04-1571 Merck Millipore) and anti-AMA-1 1/2000 (ab150113 abcam). The slides are then washed three times with PBS-T and incubated with secondary antibody for 1 hour at room temperature, in the dark. The secondary antibodies used for the L1 staining: anti-rat Alexa488 1/2000 (ab150157) and anti-rabbit Alexa594 1/2000 (ab150080). The secondary antibodies used for the embryo staining: anti-rat Alexa488 1/2000 (ab150157) and anti-mouse Alexa594 1/2000 (ab150116). The slides are then washed twice in PBS-T, once in PBS and quickly rinced with water before mounting using Fluoroshield + DAPI (Sigma F6057).

17. Imaging

Slides were examined on a Zeiss Axio Imager Z1 microscope equipped with an Hamamatsu Digital Camera Orca Flash 2.8 C11440 using the Zeiss imaging software Zen. The exposure parameters were as follow for all pictures: Alexa488 exposure 2s; Alexa594 exposure 0.2s and DAPI exposure 5ms.

VIII. References

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