Regulation of the self-renewal vs. differentiation decision in the C.elegans germline

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Ich erkläre, dass ich die Dissertation „Regulation of the self–renewal vs. differentiation decision in the *C.elegans* germline“ nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Universität und keiner anderen Fakultät der Universität Basel eingereicht habe.

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Table of Content

Summary

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Adult stem cells are the basis of our reproductive potential, of tissue growth and maintenance, and of wound healing and regeneration. Ageing phenotypes such as hair loss or brittle bones in fact reflect the gradual decline of the proliferative capacities of adult stem cells with time. On the other hand, stem cells that proliferate too fast provoque diseases such as cancer. Understanding the molecular players regulating the decision of stem cells to divide or differentiate is therefore of prime importance to treat conditions caused by stem cell dysfunction. Many pathways that regulate the maintenance and differentiation of stem cells, as well as features distinguishing stem cells from their more committed progeny, are known today, among them the conserved Notch signaling pathway. How these molecules instruct stem cell identity is however largely unknown.

C.elegans germline stem cells, which are maintained by Notch signaling, constitute an excellent model to study the regulation of stem cell identity. *C.elegans* is amenable to genetic manipulation, to gene knockdown by RNAi, and to transgenesis. Additionally, the *C.elegans* germline can be readily monitored in live animals through the transparent cuticle as well as dissected for gene expression studies or immunofluorescence.

Here, we use these tools to identify genes that respond to Notch signaling in germline stem cells. We find that many Notch responsive genes reside on the X–chromosome. In the following, we uncover a crosstalk between Notch signaling and the *C.elegans* Polycomb proteins MES–2,–3,–4, and –6, which are known to repress X–linked genes in the germline.

Additionally, we identify a role of the CIP/KIP cell cycle inhibitor CKI–2 in the self–renewal versus differentiation decision of *C.elegans* germline stem cells. We find that CKI–2 is a key target of two conserved stem cell regulators, the RNA binding proteins FBF/Pumilio and MEX–3. Particularly FBF/Pumilio proteins have been implied in stem cell regulation in many organisms, and in *C.elegans* are required to maintain stem cells during adulthood. Key targets among the mRNAs targeted by FBF/Pumilio proteins have however remained elusive. We demonstrate that both MEX–3 and FBF/Pumilio proteins associate with the *cki-2* 3'UTR, that FBF/Pumilio binding elements are required for 3'UTR mediated regulation *in vivo*, and that regulation of CKI–2 by FBF/Pumilio contributes to the maintenance of germline stem cells.

Introduction

1. Why are stem cells fascinating?

1.1 Hunting the origins

Humans have always been curious about origins. *What was the origin of the universe? How was our planet formed? And how come there is life, how come we are here?* Every time and culture found their own answers and passed them on in their myths and religious beliefs. Modern mankind, despite an increasing unwillingness to believe in tranditional explanations, still faces the same questions. This is why scientific news that deal with our history such as the birth of a solar system millions of lightyears away are as fascinating to the general reader as the discovery of a human ancestor or the extraction of mammoth genetic material. On the personal level, we similarly set high value in knowing our roots. This seems essential even more in today's mobile and globally connected society. We define ourselves (and others) by looking at ethnic and social background. Knowing our personal origins is an existential need, demonstrated by the desire of adopted kids to face their genetic parents to whom they have no personal connection at all. On an even smaller scale, we chase our personal genesis. We try to understand who we are by looking back at childhood experiences, nourishing armadas of therapists on the way. We trace times we cannot remember with the help of stories and pictures, and ultimately end up at the earliest of events, our personal stem: *Really, "me" all began with two cells?*

The desire to trace ourselves as individuals, as communities, to trace the existence of life and everything around us may reflect our desire to understand existence. If we know where we come from, how we were made, we know why we are here... don't we?

1.2 Chasing immortality

Another obsession of humanity that plays into stem cell research, equally strong and existential as our drive to unearth our roots, is concerned with death/finiteness and its pretty sister eternity. The concept of an absolute and irreversible end exceeds human imagination. Just like for origins, virtually every human culture has developed myths about continuity after death. To bury or conserve the dead and supply them with grave

goods, a habit that implies belief in some kind of afterlife, is generally considered a transition to a higher cultural level in the history of mankind. Virtually all religions are based on the concept of infiniteness, be it resurrection, passage of the dead into another world, or reincarnation.

The pursuit of eternal life, however, is not exclusively religious. In fact, science as we know it today is a side product of the efforts of medieval alchemists to synthesize the Philosopher's Stone, which would not only turn worthless materials into noble metal, but also liquids into potions of strong healing powers and rejuvenating power. The desire for an elixir of life that confers immortality recurs in myths as greek Ambrosia and Hindu Amrit, the drinks of the immortal gods. Obviously, the Elixier of Life, although feverishly sought after, remained elusive. However, it did not escape human cognition that some organisms possess regenerative capabilities that approximate immortality. Being chopped in half, a deadly assault for the pride of creation, primitive earthworms would not only survive but double. Again, mythology reflects mankind's fascination with regeneration. Hydra regrows not only one, but two heads for every one scythed by Herakles' sword, and Prometheus' liver feeds the eagle day after day.

Today's research on regeneration and stem cells is just another version of the hunt for the Elixir of life. Why can't we, like Axolotl, regrow entire body parts upon amputation, when we are comparatively similar in body morphology and genetic makeup? And if we knew – could we eventually defeat death? The molecular and mechanistic basis of stem cell capacities are intensely investigated today. However, the picture is far from complete, and common themes are only starting to emerge. Stem cells retain their mystical, mythical and magical image, an image reflected in the naming of the stem cell factor Nanog after Tir Na Nog, the land of eternal youth in celtic mythology (MacKillop 2004).

These two metaphorical implications of stem cell research – our desire to understand origins on one hand and our desire to defeat mortality on the other hand – fuel stem cell research to this hour. They contribute to researchers' motivation, they stimulate funding sources to invest in stem cell research, and they fascinate the broader public. Stem cell research promises benefits that exceed the mere satisfaction of knowledge

acquisition, since stem cell based applications promise relief from our existential fears of losing beauty, youth, friends, and ourselves.

2. What are stem cells?

2.1 Defining “Stem Cell”

What do we mean when we call something a stem cell? Semantically, the term “stem cell” has a hierarchical connotation and implies linear succession. Today’s functional definitions are however much more narrow. “Stemcellness” entails, in the classical view, proliferative capacity, selfrenewal, clonality, and potency. Stem cell systems conform to these criteria to varying extents. Sensu strictu, although the most potent cells imaginable, the earliest cells of an embryo are NOT self renewing, since they cannot balance the decision to self renew or differentiate. Yet, *in vitro*, the very same cells (embryonic stem cells, ESCs) have the potential to self–renew indefinitely. What is called “stem cell” thus depends on the context, and a definition based on functionality seems most appropriate. According to this criterion, a stem cell – no matter whether embryonic stem cell, progenitor cell, founder cell, precursor cell or transit amplifying cell – has the ability to reconstitute, in dependence of its potency, all or parts of a given tissue. Early blastomeres, the *in vivo* equivalent to ESCs, are the most potent cells in an organism. These cells will not only contribute to all tissues of the next generation, but will also produce extraembryonic tissues. Embryonic Cancer Cells (ECCs), which are derived from germ cell cancers (teratomas), compare to ESCs in potency. ECCs differentiate into all three germlayers and were used heavily in the early days of in vitro stem cell research because they are easy to handle and meet no ethical objections,. Since the advent of ESCs and induced pluripotent cell lines (iPSCs), ECCs have however mostly disappeared from the labs. Truly totipotent *in vivo*, though less well amenable to research, are germ cells (GCs). They will give rise to all tissues of the next generation, and thus are the only totipotent cell population in adult organisms. Otherwise, stem cell identity in the adult is restricted to adult stem cells (ASCs). Whether ASCs never completely lost potential during development, or regained potency at a later stage, is not known. ASCs maintain and repair tissues, and therefore ASC failure results in impaired wound healing and aging, while uncontrolled ASC

proliferation has been proposed as a cause of cancer. Finally, cancer stem cells (CSCs), the mere existence of which is still debated, have achieved dubious fame. CSCs may not only seed tumors, but may also display particular resistance to therapy. Relapse after treatment is therefore blamed on this poorly defined cell population (Visvader 2011). Today, we know how to artificially induce stem cell behaviour in terminally differentiated cells to produce induced pluripotent cells (iPSCs) (reviewed by Stadtfeld and Hochedlinger 2010). Such reprogramming experiments will be crucial for understanding how cell fate decisions are regulated. However, iPSCs do acquire new traits such as mutations during reprogramming and also do not entirely eradicate their previous identity (Pera 2011), challenging their applicability in the clinic.

2.2 Evolutionary origins of stem cells

Stem cell research on a variety of models underlined their diversity rather than unifying principles in the regulation of stemcellness. One possible approach towards identifying core stem cell regulators and properties is therefore to look at the evolution of stem cells and at the requirements of stem cells in evolutionarily ancient organisms.

Tissue specification implies the existence of a progenitor cell during development. In an evolutionary sense, stem cells therefore emerged concomitantly with the appearance of specialized tissues (in the most simple scenario, soma and germline). The most prominent example for early multicellularity involving progenitor cells is found in subspecies of the green algae Volvox. Volvox are considered the most ancient truly multicellular organism by the criteria of synergism / division of labor, dispensability of individual cells, and reproductive altruism.[1] In Volvox, a gene also found in unicellular algae (*regA*) was adapted for a multicellular lifestyle. *regA*, which

[1] Although prokaryotic Myxobacteria and eukaryotic slime molds (such as Dictyostelium) are capable of impressive and complex multicellular behaviour, including functional specialisation of cells and reproductive altruism (reviewed in Gross, J. D. (1994). "Developmental decisions in Dictyostelium discoideum." Microbiol Rev **58**(3): 330–51. and in Shimkets, L. J. (1990). "Social and developmental biology of the myxobacteria." Microbiol Rev **54**(4): 473–501), their take on multicellularity neither involves kinship of cells (clonality) nor progressive restriction of developmental potential along a cell lineage, and therefore represents a sophisticated form of colonialism.

in a unicellular context directs temporal alternation of reproductive and vegetative lifestyle, directs reproductive and vegetative lifestyle in a spatial context in Volvox (reviewed in Michod 2007). *RegA* promotes a non–dividing, flagellated cell fate (terminal differentiation). Cells which do not express *regA* grow in size beyond the threshold required for cell divisions and thus assume reproductive fate. Reproductive cells of Volvox give rise to both types of cells in the next generation and can therefore be considered developmental stem cells.

Animal multicellularity supposedly arose through a similar colonialisation–driven process. The development of cell–cell–communication and nutrient exchange among unicellular flagellated choanocytes likely enabled the development of cells specialized in reproduction and / or proliferation versus feeding (Nielsen 2008). This idea is supported by the build of sponges, which by several criteria (sponges dispose of very few cell types, do not obviously gastrulate (although this is a matter of debate: (Muller 2006) and (Nielsen 2008)), and lack true epithelia connected by tight cell junctions as well as true and clustered HOX genes) reside at the root of the metazoan tree (Halanych 2004; Muller 2006) and resemble a permanent choanoflagellate colony. How sponges maintain stem cells may therefore tell about universal and ancient stem cell requirements. Sponge blast cells and adult stem cells, so called archaeocytes, can be identified based on the expression of genes identified as stem cell markers in higher organisms, such as the Polycomb group gene *EED* (Muller 2006), *MsCP1*, *Noggin*, and *glia maturation factor* (Muller, Korzhev et al. 2003), indicating that the molecular machinery promoting stemness is both evolutionarily ancient and highly conserved. Also, sponge stem cells depend on interactions with surrounding tissue for their proliferative capacity and immortality; as single cells, they arrest proliferation and lose telomerase expression (Koziol, Borojevic et al. 1998). Organismal control of the proliferative capacities of the stem cell pool through niche–like interactions is therefore an ancient concept.

Interestingly, in the organism Trichoplax, an organism that competes with sponges for the most basal position in the metazoan phylum, the ParaHox gene *Trox-2* has been ascribed a function in tissue maintenance (Jakob, Sagasser et al. 2004). In Cnidarians and Bilateria, Hox genes control the patterning of the body axis; duplications and differential regulation of Hox genes are considered a major driving force of macroevolutionary innovation. Trichoplax is, however, a non–axial organism

composed of an upper, ciliated epithelium and a lower, digestive epithelium. *Trox-2* is expressed in a ring of cells around the junction of the two epithelia and is required for continuous growth and fission of the organism (Jakob, Sagasser et al. 2004). Hox genes might therefore represent another class of ancient genes originally involved not only in patterning, but also stem cell biology.

Next to sponges and Placozoa, Ctenophores are considered closest to a common ancestor of all metazoa, and might therefore be useful to determine the origin of metazoan stem cells and universal stem cell properties (Halanych 2004; Dunn, Hejnol et al. 2008). Ctenophores are, although still diploblastic, much more complex in body morphology than sponges and dispose of several highly specialized cell types. Expression analyses of the stem cell marker genes of the SOX family have identified several cell populations in Ctenophores, among them a group of cells at the base of the tentacle which is continuously supplying cells to that organ (Jager, Queinnec et al. 2008). These cells therefore represent the most ancient adult stem cell population identified to date which is dedicated to maintain homeostasis in a specialized tissue.

A fascinating hypothesis on the evolutionary origin of adult stem cells is based on the observation that many primitive organisms, including sponges and ctenophores, reproduce through a larval stage (Arenas–Mena 2010). Evidently, organisms with biphasic development set aside plastic cells during larval stages to support metamorphosis into the adult animal. Although this practice is obsolete in uniphasically developing organisms such as vertebrates, the allocation of plastic cells according to the metamorphosis model has been retained during evolution. Our adult stem cell pools may thus originate from the metamorphic lifestyle of our sponge–like ancestors.

Molecules expressed in embryonic and adult stem cells in diverse organisms are often highly conserved, such as *Sox2* or the Puf protein family (Spassov and Jurecic 2003; Jager, Queinnec et al. 2008), supporting the idea that stemcellness may have universal traits, and that stem cells across organisms and tissues may have very similar requirements. It is therefore noteworthy that the prominent mammalian stem cell factor *Oct4* is an evolutionarily novel acquisition. Invertebrate genomes do not contain related sequences, and the related *pou2* gene of egg–laying vertebrates such as fish, xenopus or axolotl cannot substitute for *Oct4* function in mouse ES cells. The first

gene resembling *Oct4*, *Pou5f1*, appears in the order of monotremes along with the first appearance of rudimentary placental structures. *Oct4*, although known to us as paradigm pluripotency factor, is therefore a byproduct of placenta evolution (Niwa, Sekita et al. 2008).

2.3 The origins of stem cell research

A 21st century stem cell researcher ought to give credit to the giants whose shoulders she is standing on. The idea of dividing cells as smallest unit of growth and regeneration seems natural to a contemporary biologist, but in fact, this idea was novel and exciting only two hundred years ago. Lizard tail regeneration, which we today recognize as prime example of adult stem cell capacitiy readout, was already documented by Aristotle in his Historia Animalium (Aristotle, Balme et al. 2002). However, these observations got unearthed only during Renaissance. A seminal publication by the french natural scientist René–Antoine Ferchault de Réaumur (Reaumur 1712) re–initiated the field and was followed by multiple observations on the regenerative capabilities of diverse organisms. Around the same time, microscopes initially devised by Galileo and Janssen were improved by Huygens and Leeuwenhook, and scientists became more and more aware that tissues consisted of smaller units of some sort. However, it took more than a hundred years from the first observation of cellularity of cork by Hooke in 1665 until Dutrochet, Milne–Edwards and Raspail between 1824 and 1833 came to the conclusion that all animals and plants have a similar cellular structure. In 1839, Schwann finally stated once and for good that all organisms were constructed from cells. Only then could any debate on the origin of those cells initiate. Schwann himself, though today considered the father of the cell theory, was an adept of the exony concept which stated that cells would arise from matter outside of cells. Another idea at the time put forward by Mohl in 1837 was that organisms may be born with the full complement of cells which would over time enlarge and rearrange. Today we know that this is indeed the case for organisms such as *C.elegans*. Although at Mohr's lifetime, cell divisions had actually already been observed in algae and embryos, the issue was only resolved in 1858 to a point where Virchow could make is famous statement "*omnis cellula e cellula*" (Virchow 1859) – *every cell stems from another one*, a seminal realisation for stem cell research.

Research on adult stem cells as we know it today was fueled by the collision of the discovery of radioactivity late in the 19th century and global conflicts in the first half of the 20th century. During and after World War II, the protective effects of spleen or marrow cells against lethal doses of irradiation, based on their ability to reconstitute the damaged hematopoietic system, became apparent. Long term successful bone marrow transplants were however achieved only in the late 50ies (Humble and Newton 1958). Based on these transplantation experiments, hierarchies of blood precursor cells could be established (Till and Mc 1961), followed by lineage analysis of other accessible regenerating tissues, such as gut and epidermis (Withers and Elkind 1969). Finally, the discovery of totipotent cells in embryocarcinomas that could be propagated *in vitro*, ECCs (embryonic carcinoma cells), opened up experimental avenues that laid the foundation for today's definition of stemcellness in terms of potency: the ability to reconstitute all or parts of an organism. ESCs replaced ECCs in 1981 (Martin 1981). Over the years, culture conditions and factors required to keep nontransformed cells in culture and maintain their potency have been identified and applied inversely to induce stem cell identity in terminally differentiated cells. In parallel, genetically amenable model organisms such as *C.elegans* and *Drosophila* have facilitated the identification of molecular players maintaining stem cells *in vivo*, so that by today, approximately 26.000 Pub–Med indexed publications carry "stem cell" in the title. And yet, after a century of research efforts, we still lack universal laws concerning the requirements of stem cells.

3. How is stem cell identity maintained?

Certainly, we do know a lot more about stem cells today then when first bone marrow transplantation experiments were performed. The model organisms *Drosophila* and *C.elegans*, later joined by zebrafish, mouse and novel models such as sponges and planaria, allowed the characterisation of pathways required to maintain stem cell populations. In parallel, it has become clear that stem cells share particular features, such as open or bivalent chromatin conformation, a preference for low oxygen conditions, and a G1–S heavy cell cycle profile (Lako, Neganova et al. 2009; Edel and Belmonte 2010; Lange and Calegari 2010; Rehman 2010; Gaspar–Maia, Alajem et al. 2011). However, it is largely unknown how stem cell factors induce such stem cell

features. Forward approaches using the germlines of *C.elegans* and *Drosophila* have contributed tremendously to the identification of stem cell factors. As shortlived organisms – a few weeks at maximum – worms and flies are largely independent from somatic adult stem cells, with the exception of the *Drosophila* intestine (Ohlstein and Spradling 2006); both however contain powerful stem cell populations in their germlines. Compromised fertility is therefore a straightforward readout of germline stem cell defects in these organisms and facilitated large scale screening efforts.

Conserved **signaling pathways** are among the stem cell regulators identified in worms and flies. The first germ line proliferation defective mutants in *C.elegans* were identified by Austin and Kimble in 1987 (Austin and Kimble 1987). The affected transmembrane protein was later found to be orthologous to *Drosophila* Notch (Yochem and Greenwald 1989). In *Drosophila*, female GSCs depend on BMP signaling, and male GSCs on Jak–Stat signaling (Fuller and Spradling 2007). Other signaling pathways have been implied in vertebrate stem cell maintenance, such as Wnt signaling in the intestine (reviewed by Lowry and Richter 2007). Conserved **RNA regulatory proteins** (again originally identified in worms and flies) have also been implied in stem cell maintenance. Prominent members of this group include Nanos, Pumilio, and small RNA pathway components (Spassov and Jurecic 2003; Samji 2009; Saga 2010). Finally, cell culture experiments paint a picture of **transcriptional regulation** of pluripotency that involves the transcriptional regulators Oct4, Sox2, Myc, and Klf4 (Chambers and Tomlinson 2009; Pei 2009). It is intuitive that cells cultured *ex vivo* in a petri dish do not rely on cell–cell signaling to maintain stem cell properties. However, whether signaling pathways identified *in vivo* converge on the same genes or at least regulate the same properties as transcription factors identified *in vitro* remains to be determined.

For the sake of this work, the C.elegans germline and three stem cell factors that contribute to stem cell maintenance in the *C.elegans* germline will be introduced in more detail: Notch as an example of a signaling pathway, and MEX–3 and FBF–1 / FBF–2 as translational regulators involved in stem cell regulation.

3.1 Stem cell maintenance in the *C.elegans* germline

In stem cell research just like in other areas of research, model organisms have tremendously helped to outline basic concepts. Both *C.elegans* and *Drosophila* are limited in their somatic regeneration capacities. Only recently, somatic stem cells were identified in *Drosophila* (Ohlstein and Spradling 2006); *C.elegans* is entirely predetermined and cannot replace cells during adulthood at all. Other model organisms with remarkable somatic regenerative capabilities such as planaria or axolotl are much less amenable genetically and experimentally. Both worms and flies however dispose of extremely powerful germline stem cell pools. Stem cell compartments in both organisms display similarities to vertebrate adult stem cell compartments such as gut crypts, and rely on highly conserved molecules to maintain stem cell proliferation. For these reasons, *C.elegans* and *Drosophila* germlines have a longstanding tradition in adult stem cell research.

In *C.elegans*, stem cells are located in the distal ends of two gonad arms. Along the distal–to–proximal axis of each arm, germ cells develop in a linear, assembly–line fashion from germline stem cells to sperm (during juvenile stages) and oocytes (during adulthood; Fig. 1A). Oocytes then arrest in meiotic prophase I until fertilisation. Stem cell proliferation in the distal gonad is induced by the niche cell (Distal Tip Cell, DTC; Kimble and White 1981). The DTC caps the distal end of the gonad and is part of the somatic gonad tissue. Germ cells that move away from the DTC lose stem cell identity and initiate meiotic differentiation. The DTC extends filamentous processes along the mitotic zone and expresses the Notch ligand LAG–2 (Henderson, Gao et al. 1994; Hall, Winfrey et al. 1999). LAG–2 activates the Notch receptor GLP–1 expressed on distal germ cells (Crittenden, Troemel et al. 1994; Henderson, Gao et al. 1994). The signal is then transmitted through the nuclear cofactors LAG–1 and LAG–3 to promote a transcriptional profile that supports stem cell proliferation (Christensen, Kodoyianni et al. 1996; Petcherski and Kimble 2000). Stem cell proliferation and stem cell identity cannot be uncoupled at the level of Notch signaling. *Loss-of-function* mutations in the LAG–2 ligand, the GLP–1 receptor or the LAG–1 and LAG–3 nuclear cofactors cause cell cycle exit and terminal differentiation of all stem cells (Austin and Kimble 1987). *Gain-of-function* mutations in the GLP–1 receptor on the other hand induce stem cell tumors (Gustafsson, Zheng et al. 2005).

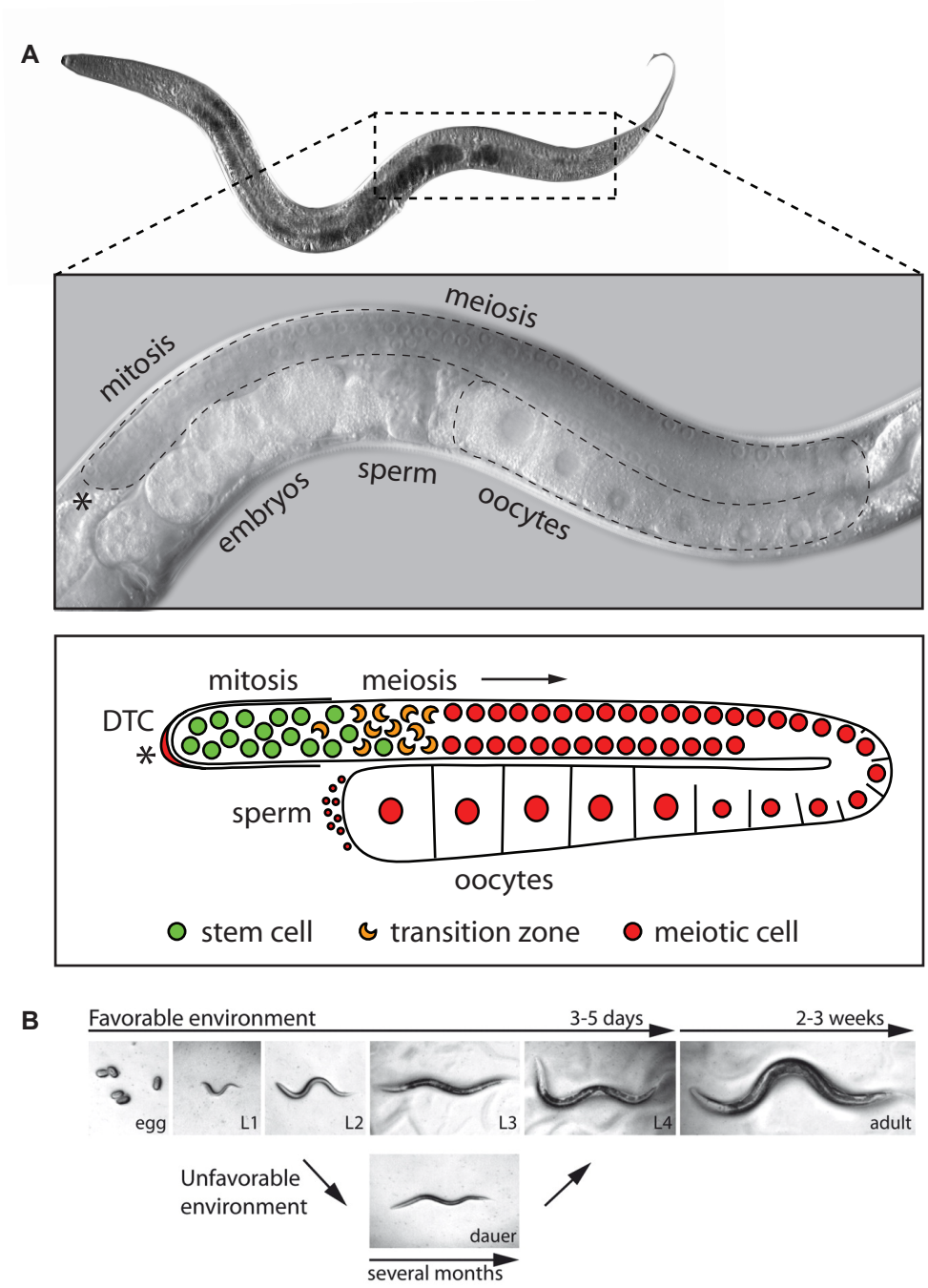


Figure 1. ***C.elegans* germline and development.**

(A) Live *C.elegans* hermaphrodite, approximate length: 1mm. One gonad arm is magnified and outlined below (top panel) as well as depicted schematically (bottom panel). Stem cells proliferate in the distal germline under the influence of Notch signaling from the distal tip cell (DTC). When germ cells lose contact with the DTC, they enter meiosis in the transition zone, characterized by crescent-shaped morphology in DAPI stainings. More proximmally, they differentiate as sperm (juvenile stages) or oocytes (during adulthood). The distal end of the gonad is marked by an asterisk. (B) Under favorable conditions, *C.elegans* development proceeds from embryo to adult through four larval stages. When larvae hatch in unfavorable conditions, for example in the absence of food, they enter the Dauer stage and survive without food for prolonged periods. When conditions improve, development resumes. Reproduced from Fielenbach and Antebi, *C. elegans dauer formation and the molecular basis of plasticity,* 2008.

It is unclear to date how Notch signaling promotes stem cell identity. Partly, it may excert its function through conserved RNA regulators of the Pumilio family, FBF–1 and FBF–2 (collectively referred to as FBF). These are expressed in the distal germline and reported to act downstream of Notch signaling (Lamont, Crittenden et al. 2004; Thompson, Bernstein et al. 2005). However, *fbf* mRNA expression does not strictly require Notch signaling – it is expressed in *gld-1 gld-2; glp-1* mutant germline tumors in which Notch is lacking (own observation; see Figure 22). Since FBF mutants lose stem cells only as adults, additional unidentified pathways are clearly operating in parallel to FBFs downstream of Notch signaling (Crittenden, Bernstein et al. 2002), during juvenile stages. MEX–3 and PUF–8, two other conserved RNA regulators, have also been implicated in germline stem cell proliferation and are redundantly required for juvenile germ cell proliferation. However, in their absence, stem cells do not terminally differentiate into mature gametes, but rather arrest in an undifferentiated state.

FBFs, MEX–3, and PUF–8 are RNA binding proteins and translationally repress mRNAs by attaching to the 3'UTR of their targets. The best characterized target of FBF is GLD–1, itself an RNA binding protein. FBF–mediated repression prevents GLD–1 expression in the stem cell compartment (Crittenden, Bernstein et al. 2002). More proximally, GLD–1 is upregulated and together with the poly(A)polymerase GLD–2 and its adaptors, GLD–3, GLD–4, and RNP–8 (Kadyk and Kimble 1998; Kim, Nykamp et al. 2009; Schmid, Kuchler et al. 2009; Kim, Wilson et al. 2010) promotes meiotic progression of germ cells (Francis, Barton et al. 1995). Recent years have seen large scale mRNA–coprecipitation attempts to identify bulk targets for several proteins governing the mitosis – meiosis decision, for FBF, GLD–1, GLD–2, RNP–8, and GLD–3 (Kershner and Kimble 2010; Kim, Wilson et al. 2010; Wright, Gaidatzis et al. 2010). Among hundreds of putative targets identified for each, the very factors mediating the stem cell maintenance versus meiotic entry decision have however remained elusive, with one exception: a combined large scale association – candidate approach in our lab has recently identified the first GLD–1 target involved directly in the self–renewal versus differentiation transition, CYE–1/Cyclin E (Biedermann, Wright et al. 2009). Among 948 putative GLD–1 targets (Wright, Gaidatzis et al. 2010), *cye-1* repression is crucial to maintain mitotic quiescence in the meiotic germline. For the first time, a master regulator of the mitosis – meiosis decision of *C.elegans* germline stem cells has

thus been directly and functionally linked to the executive machinery governing an individual cell's decision to self–renew or to maintain quiescent.

Germline stem cell proliferation also depends on somatic cues. Certainly, it is a good idea to adapt the number of progeny and the energy invested in germ cell production to nutrient availability. *C.elegans* during early development has the option to arrest development after hatching ("L1 diapause") or during the L2 larval stage ("Dauer"; Fig. 1B; Riddle and Albert 1997; Fukuyama, Rougvie et al. 2006; Fielenbach and Antebi 2008). These arrests are induced by starvation and, in the case of Dauer, also by heat stress or crowding. Dauer larvae survive for weeks without food and will resume normal development as soon as environmental conditions improve. During both L1 diapause and Dauer stage, germ cell proliferation ceases. Stem cell identity is however maintained, since germline development will resume after exit from the dauer stage just normally. During later stages, starvation elicits a similar program. Adult worms deprived of food will not only arrest germline stem cell proliferation, but use material from the germline as energy source until only a few distal germ cells remain. These are however capable of setting up a germline again when food is provided, and worms that have gone through "adult reproductive diapause" (ADR) produce an almost wild type complement of progeny (Angelo and Van Gilst 2009). During developmental arrests, germline stem cell proliferation and identity can thus be uncoupled. The communication of nutritional status from soma to germline requires the Insulin signaling pathway, but it is not clear to date whether Insulin signaling regulates germ line stem cell proliferation through the known complement of stem cell factors (Fukuyama, Rougvie et al. 2006; Narbonne and Roy 2006; Michaelson, Korta et al. 2010).

3.2 Notch signaling

Notch signaling is a highly conserved pathway mediating communication between neighbouring cells (Maine, Lissemore et al. 1995; Lissemore and Starmer 1999; reviewed by Bray 2006). It is required for proper development of most tissues (for a review of mouse models, see Hansson, Lendahl et al. 2004) and is particularly famous for regulating cell fate choices. During development, Notch signaling mediates both binary fate decisions, where one cell out of two or more with equivalent developmental

potential is singled out to adopt one of two alternative identities (examples: *Drosophila melanogaster* bristle development (Guo, Jan et al. 1996), cell fate specification in the *C.elegans* vulva (Greenwald, Sternberg et al. 1983)), as well as inductive events, where cell populations receive a fate–instructing cue from an unaffected signal source (examples: developmental boundary formation events (reviewed by Irvine 1999) or, of relevance to this work, *C.elegans* germline stem cell proliferation (Austin and Kimble 1987)).

As a universal regulator of cell fate choice, Notch signaling has naturally been implied in the maintenance and regulation of stem cells (reviewed by Chiba 2006). The most straightforward and historically prime example of Notch–dependent stem cell maintenance is Notch requirement and sufficiency in *C.elegans* germline stem cell identity (Austin and Kimble 1987; Berry, Westlund et al. 1997). Notch signaling has in the following received attention also in vertebrate stem cell contexts and was found to contribute to the maintenance of

- *CNS stem cells* (Nakamura, Sakakibara et al. 2000; Solecki, Liu et al. 2001; Gaiano and Fishell 2002; Hitoshi, Alexson et al. 2002; Hitoshi, Seaberg et al. 2004);
- *Intestinal stem cells* of the transit amplifying compartment, which require Notch signaling to prevent differentiation along the goblet cell lineage (van Es, van Gijn et al. 2005), an effect supported by gamma–secretase inhibitor trials (Milano, McKay et al. 2004; Wong, Manfra et al. 2004) and Notch overexpression experiments (Fre, Huyghe et al. 2005);
- *Melanocyte stem cells* (Moriyama, Osawa et al. 2006);
- *Pancreatic precursor cells* (Apelqvist, Li et al. 1999; Jensen, Heller et al. 2000; Murtaugh, Stanger et al. 2003);
- and *Skin/hair stem cells* (Yamamoto, Tanigaki et al. 2003);

Given its widespread requirement in cell fate regulation, it is not surprising that defects in Notch signaling contribute to diseases. Notch–related inherited conditions, such as Alagille syndrome (Artavanis–Tsakonas 1997; Li, Krantz et al. 1997; Oda, Elkahloun et al. 1997), tetralogy of fallot, syndactyly, spondylocostal dysostosis (reviewed by Gridley 2003), CADASIL (Joutel, Corpechot et al. 1996; Joutel, Andreux et al. 2000; Karlstrom, Beatus et al. 2002; Louvi, Arboleda–Velasquez et al. 2006) and

familial aortic valve disease (Garg, Muth et al. 2005) reflect the role of Notch signaling during development. In addition, Notch signaling has been implied in various cancers. Affected tissues reflect the panel of organs where Notch signaling has been implied in development and maintenance and include:

- *the hematopoietic system*, particularly T–cells; historically, human Notch was actually identified as the gene residing at the breakpoint in a subset of chromosomal translocations causing T–cell leukemias (Ellisen, Bird et al. 1991). Oncogenic activity of the resulting fusion proteins was confirmed using mouse models (O'Neil, Calvo et al. 2006), but in humans, rearrangements turned out to be rare. Notch overexpression or mutation however appears to be common in leukemia (Jundt, Anagnostopoulos et al. 2002; Bellavia, Campese et al. 2003; Chiaramonte, Calzavara et al. 2003; Weng, Ferrando et al. 2004).
- *the intestine*, where inhibition of Notch signaling promotes the terminal differentiation of adenoma cells along the secretory lineage (van Es, van Gijn et al. 2005);
- *the mammary gland*; mouse mammary tumor virus provoques cancer due to insertions in Notch receptor genes that cause the expression of truncated versions (Gallahan, Kozak et al. 1987; Uyttendaele, Marazzi et al. 1996; Dievart, Beaulieu et al. 1999). Evidence implying hyperactive Notch signaling in human breast cancer is however only correlative (Jhappan, Gallahan et al. 1992; Pece, Serresi et al. 2004; Stylianou, Clarke et al. 2006; Dickson, Mulligan et al. 2007; Reedijk, Pinnaduwage et al. 2008; Rizzo, Miao et al. 2008).
- *pancreas*; in pancreatic cancer, aberrant Notch signaling may be involved in the initiation stages and cooperate with TGF–beta signaling (Hingorani, Petricoin et al. 2003; Miyamoto, Maitra et al. 2003; Wang, Banerjee et al. 2006; Kimura, Satoh et al. 2007).
- *prostate* (Santagata, Demichelis et al. 2004)
- *skin/melanocytes* (Balint, Xiao et al. 2005; Nickoloff, Hendrix et al. 2005; Liu, Xiao et al. 2006);
- *lung* (Dang, Gazdar et al. 2000);
- *cervix* (Zagouras, Stifani et al. 1995);
- *brain*; Notch signaling promotes proliferatoin and invasiveness of glioma cells (Pierfelice, Schreck et al.; Shih and Holland 2006; Zhang, Zheng et al. 2008). In

the childhood tumor medulloblastoma, expression of the Notch target gene Hes1 equals poor prognosis, and Notch signaling is required for unlimited growth of medulloblastoma cells and tumor engraftment (Fan, Mikolaenko et al. 2004; Hallahan, Pritchard et al. 2004; Fan, Matsui et al. 2006).

How Notch signaling contributes to tumor development is not quite clear. In a more general manner, Notch signaling acts anti–apoptotic (Rangarajan, Syal et al. 2001; Nair, Somasundaram et al. 2003), pro–transformative (Wang, Banerjee et al. 2006), pro–invasion (Sarmento, Huang et al. 2005), pro–proliferative (Zeng, Li et al. 2005; Hellstrom, Phng et al. 2007; Siekmann and Lawson 2007), and pro–angiogenetic (Klinakis, Szabolcs et al. 2006; Palomero, Lim et al. 2006; Sharma, Calvo et al. 2006; Weng, Millholland et al. 2006; Efstratiadis, Szabolcs et al. 2007) and may thus contribute to tumor formation. Proposed tumor promoting–mechanisms also include crosstalk to known oncogenes such as myc (Mungamuri, Yang et al. 2006), mTOR/p53 (Miyamoto, Maitra et al. 2003), and TGF–beta (Nickoloff, Osborne et al. 2003). Naturally, cancer therapies targeting Notch signaling have been proposed (Noguera–Troise, Daly et al. 2006; Ridgway, Zhang et al. 2006; Wang, Zhang et al. 2006; Wang, Zhang et al. 2006). While various inhibition strategies aiming at diverse pathway components, such as ligand depletion, have been explored (Curry, Reed et al. 2005; Paris, Quadros et al. 2005; van Es, van Gijn et al. 2005; Rizzo, Miao et al. 2008), gamma secretase inhibitors (gamma secretase mediates cleavage of the Notch receptor upon ligand binding, see below) appear most relevant for clinical application (Nicolas, Wolfer et al. 2003). Since Notch signaling is required for proper tissue homeostasis, systemic administration of Notch inhibitors necessarily provokes adverse effects.

Given the very general requirement of Notch signaling in cell fate decisions and disease, it is of prime importance to understand how Notch signaling affects target genes, and how these genes in turn affect cellular identity. However, despite the seemingly simple pathway architecture distinguishing Notch signaling from other “ivy league” signaling pathways such as MAP kinase, BMP or Sonic hedgehog – the Notch signal is directly relayed from the plasma membrane to the nucleus without second messengers, since a fragment of the receptor itself acts as transcription factor – few Notch target genes have so far been identified. This is even more surprising in light of the fact that the name–giving phenotype – notched *Drosophila* wings (Fig. 2A) – was

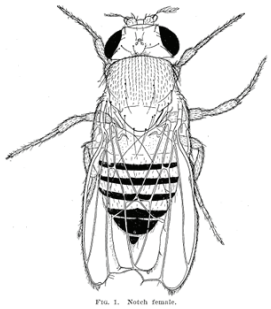
first observed almost a hundred years ago (Wharton, Johansen et al. 1985). In *C.elegans*, the Notch receptor was independently identified and cloned as vulval determinant, embryonic determinant, and germline stem cell factor (Fehon, Kooh et al. 1990; Rebay, Fleming et al. 1991; Henderson, Gao et al. 1994; Mello, Draper et al. 1994). Subsequent studies in *Drosophila* and *C.elegans* identified conserved core components such as ligands (Pan and Rubin 1997; Sotillos, Roch et al. 1997; Wen, Metzstein et al. 1997), receptor processing enzymes (Fortini and Artavanis–Tsakonas 1994; Christensen, Kodoyianni et al. 1996), the corepressor/coactivator CSL (Doyle, Wen et al. 2000; Petcherski and Kimble 2000) and the coactivator Mastermind (Fehon, Kooh et al. 1990; Rebay, Fleming et al. 1991; Henderson, Gao et al. 1994), and linked them mechanistically into a pathway (Figs. 2B and C).

The Notch signaling pathway is heavily regulated by endocytic processes and posttranslational modifications (Haines and Irvine 2003; Le Borgne, Bardin et al. 2005). However, following sections will focus on the relay of the signal from plasma membrane to target genes and the regulation of target gene expression, since these aspects are most relevant for this work.

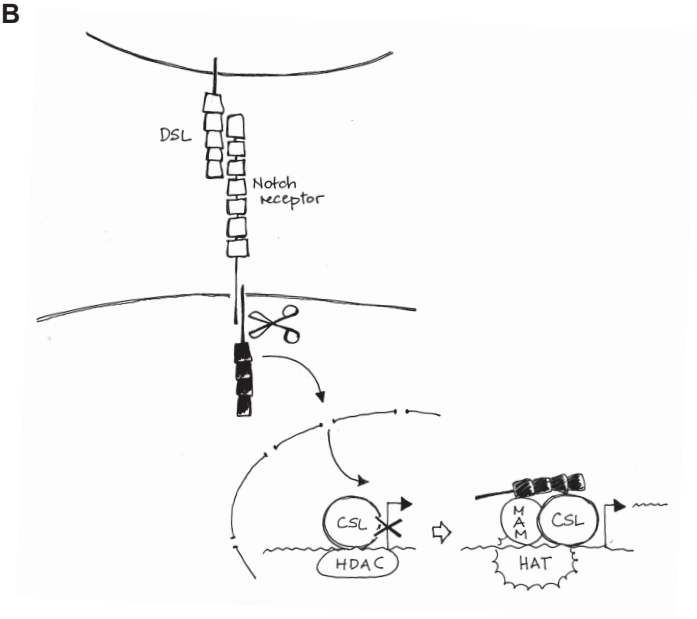
Both the Notch receptor and its ligands, the DSL (Delta–Serrate–LAG–2) proteins (Yochem and Greenwald 1989), are transmembrane proteins (Chen and Greenwald 2004; Komatsu, Chao et al. 2008), although secreted DSL variants (D'Souza, Miyamoto et al. 2008) as well as non–DSL ligands have been described (reviewed by Schroeter, Kisslinger et al. 1998; De Strooper, Annaert et al. 1999). Upon ligand binding, the intracellular portion of the receptor (NICD, Notch Intracellular Domain) is proteolytically cleaved off by gamma–secretases (Kidd, Lieber et al. 1998; Struhl and Adachi 1998) and translocates to the nucleus to affect transcription of target genes (Lai 2002).

In the absence of signal, Notch target genes are considered to be constitutively repressed by the DNA–binding protein CSL (named after CBF1/RBP–J, Su(H), and LAG–1; reviewed by Morel, Lecourtois et al. 2001). CSL recruits chromatin modifiers and establishes a repressive environment. In *Drosophila*, the adaptor Hairless tethers the repressors Groucho and dCtBP, which in turn recruit histone deacetylases (Kao, Ordentlich et al. 1998), to CSL (Barolo, Stone et al. 2002; Nagel, Krejci et al. 2005). Hairless appears to be unique to flies; in mammals, CSL recruits CtBP (Oswald, Winkler et al. 2005), HDAC1 (Kao, Ordentlich et al. 1998) and other corepressors such as

A



B



C

Component type	*Drosophila melanogaster*	Vertebrates and mammals	*Caenorhabditis elegans*
Receptor	Notch	Notch1–4	LIN-12, GLP-1
Ligand	Delta, Serrate	Delta1–4/A–D, Serrate, Jagged1–2	APX-1, LAG-2, ARG-1, DSL-1
CSL DNA-binding protein	Su(H)	CBF1/RBPkJ	LAG-1
Co-activator	Mastermind	Mastermind1–3	LAG-3
Co-repressor	Hairless, SMRTR*	SMRT	
γ-secretase complex	Presenilin, nicastrin, APH1, PEN2	Presenilin1–2, nicastrin, APH1, PEN2	SEL-12/presenilin, APH-2/nicastrin, APH-1, PEN-2
Glycosyl transferase	Fringe	Lunatic Fringe, Radical Fringe, Manic Fringe	
Metalloprotease, receptor cleavage	Kuzbanian, Tace CG7908*	ADAM10, TACE/ADAM17	SUP-17/Kuzbanian, ADM-4/TACE
Metalloprotease, receptor cleavage	Kuzbanian-like		
Ring finger E3 (ligand regulation)	Mind bomb 1	Mind bomb 1–2	
Ring finger E3 (ligand regulation)	Neuralized	Neuralized1–2	F10D7.5*
Ring finger E3 (receptor regulation)	Deltex	Deltex	
HECT domain E3 (receptor regulation)	Su(dx), NEDD4	Itch, NEDD4*	WWP-1
F-box E3 (nuclear)	Archipelago*	FBW7/SEL10	SEL-10
Numb, cytoplasmic Notch inhibitor	Numb	Numb, Numb-like	
Numb-associated kinase	Numb-associated kinase	AP2-associated kinase	SEL-5
4-pass transmembrane protein, positive regulator	Sanpodo		
Immunoglobulin C2-type cell-adhesion molecule	Echinoid		IGMC-1*
bHLH repressors, target genes	E(spl)bHLH	HES/ESR/HEY	REF-1
Neuralized E3 inhibitors	Bearded, Tom, M4		

*Protein homologues that have not yet been tested for roles in Notch signalling. Where no protein is listed, relatives have not been identified. AP2, adaptor protein-2; CSL, CBF1, Su(H) and LAG-1; E3, ubiquitin ligase; bHLH, basic-helix–loop–helix.

Figure 2. **Notch signaling.**

(A) Dorsal view of a *Drosophila* Notch receptor mutant female. The characteristic notched appearence of the wing margin gave receptor and pathway its name. Image reproduced from: T.H.Morgan, *The theory of the gene,* 1917. (B) Illustration of the core Notch signaling pathway. Upon ligand binding, the Notch receptor is proteolytically cleaved at the plasma membrane. The intracellular fragment translocates to the nucleus, associates with CSL bound at target genes and converts a repressive complex into a transcription-activating complex. DSL, Delta-Serrate-LAG-2; CSL, CBF1-Su(H)-LAG-1; HDAC, Histone deacetylase; HAT, Histone acetyltransferase; MAM, Mastermind. (C) Table illustrating the conservation of Notch pathway components between *Drosophila*, vertebrates, and *C.elegans.* Table reproduced from S.J.Bray, *Notch signalling: a simple pathway becomes complex,* 2006

MeCP2 (Stancheva, Collins et al. 2003) and KyoT2 (Taniguchi, Furukawa et al. 1998) via the transcriptional co–repressor SMRT (Oswald, Kostezka et al. 2002) and the SMRT–associated protein SHARP (Oswald, Kostezka et al. 2002; Oswald, Winkler et al. 2005). CSL also interacts with the transcriptional regulator SKIP and the co–repressor CIR (Hsieh, Zhou et al. 1999; Zhou, Fujimuro et al. 2000; Kasturi, Zanetti et al. 2010); CIR in turns recruits HDAC2 (Goodfellow, Krejci et al. 2007). The histone chaperone Asf1 is also recruited by CSL and has been implied in repression (Olave, Reinberg et al. 1998). In addition, silencing of Notch target genes may involve direct contact of CSL with TFIID and TFIIA, components of the basal transcription machinery (Jarriault, Brou et al. 1995; Tamura, Taniguchi et al. 1995), and the recruitment of POU homeodomain proteins (Neumann and Cohen 1998).

Upon ligand binding, the intracellular part of the receptor is released by gamma secretases and translocates to the nucleus, where it joins CSL (Petcherski and Kimble 2000; Lubman, Ilagan et al. 2007; Del Bianco, Aster et al. 2008; Friedmann, Wilson et al. 2008), and recruits Mastermind (Nam, Sliz et al. 2006; Wilson and Kovall 2006). Nipped–A, a component of SAGA and Tip60 coactivator complexes, may impact the ability of CSL to recruit Mastermind, since Mastermind binding patterns are disrupted on polytene chromosomes of Nipped–A mutant flies (Gause, Eissenberg et al. 2006). Tip60 is indeed reported to attenuate Notch signaling through acetylation of NICD (Kim, Ann et al. 2007). Upon formation of the ternary complex of CSL, NICD and Mastermind, which involves conformational changes and masking of CSL DNA binding sites by NICD (Kurooka and Honjo 2000), the remodeled CSL–containing complex now recruits general transcription factors and chromatin remodelers.

Histone acetylation appears essential for Notch target gene activation. HDAC antagonizes Notch signaling in the vertebrate retina (Yamaguchi, Tonou–Fujimori et al. 2005), and the histone deacetylase Sin3a is important for Notch target gene repression (Moshkin, Kan et al. 2009). NICD and CSL together recruit the histone acetylases GCN5 and PCAF (Oswald, Tauber et al. 2001). The histone acetyltransferase p300 contributes to target gene activation (Zhou, Fujimuro et al. 2000; Fryer, Lamar et al. 2002; Hansson, Popko–Scibor et al. 2009; Saint Just Ribeiro and Wallberg 2009) by recruiting the histone acetyltransferase PCAF (Wallberg, Pedersen et al. 2002). The corepressor SMRT is displaced by NICD–recruited SKIP (Wallberg, Pedersen et al. 2002). In addition, chromatin remodeling complexes SWI/SNF (Kadam and Emerson 2003; Armstrong,

Sperling et al. 2005; Gause, Eissenberg et al. 2006) and ISWI/NURF (Kugler and Nagel 2010) are also involved in transcriptional activation of Notch target genes.

While histone acetylation status clearly affects Notch target gene regulation, far fewer reports imply histone methylation to Notch target gene repression or activation, respectively. H3K4 methylation, a mark of transcription competent chromatin, plays a role in Notch target gene activation, since target activation depends on the ubiquitin ligase Bre1, which is indirectly required for H3K4 methylation (Bray, Musisi et al. 2005). The histone demethylase LID/KDM, which removes H3K4me marks, has by genetic interaction proposed to be involved in the repression of Notch targets (Moshkin, Kan et al. 2009). KDM5A interacts with CSL and may be involved in removal of H3K4me3 from target genes after Notch signaling (Liefke, Oswald et al. 2010). Curiously, Delta overexpression, which should activate transcription, depletes H3K4me3 globally from imaginal discs (Ferres–Marco, Gutierrez–Garcia et al. 2006). Notch signaling has also been linked to the repressive H3K27 methylation mark deposited by the Polycomb Repressive Complex 2 (PRC2). Mutations in *Drosophila* Tantalus, an interaction partner of the Polycomb protein ASX (additional sex combs), elicits phenotypes reminiscent of Notch mutations and interacts with Notch genetically (Dietrich, Yang et al. 2005). Deregulation of the Polycomb proteins Pipsqueak and Lola cooperate with Delta overexpression in the formation of tumors in Drosophila (Ferres–Marco, Gutierrez–Garcia et al. 2006). However, Polycomb in these cases acts upstream of Notch signaling by deregulating core pathway components, rather than by coregulating target genes (Martinez, Schuettengruber et al. 2009; Herz, Madden et al. 2010).

Most Notch signaling events, particularly during development, are transient and require tight temporal and spatial regulation. Formation of the ternary CSL–NICD–Mastermind complex triggers NICD turnover by ubiquitin–mediated proteasomal degradation of NICD after phosphorylation by Cdk8 (Fryer, White et al. 2004), leaving the CSL corepressor behind at the target gene (Deftos and Bevan 2000; Gajewski, Sieger et al. 2003; Pourquie 2003; Giudicelli and Lewis 2004; Fior and Henrique 2005). Ubiquitin–mediated protein degradation has been more generally implied in the shutdown of Notch signaling, and mutations affecting NICD degradation have been implied in Notch–dependent cancerogenesis (Qiu, Joazeiro et al. 2000; Fryer, White et al. 2004; Tsunematsu, Nakayama et al. 2004; Weng, Ferrando et al. 2004; Chiang, Xu et al. 2006; O'Neil, Grim et al. 2007; Thompson, Buonamici et al. 2007). Notch targets

have also been reported to negatively feed back on their own transcription (Stark, Brennecke et al. 2003; Hirata, Bessho et al. 2004; Lai, Tam et al. 2005), or to be shortlived and/or targeted by miRNAs (Dou, Zeng et al. 1994; Christensen, Kodoyianni et al. 1996).

Given the apparently simple architecture of the core pathway and its significance in development and disease, it is surprising that the identification of Notch target genes lags behind. Partly, this is due to the fact that NICD efficiently activates target genes at concentrations below detection limit and additionally is turned over rapidly, hampering chromatin–immunoprecipitations to identify binding sites on DNA. Sequences that promote the association of CSL with individual promotors have been identified (Bailey and Posakony 1995; Lecourtois and Schweisguth 1995; Christensen, Kodoyianni et al. 1996) but are not sufficiently specific to be useful in target predictions.

Nonetheless, Notch responsive genes have been identified in several contexts, for example in hematopoietic development (IL6 (Kannabiran, Zeng et al. 1997; Plaisance, Vanden Berghe et al. 1997), pre–T–Cell receptor alpha (Reizis and Leder 2002), IL4 (Amsen, Blander et al. 2004)), in the nervous system (GFAP (Ge, Martinowich et al. 2002), Brain lipid–binding protein (Anthony, Mason et al. 2005), in cell cycle regulation (p21 (Rangarajan, Talora et al. 2001), CycD1 (Ronchini and Capobianco 2001), SKP2 (Sarmento, Huang et al. 2005), c–myc (Klinakis, Szabolcs et al. 2006; Palomero, Lim et al. 2006; Sharma, Calvo et al. 2006; Weng, Millholland et al. 2006) and development (Nodal (Krebs, Iwai et al. 2003), Nrarp (Krebs, Deftos et al. 2001). The best characterized and conserved targets belong to two families of bHLH genes, Hes (also E(sp)) and Herp (also Jey, Hesr, HRT, CHF, gridlock; Oellers, Dehio et al. 1994; Iso, Chung et al. 2002; Iso, Kedes et al. 2003). Hes/Herp bHLH proteins repress their target genes (Davis and Turner 2001) by recruiting Groucho/TLE proteins (Heitzler, Bourouis et al. 1996) or by sequestering transcriptional co–factors (Kuroda, Tani et al. 1999). Hes/Herp proteins mediate Notch signaling in mammalian neurogenesis (Kageyama and Ohtsuka 1999; Ohtsuka, Ishibashi et al. 1999), endocrine development (Jensen, Pedersen et al. 2000; Kita, Imayoshi et al. 2007; Raetzman, Cai et al. 2007), heart development (Donovan, Kordylewska et al. 2002; Gessler, Knobeloch et al. 2002; Sakata, Kamei et al. 2002; Fischer, Schumacher et al. 2004), pancreas development (Murtaugh, Stanger et al. 2003; Sumazaki, Shiojiri et al. 2004), and adipocyte

differentiation (Ross, Rao et al. 2004). In C.elegans, the diverged HES–family member REF–1 mediates Notch signaling events during embryonic patterning (Neves and Priess 2005).

Accessibility of target genes appears to play a major role in target activation by Notch signaling, providing an explanation for the wide and versatile use of the pathway as cell– and tissue specific activator of transcription (Iso, Chung et al. 2002; Cave, Loh et al. 2005; Ong, Cheng et al. 2006). Also, some Notch–responsive enhancers are combinatorial and require cooperation with other transcription factors (Cooper, Tyler et al. 2000; Furriols and Bray 2001; Neves, English et al. 2007). Thus, Notch signaling receptivity can be finetuned to give a very specific output depending on the signaling context. Identifying targets that play a role in stem cell maintenance in *C.elegans* therefore may or may not be telling about Notch signaling in general. Features of Notch signaling that are conserved between organisms, such as the secondary transcription factors Hes/Herp, or the similarity of both the CSL/NICD binding site (Tun, Hamaguchi et al. 1994; Christensen, Kodoyianni et al. 1996) and of CSL itself across species (CSL is 84% identical between *Drosophila* and human) suggest that extrapolations may be possible.

3.3 The FBF/Pumilio protein family

The conserved family of Pumilio/FBF (Puf) proteins (Fig. 3A) is characterized by the Pumilio homology domain / Puf domain, which consists of eight to nine consecutive sequence repeats and a flanking region (Zhang, Gallegos et al. 1997), and typically recognizes RNAs containing a UGUR (R = purine) trinucleotide followed by AU–rich sequences (Wickens, Bernstein et al. 2002; Table 1).

The structural features of the interaction of Puf proteins with their cognate RNAs has been well characterized. Alpha–helical repeats are stacked against each other and create a curved surface of alpha helices (Wang, McLachlan et al. 2002), which interacts with a 8–10 nt stretch of RNA on a one repeat – one nucleotide basis (Opperman, Hook et al. 2005; Gupta, Nair et al. 2008; Wang, Opperman et al. 2009; Fig. 3B). The ability to flip bases 4–6 away from the protein surface has been demonstrated for FBF–2 and provides flexibility in length and base composition of the target motif (Wang, Opperman et al. 2009). The amino acid residues providing nucleotide specificity

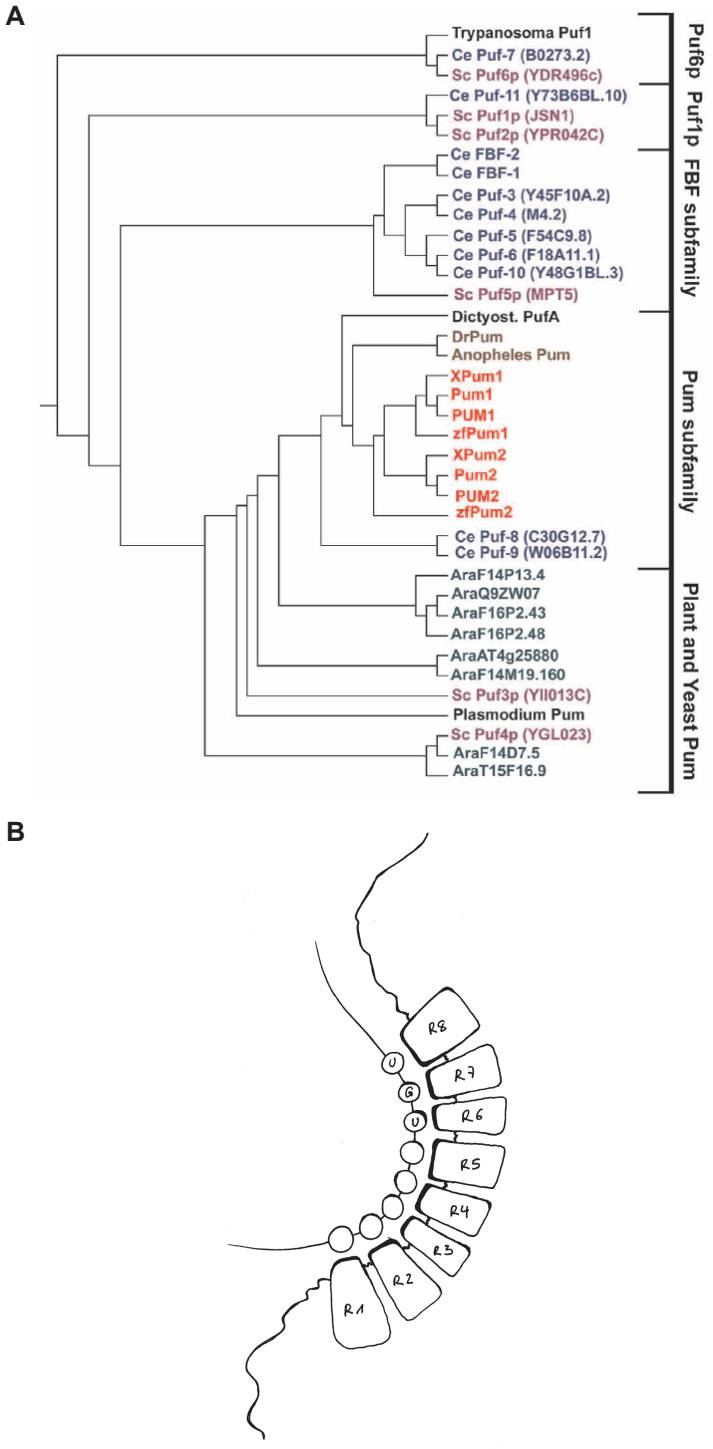


Figure 3. **Conservation and RNA recognition of the Pumilio/FBF protein family.**
(A) Phylogenetic dendrogram of the Pumilio/FBF (Puf) family of proteins. The Puf family consists of Puf6p, Puf1p, FBF, PUM and plant PUM subfamilies. Reproduced from Spassov et al, *The PUF family of RNA-binding proteins: does evolutionarily conserved structure equal conserved function?*, 2003. (B) Schematic representation of the Puf domain complexed with cognate RNA. Eight RNA-recognition helices (R1–R8) specifically recognize one nucleotide each (U1–A8). Illustration after Opperman et al, *A single spacer nucleotide determines the specificities of two mRNA regulatory proteins*, 2005.

to each repeat have been mapped (Sonoda and Wharton 1999; Opperman, Hook et al. 2005; Miller, Higgin et al. 2008; Wang, Opperman et al. 2009). Modifications that introduce key residues of other family members induce the predicted specificity changes (Cheong and Hall 2006; Koh, Opperman et al. 2009). The Pumilio domain is therefore being promoted as attractive candidate for an RNA recognition adaptor that could be engineered to the researchers needs (Satou 1999; Schoppmeier, Fischer et al. 2009; Takada, Kawana et al. 2009).

Table 1. *Recognition motifs of Puf proteins*

protein	recognition sequence	determined by	publication
Pumilio	HHUGUAHAUAHDHWDD	RIP–chip, MEME	(Gerber, Luschnig et al. 2006)
Puf1p Puf2p Puf3p Puf4p Puf5p	none identifyable none identifyable YHUGUAHAUA HNUGUAHAHUA WUGUAWYWDUA	RIP–chip, MEME	(Gerber, Herschlag et al. 2004)
Pum1 Pum2	NNUGUAHAUANN NNUGUAHAUANW	RIP–chip, MEME	(Galgano, Forrer et al. 2008)
Pum2	UGUANAUARNNNNBBBBSCCS	SELEX	(White, Moore–Jarrett et al. 2001)
Pum1	NNNNNUDUAHAWANN	RIP–chip, MEME	(Morris, Mukherjee et al. 2008)
PUF–8 FBF	UGURHRDW UGUAHHAU	Y3H	(Opperman, Hook et al. 2005)
PUF–5	NNHUGUHNBBDNN	Y3H	(Stumpf, Kimble et al. 2008)
PUF–11	HHUGURAAUR HDYUGURYHHKRW HHHUGUHRDRWND	Y3H	(Koh, Opperman et al. 2009)
FBF–1	NVNDNNHNUKHDHNDDN	Y3H	(Bernstein, Hook et al. 2005)

R = A,G; K = G,U; Y = C,U; W = A,U; V = A,C,G; D = A,G,U ; H = A,C,U ; N = any base

Puf proteins owe their name to two prominent family members, *Drosophila* Pumilio and *C.elegans* FBF (Nusslein–Volhard, Frohnhofer et al. 1987; Zhang, Gallegos et al. 1997). *Drosophila* Pumilio was originally identified in screens for maternal effect lethal mutations affecting embryonic patterning conducted by Ruth Lehmann and

Christiane Nüsslein–Volhard. Pumilio mutant *Drosophila* embryos essentially lack posterior structures and are therefore smaller than average (hence the name from latin pumilius pumilio, dwarf). The isolation of zygotic lethal alleles in the same screen pointed towards an additional function of *pumilio* in adult flies. Indeed, other independently isolated alleles ("*bemused*", "*pumuckl*", and "*ovarette*") have behavioural, locomotory, or adult germline defects (Stern, Blake et al. 1995; Lin and Spradling 1997; Schmucker, Jackle et al. 1997), uncovering the requirement of Pumilio in the nervous system and in germline stem cells.

The identity of Pumilio as RNA binding protein targeting the hunchback mRNA was confirmed in 1995 by Murata (Murata and Wharton 1995). The first *C.elegans* Puf family member, FBF, was isolated based on its RNA binding property by a reverse approach starting from the *fem-3* mRNA – hence the name *fem-3* mRNA binding factor (Zhang, Gallegos et al. 1997). The, retrospectively, main and conserved role of FBF – *C.elegans* germline stem cell maintenance – was described only after simultaneous disruption of both FBF proteins, FBF–1 and FBF–2, which are almost identical in sequence and are redundantly required for continued stem cell proliferation in the germline (Crittenden, Bernstein et al. 2002).

Since then, the Pumilio family as well as their RNA target list have kept expanding. With the availability of genome sequences, Puf proteins have been identified in all eukaryotic organisms, including plants (Fig. 3A). While *Drosophila* and Vertebrates dispose of one and two Puf proteins, respectively, five members have been described in yeast, twelve in *C.elegans* and more than twenty in Arabidopsis thaliana (Spassov and Jurecic 2003; Abbasi, Park et al. 2011). These expansion of the gene family confers robustness to the system and at the same time allows the evolution of distinct RNA binding properties and functions. In *C.elegans*, Puf proteins often come in duplicated, redundant gene pairs, and even mouse PUM1 and PUM2 target largely overlapping populations of mRNAs (Galgano, Forrer et al. 2008). Puf proteins therefore exemplify the importance to choose the least complex model possible – discovery in *Drosophila* was possible because there is just one Puf protein in flies. Puf proteins are at the same time an example that sometimes, "horizontal" extrapolation between model organisms makes sense. The role of Puf proteins as conserved stem cell regulators was eventually disclosed in *C.elegans* (Crittenden, Bernstein et al. 2002), and taking a step down the evolutionary ladder to Planaria has prompted a revised look at

the Puf family as ancient stem cell regulators that only later evolved additional tasks in higher organisms (Salvetti, Rossi et al. 2005).

While Puf function in stem cells and the nervous system appears conserved, this is not the case for Puf targets (Morris, Mukherjee et al. 2008). For *yeast* Puf1–5p, *Drosophila* Pumilio, human Pum1 and Pum2, and *C.elegans* FBF, targets have been determined large scale by RNA–co–IP (White, Moore–Jarrett et al. 2001; Gerber, Herschlag et al. 2004; Fox, Urano et al. 2005; Gerber, Luschnig et al. 2006; Galgano, Forrer et al. 2008; Morris, Mukherjee et al. 2008; Kershner and Kimble 2010). Each protein associates with hundreds of mRNAs, which frequently belong to functional units. For example, *yeast* Puf3p preferentially associates with mRNAs involved in mitochondrial metabolism, while *C.elegans* FBF targets entire signaling pathways and functional units, such as the MAP Kinase pathway or the apoptotic machinery (Kershner and Kimble 2010). This strategy of coregulation of a substantial fraction of the transcriptome has been termed RNA regulon or RNA operon concept (Keene 2007). Possibly, Puf proteins may have preceeded chromatin as global regulatory mechanism of mRNA expression. This ancient function may be preserved in stem cells. Stem cell chromatin has been described as comparably transcription–competent (Gaspar–Maia, Alajem et al. 2011), and Puf proteins together with other translational regulators may globally govern gene expression of stem cells until chromatin–based mechanisms of regulation take over during differentiation.

Large scale target identification efforts have permitted the computation of motifs correlating with Puf binding. In most cases, the 3'UTRs of Puf targets are enriched for a UGU trinucleotide motif, which is followed by a more flexible two– to four nucleotide long spacer and an AU–rich region. Bases outside the core motif also contribute to affinity (Zhu, Stumpf et al. 2009). Interestingly, human Pum1 and *Drosophila* Pumilio, although they have identical binding preferences, associate with very different mRNA pools, indicating that Puf proteins may be rapidly adapted for new purposes by target site evolution (Morris, Mukherjee et al. 2008).

Pumilio proteins have been reported to destablize their associated mRNAs by recruiting the deadenylation machinery and the exosome (Wreden, Verrotti et al. 1997; Olivas and Parker 2000; Goldstrohm, Hook et al. 2006; Goldstrohm, Seay et al. 2007; Ulbricht and Olivas 2008; Chritton and Wickens 2010). However, Puf targets in Xenopus oocytes and in neurons are not degraded, but rather stored and/or

transported in a deadenylated state to be available for translation upon oocyte maturation and synaptic remodeling, respectively (Chen, Li et al. 2008; Pique, Lopez et al. 2008; Ota, Kotani et al. 2011). Also, since mRNAs associated with Puf proteins can be coimmunoprecipitated (Gerber, Herschlag et al. 2004; Fox, Urano et al. 2005; Gerber, Luschnig et al. 2006; Galgano, Forrer et al. 2008; Morris, Mukherjee et al. 2008; Kershner and Kimble 2010), Puf proteins clearly do not induce immediate degradation of all associated targets, and under certain circumstances may even promote translation, as reported for FBF–1 and its target *egl-4* (Kaye, Rose et al. 2009).

Differential effects of Puf proteins on their targets – degradation versus storage – may reflect the habit of Puf proteins to cooperate with other proteins on the same mRNA. For example, *hunchback* regulation in the *Drosophila* embryo requires the recruitment of the conserved meiotic regulator Nanos by Pumilio (Sonoda and Wharton 1999). The interaction and cooperation of Pumilio and Nanos in the germline is conserved in worms and vertebrates (Kraemer, Crittenden et al. 1999; Moore, Jaruzelska et al. 2003). Regulation of the *bicoid* mRNA on the other hand does not require Nanos (Gamberi, Peterson et al. 2002). Another conserved meiotic regulator, the DAZL/Boule protein family, also co–occupies mRNAs with Pumilio (human Pum2) (Xu, Moore et al. 2001). Interestingly, Boule–Pum2 targets do not overlap with Pum2–only targets (Urano, Fox et al. 2005), suggesting that cooperating proteins may not serve as combinatorial code to subset the total target pool, but may actually affect the mRNA recognition properties of Puf proteins. Considering the spring–like architecture of the Pumilio RNA binding domain, it is easily conceivable how a protein interacting with the backbone could distort the RNA–interface to accomodate slightly different recognition elements. Inversely, RNA binding may expose or occlude protein interaction sites on the protein surface such that the target mRNA may determine its own fate in dependence of the precise binding motif – deadenylation, storage, degradation, or transport, depending on the protein sets Pumilio could or could not accomodate when bound to a certain motif.

Finally, several pieces of evidence link Puf proteins to the miRNA machinery. *Drosophila* Pumilio recruits the TRIM protein Brat, a component of the miRNA machinery, to the hunchback mRNA (Sonoda and Wharton 2001). Interestingly, human TRIM proteins do interact with Puf proteins (Inga Loedige, FMI, personal communication), and many human Pum1 and Pum2 targets are also predicted miRNA

targets (Galgano, Forrer et al. 2008; Kedde, van Kouwenhove et al. 2010; Leibovich, Mandel–Gutfreund et al. 2010). At least in the case of human *p27* mRNA, recognition of a miRNA target site by its cognate miRNA depends on Puf, which upon binding unfolds a secondary structure in the 3'UTR which otherwise masks the site (Kedde, van Kouwenhove et al. 2010). Whether this is a general feature of the Puf–miRNA axis remains to be determined.

3.4 MEX-3

Like Puf proteins, MEX–3 proteins are conserved among metazoans (Buchet–Poyau, Courchet et al. 2007). They belong to the KH family of RNA binding proteins and additionally contain a C–terminal RING finger domain, the function of which is not clear to date. Curiously, the prototypical and name–giving *C.elegans* MEX–3 (for Muscle EXcess – *C.elegans* MEX–3 prevents muscle fate in non–muscle lineages in the embryo) lacks the RING finger, and no interaction partner reported to date contains such a domain (Huang, Mootz et al. 2002). According to the prediction programs TargetScan and NetPhos, *C.elegans* MEX–3 is likely posttranslationally modified and may interact with 14–3–3 proteins and several protein kinases (Figs. 4 and 5; Blom, Gammeltoft et al. 1999; Obenauer, Cantley et al. 2003). Indeed, vertebrate MEX–3 is reported to be a phosphoprotein and to bind to 14–3–3 proteins and Argonautes in dependence of its phosphorylation status (Courchet, Buchet–Poyau et al. 2008).

Developmental function of MEX-3: cell fate specification

In anuran amphipians, nematodes, ascidians, and ecdysozoa, maternally provided MEX–3/PEM–3 (Posterior End Mark, the ascidian orthologue; Satou 1999) contributes to embryonic patterning. MEX–3 prevents expression of the posterior determinant Caudal/PAL–1 in the anterior of the embryo (Draper, Mello et al. 1996; Hunter and Kenyon 1996; Satou 1999; Schoppmeier, Fischer et al. 2009). Lack of Caudal in the posterior or inappropriate expression in the anterior part of the embryo results in cell fate transformations, such as ectopic muscle in the case of *C.elegans mex-3* embryos (Draper, Mello et al. 1996). Interestingly, the paradigm organism for embryonic patterning, *Drosophila*, uses Bicoid instead of MEX–3 to regulate *caudal* (Dubnau and

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MPVVSVRPFSMRNEGFSNGHNTWNDAEFWKPMKEEQIAYKLPGAWYYEEDTASCSPVSDPEDIAQFLNYRTSIGVQNVTE      80
SVEVPTSEHVAEIVGRQGCKIKALRAKTNTYIKTPVRGEDPIFVVTGRLEDVNEAKREIDCAAEHFTQIRASRRHTQGAH     160
APGQITSYVRVPLRVVGLVVGPKGATIKRIQQDTHTYIITPSREREPVFEVTGLPHNVEAARKEIETHIFQRTGNLPETD     240
NDFAGQLAGVSLMVQKQQQAQQQMQEAQQQSMFYRRAFGNSNPFNQKEMSSSPFGMESSLGLDALLRSFPSMRSSLTPES     320
LSGTGLSSRPSLGGGQSAKQDLPTYDYWGTNNSLNDIMENEILSRKYDALSAWSSMGLEKREESPTNGLMSSLKGTSAGF     400
GLLSTIWSGGNMNLSPGSLASASASPTSSTCDHNDHTLVPING                                          480
.........S......S....T.............Y..........T...S..S.....................          80
S.....S.....................Y..T..........T.....................S...T....          160
.....T.Y.........................Y..T.S.....................................          240
.................................................S..............S...S.T..S          320
.S....S...S............Y..................S..................S......SS....S...          400
.............S.......S.S..SS..............                                          480

Phosphorylation sites predicted:    Ser: 25  Thr: 8   Tyr: 5
```

Serine predictions

Name	Pos	Context (v)	Score	Pred
Sequence	5	MPVVSVRPF	0.202	.
Sequence	10	VRPFSMRNE	0.996	*S*
Sequence	17	NEGFSNGHN	0.714	*S*
Sequence	53	EDTASCSPV	0.028	.
Sequence	55	TASCSPVSD	0.803	*S*
Sequence	58	CSPVSDPED	0.981	*S*
Sequence	72	NYRTSIGVQ	0.120	.
Sequence	81	NVTESVEVP	0.875	*S*
Sequence	87	EVPTSEHVA	0.877	*S*
Sequence	152	QIRASRRHT	0.997	*S*
Sequence	167	GQITSYVRV	0.028	.
Sequence	202	IITPSRERE	0.948	*S*
Sequence	251	LAGVSLMVQ	0.018	.
Sequence	271	AQQQSMFYR	0.010	.
Sequence	281	AFGNSNPFN	0.010	.
Sequence	290	QKEMSSSPF	0.447	.
Sequence	291	KEMSSSPFG	0.054	.
Sequence	292	EMSSSPFGM	0.939	*S*
Sequence	298	FGMESSLGL	0.023	.
Sequence	299	GMESSLGLD	0.006	.
Sequence	308	ALLRSFPSM	0.025	.
Sequence	311	RSFPSMRSS	0.703	*S*
Sequence	314	PSMRSSLTP	0.064	.
Sequence	315	SMRSSLTPE	0.949	*S*
Sequence	320	LTPESLSGT	0.902	*S*
Sequence	322	PESLSGTGL	0.934	*S*
Sequence	327	GTGLSSRPS	0.667	*S*
Sequence	328	TGLSSRPSL	0.086	.
Sequence	331	SSRPSLGGG	0.980	*S*
Sequence	337	GGGQSAKQD	0.132	.
Sequence	353	GTNNSLNDI	0.027	.
Sequence	364	NEILSRKYD	0.862	*S*
Sequence	371	YDALSAWSS	0.382	.
Sequence	374	LSAWSSMGL	0.307	.
Sequence	375	SAWSSMGLE	0.025	.
Sequence	384	KREESPTNG	0.997	*S*
Sequence	391	NGLMSSLKG	0.520	*S*
Sequence	392	GLMSSLKGT	0.591	*S*
Sequence	397	LKGTSAGFG	0.669	*S*
Sequence	404	FGLLSTIWS	0.095	.
Sequence	408	STIWSGGNM	0.005	.
Sequence	415	NMNLSPGSL	0.848	*S*
Sequence	418	LSPGSLASA	0.050	.
Sequence	421	GSLASASAS	0.443	.
Sequence	423	LASASASPT	0.726	*S*
Sequence	425	SASASPTSS	0.982	*S*
Sequence	428	ASPTSSTCD	0.924	*S*
Sequence	429	SPTSSTCDH	0.981	*S*

Threonine predictions

Name	Pos	Context (v)	Score	Pred
Sequence	22	NGHNTWNDA	0.731	*T*
Sequence	51	YEEDTASCS	0.626	*T*
Sequence	71	LNYRTSIGV	0.031	.
Sequence	79	VQNVTESVE	0.042	.
Sequence	86	VEVPTSEHV	0.331	.
Sequence	108	LRAKTNTYI	0.017	.
Sequence	110	AKTNTYIKT	0.178	.
Sequence	114	TYIKTPVRG	0.667	*T*
Sequence	126	IFVVTGRLE	0.700	*T*
Sequence	147	AEHFTQIRA	0.092	.
Sequence	156	SRRHTQGAH	0.663	*T*
Sequence	166	PGQITSYVR	0.719	*T*
Sequence	186	PKGATIKRI	0.488	.
Sequence	194	IQQDTHTYI	0.022	.
Sequence	196	QDTHTYIIT	0.010	.
Sequence	200	TYIITPSRE	0.504	*T*
Sequence	212	VFEVTGLPH	0.060	.
Sequence	227	KEIETHIFQ	0.016	.
Sequence	233	IFQRTGNLP	0.038	.
Sequence	239	NLPETDNDF	0.105	.
Sequence	317	RSSLTPESL	0.723	*T*
Sequence	324	SLSGTGLSS	0.015	.
Sequence	344	QDLPTYDYW	0.005	.
Sequence	350	DYWGTNNSL	0.062	.
Sequence	386	EESPTNGLM	0.008	.
Sequence	396	SLKGTSAGF	0.129	.
Sequence	405	GLLSTIWSG	0.019	.
Sequence	427	SASPTSSTC	0.031	.
Sequence	430	PTSSTCDHN	0.085	.
Sequence	437	HNDHTLVPI	0.032	.

Tyrosine predictions

Name	Pos	Context (v)	Score	Pred
Sequence	39	EQIAYKLPG	0.535	*Y*
Sequence	46	PGAWYYEED	0.030	.
Sequence	47	GAWYYEEDT	0.158	.
Sequence	69	QFLNYRTSI	0.010	.
Sequence	111	KTNTYIKTP	0.751	*Y*
Sequence	168	QITSYVRVP	0.865	*Y*
Sequence	197	DTHTYIITP	0.789	*Y*
Sequence	274	QSMFYRRAF	0.008	.
Sequence	345	DLPTYDYWG	0.507	*Y*
Sequence	347	PTYDYWGTN	0.253	.
Sequence	367	LSRKYDALS	0.039	.

Figure 4. **Several residues of C.elegans MEX-3 are predicted to be phosphorylated.**
C.elegans MEX-3 protein sequence and, below, phosphorylateable residues therein (Serine, S; Threonine, T; Tyrosine, Y). The likelyhood that an individual residue is phosphorylated as determined by the NetPhos 2.0 software (*http://www.cbs.dtu.dk/services/NetPhos/*) is indicated in the bottom panel by a score between 0 (unlikely) and 1 (very likely). More than ten residues received a probability score greater than 0.9. Scores above the threshold of 0.500 are assigned as '*S*', '*T*', or '*Y*.

Phosphoserine/threonine binding group (pST_bind)				
14-3-3 Mode 1			Gene Card YWHAZ	
Site	Score	Percentile	Sequence	SA
T156	0.4712	0.955 %	IRASRRHTQGAHAPG	1.526
Src homology 2 group (SH2)				
PLCg C-terminal SH2			Gene Card PLCG1	
Site	Score	Percentile	Sequence	SA
Y197	0.4342	0.566 %	IQQDTHTYIITPSRE	0.500
Basophilic serine/threonine kinase group (Baso_ST_kin)				
PKC delta			Gene Card PRKCD	
Site	Score	Percentile	Sequence	SA
T110	0.4507	0.765 %	ALRAKTNTYIKTPVR	1.751
PKC alpha/beta/gamma			Gene Card PRKCA	
Site	Score	Percentile	Sequence	SA
S311	0.4579	0.680 %	ALLRSFPSMRSSLTP	1.068
Protein Kinase A			Gene Card PRKACG	
Site	Score	Percentile	Sequence	SA
T156	0.4754	0.887 %	IRASRRHTQGAHAPG	1.526
PKC alpha/beta/gamma			Gene Card PRKCA	
Site	Score	Percentile	Sequence	SA
T186	0.4766	0.996 %	VVGPKGATIKRIQQD	0.908
DNA damage kinase group (DNA_dam_kin)				
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
T317	0.4814	0.365 %	PSMRSSLTPESLSGT	1.312
Acidophilic serine/threonine kinase group (Acid_ST_kin)				
Casein Kinase 1			Gene Card CSNK1G2	
Site	Score	Percentile	Sequence	SA
S53	0.3920	0.292 %	YYEEDTASCSPVSDP	0.497
Proline-dependent serine/threonine kinase group (Pro_ST_kin)				
Cdk5 Kinase			Gene Card CDK5	
Site	Score	Percentile	Sequence	SA
T114	0.4232	0.294 %	KTNTYIKTPVRGEDP	1.042
Cdk5 Kinase			Gene Card CDK5	
Site	Score	Percentile	Sequence	SA
T200	0.4651	0.573 %	DTHTYIITPSREREP	0.660
Cdc2 Kinase			Gene Card CDC2	
Site	Score	Percentile	Sequence	SA
T114	0.4759	0.347 %	KTNTYIKTPVRGEDP	1.042
Cdc2 Kinase			Gene Card CDC2	
Site	Score	Percentile	Sequence	SA
T200	0.5259	0.786 %	DTHTYIITPSREREP	0.660
Erk1 Kinase			Gene Card MAPK3	
Site	Score	Percentile	Sequence	SA
S55	0.5310	0.806 %	EEDTASCSPVSDPED	0.339
Erk1 Kinase			Gene Card MAPK3	
Site	Score	Percentile	Sequence	SA
T200	0.5382	0.896 %	DTHTYIITPSREREP	0.660
Kinase binding site group (Kin_bind)				
PDK1 Binding			Gene Card PDPK1	
Site	Score	Percentile	Sequence	SA
D346	0.5677	0.343 %	KQDLPTYDYWGTNNS	1.411
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
V180	0.6816	0.943 %	LRVVGLVVGPKGATI	0.319

Figure 5. **Predicted MEX-3 interacting proteins and modification sites.**
Shown are potential interactors and modifiers of *C.elegans* MEX-3 and their respective interaction sites as determined by the MotifScan software (Obenauer et al, *Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs*, 2003). A "Score" value of 0.2 indicates that the indicated motif is among the 0.2% best matches in the database.

Struhl 1996; Rivera–Pomar, Niessing et al. 1996). *Drosophila* MEX–3 is only expressed during late embryonic development in the nervous system (Schoppmeier, Fischer et al. 2009). GLP–1/Notch, another gene involved in patterning the *C.elegans* embryo, is also regulated by MEX–3, but globally rather than in specific cells (Pagano, Farley et al. 2009). *mex-3* mutant embryos also display lineage defects in the germline. They contain excess germline precursor cells (Draper, Mello et al. 1996). Possibly, these are induced by ectopic NOS–2, a germline–specific protein which is ectopically expressed in somatic cells in *mex-3* mutants (Jadhav, Rana et al. 2008).

The role of mammalian MEX–3 in development has not yet been adressed, since vertebrate genomes encode four MEX–3 proteins (MEX–3A–D; Buchet–Poyau, Courchet et al. 2007), which may function redundantly. Mammalian embryos are not prepatterned and do not depend on maternal factors for germ layer specification, but MEX–3 may still contribute to aspects of embryonic development.

Adult function of MEX-3: maintenance of stem cell proliferation

Adult functions of MEX–3 have so far been adressed only in *C.elegans*, but expression of mouse MEX–3 in brain and testis suggest a function for MEX–3 in adult mammals (Buchet–Poyau, Courchet et al. 2007). *C.elegans* MEX–3 is specific to the germline. While *mex-3* RNA is expressed throughout the germline, MEX–3 protein is repressed in the central germline by GLD–1 and accumulates in the proximal germline in oocytes. Upon sperm depletion or heat stress, MEX–3 relocalises from the oocyte cytoplasm to granular structures (Jud, Czerwinski et al. 2008). The significance of this relocalisation, as well as a role for MEX–3 in the proximal germline (except for maternal contribution to the embryo) have not been addressed. MEX–3 is also expressed at low levels in stem cells in the distal gonad (Ciosk, DePalma et al. 2004 and Fig. 24). In stem cells, MEX–3 contributes to proliferation, since mutants have slightly fewer germ cells than the wild type (Ariz, Mainpal et al. 2009). However, MEX–3 functions redundantly with the Puf protein PUF–8, since the double mutant, but not either single mutant has severe germline proliferation defects (Ariz, Mainpal et al. 2009). MEX–3 is also required for germ cell proliferation and totipotency in a compound background lacking Notch signaling (own unpublished observations; Fig. 13). mRNAs targeted by MEX–3 in the germline stem cell compartment other than *rme-2* have not yet been identified. A

bipartite MEX–3 recognition element (MRE) has been determined in vitro (Pagano, Farley et al. 2009; A/G/U)(G/U)AGN(0–8)U(U/A/C)UA; N = any nucleotide). However, this sequence occurs too frequently in the genome to allow in silico target predictions – 2834 genes contain MREs in their 3'UTR. Even when assuming combinatorial regulation by two proteins, GLD–1 and MEX–3, on germline specific RNAs, only 2 out of 10 predicted targets (*pal-1* and *glp-1*) were really regulated by MEX–3 (Pagano, Farley et al. 2009). RNA–co–IPs have not been successful so far (own observation). Possibly, MEX–3 associates with mRNAs only in a short developmental time window, in a very small set of cells, or available reagents were simply not suited for RIP–Chip experiments.

The MEX–3 protein appears to be regulated on several levels. In the germline, *mex-3* mRNA is targeted by GLD–1. Like other maternal proteins, MEX–3 protein in the embryo is destabilized by OMA–1 (Lin 2003). Considering the high–low–high germline expression pattern, the stability of germline–expressed MEX–3 must also be regulated. Ubiquitous expression of MEX–3 in *par-3* mutant embryos (Bowerman, Ingram et al. 1997) does not result in ubiquitous repression of the MEX–3 target *pal-1*, indicating the requirement for modification or cofactors in MEX–3 dependent repression (Huang, Mootz et al. 2002). Indeed, human MEX–3 proteins are phosphorylated (Buchet–Poyau, Courchet et al. 2007; Courchet, Buchet–Poyau et al. 2008), and the interaction of human MEX–3 with 14–3–3 proteins is phosphorylation dependent (Courchet, Buchet–Poyau et al. 2008). In *C.elegans*, interacting proteins (SPN–4, MEX–5, and MEX–6; Huang, Mootz et al. 2002; Table 2) contribute to MEX–3 mediated regulation of *pal-1* in the embryo, but how they do so is not clear. Localisation of human MEX–3 is also regulated, since it shuttles between cytoplasm and nucleus via the CRM–1 export machinery (Buchet–Poyau, Courchet et al. 2007).

MEX–3 proteins have thus been implied in vital processes such as stem cell proliferation and embryonic cell fate determination. Yet, very few of their mRNA targets are confirmed (Table 3), and in *C.elegans* stem cells and in vertebrates MEX–3 is somewhat in search for a function. MEX–3 expression in the stem cell compartment and its requirement for stem cell proliferation under certain conditions (Ariz, Mainpal et al. 2009) and this work) make MEX–3 an stem cell maintaining mystery.

Table 2. *Protein interaction partners of MEX-3.*

interacting protein	**organism**	**publication**
Argonaute 1 and 2	*H.sapiens*	Buchet–Poyau, Courchet et al. 2007
14–3–3	*H.sapiens*	Courchet, Buchet–Poyau et al. 2008
POS–1, SPN–4, UNC–57, PABP–2, K07H8.10, MEX–5/6, B0250.1, F25B4.2, C43E11.9, F40F8.5, CPG–8, FUST–1, E02D9.1	*C.elegans*	Huang, Mootz et al. 2002

Table 3. *mRNAs targeted by MEX-3.*

interacting mRNA	**organism**	**publication**
bcl-2	*H.sapiens*	Donnini, Lapucci et al. 2004
glp-1	*C.elegans*	Pagano, Farley et al. 2009
nos-2	*C.elegans*	Jadhav, Rana et al. 2008; Pagano, Farley et al. 2009
pal-1	*C.elegans*	Draper, Mello et al. 1996; Hunter and Kenyon 1996

Open questions

One aim of stem cell research is to identify universal features of “stemcellness”, to identify the fundamental differences between cells that have and those that have not yet assumed a terminal identity. The goal is, of course, to understand these processes to a point where they can be manipulated and exploited for therapeutic purposes. Stem cell maintaining pathways and molecules have been identified – Wnt or Notch signaling, or Pumilio proteins, to name a few. Most are highly conserved. We know that these molecules govern cell fate decisions, but in most cases we do not know how. Stem cell maintaining molecules usually affect the activity of many genes and change the status of a cell as a whole. What a stem cell needs to remain proliferative and uncommitted is unclear.

If the general status of a cell is important for the self–renewal versus differentiation decision – what are the most distinguishing features between stem cells and committed cells? Certainly, their cell cycle profile is quite unique, since in the end, the self–renewal versus differentiation decision boils down to whether a cell opts to exit the cell cycle or to divide again (or at least remain competent to do so). This is the decision we really need to understand if we want to be able to manipulate stem cells for therapeutic purposes; all stem cell factors, as different as they may be, in the end affect that one decision.

In the *C.elegans* germline, Notch signaling and FBF proteins were found twenty and ten years ago, respectively, to be essential for germline stem cell maintenance. Although both have since been implied in stem cell biology in many other model systems, including vertebrates, it is not clear to date how they affect an individual stem cell’s decision to continue proliferation or to differentiate meiotically. Since particularly Notch signaling is highly conserved and required for stem cell maintenance in several contexts, it is highly desireable to understand the impact of Notch signaling on the self–renewal versus differentiation decision.

Scope of the thesis

When I started my work at FMI, little was known about how cell fate switches can occur in the C.elegans germline, and about the role of conserved stem cell factors in that process. However, we knew that in the compound mutant background *gld-2 gld-1; glp-1*, in which germline stem cells proliferate incessantly because, due to mutations in the meiotic factors GLD–1 and GLD–2, they cannot enter meiosis though they lack the stem cell factor GLP–1/Notch, MEX–3 protein is crucial to maintain both stem cell divisions and stem cell identity (see scheme below; a and b). We also knew at that point that MEX–3 was NOT required for proliferation and stem cell identity when in the identical background GLP–1/Notch was constitutively active (c and d).

A.	(a)	gld–1 gld–2; glp–1(–)		stem cell tumor
	(b)	gld–1 gld–2; glp–1(–)	– MEX–3	differentiation
B.	(c)	gld–1 gld–2; glp–1(++)		stem cell tumor
	(d)	gld–1 gld–2; glp–1(++)	– MEX–3	stem cell tumor

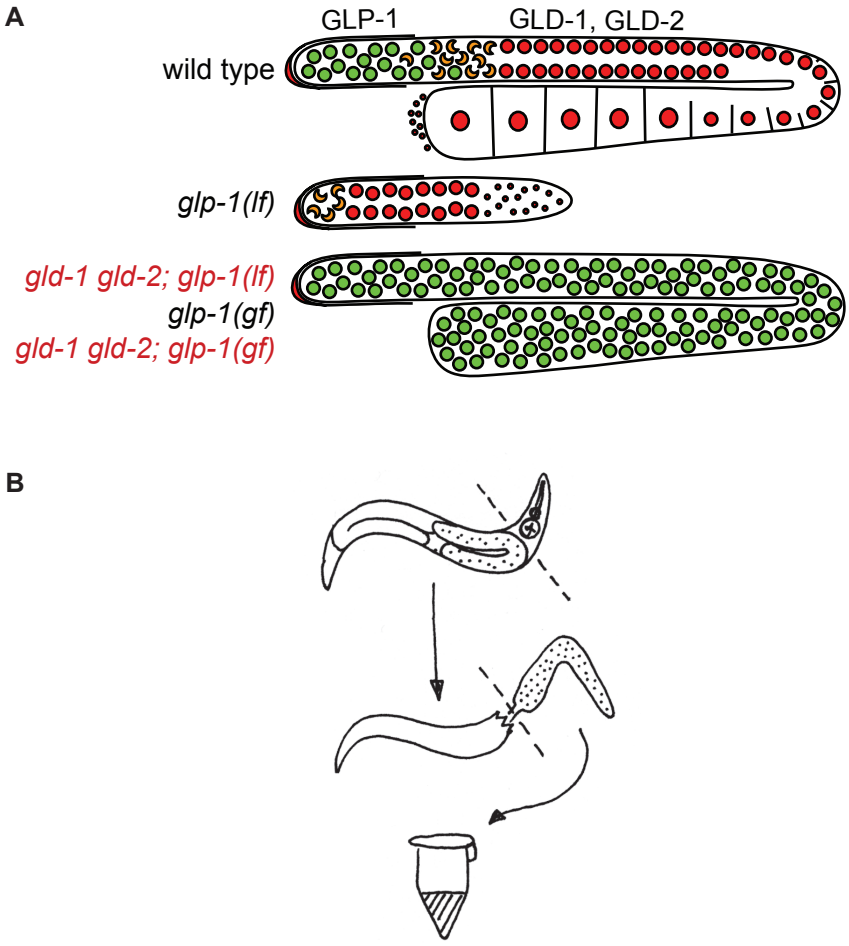
Stem cell proliferation in case (a) is thus MEX–3 dependent; stem cell proliferation in case (d) is thus GLP–1/Notch–dependent. It was therefore my aim to identify how Notch signaling and MEX–3, respectively, protect stem cells. The idea was to identify genes that are regulated on the mRNA level by Notch signaling, and to continue from whatever we found. Among other genes, we found that Notch signaling downregulates the cell cycle inhibitor CKI–2. Coincidentally, it turned out that CKI–2 was the key target of MEX–3 in scenario (a). This prompted us to look at CKI–2 function in the germline more globally, and led to the next coincidental discovery that in a wild type background, it is not MEX–3, but another highly conserved stem cell factor that regulates CKI–2 expression to maintain stem cells: the Pumilio proteins FBF. In addition, we found that Notch signaling primarily affects genes that are also regulated by the *C.elegans* equivalent of the Polycomb Repressive Complex 2 (PRC2), by the MES–proteins. The MES proteins are epigenetic modifiers involved in X–chromosome silencing in the germline and are required in a transgenerational manner for germ line stem cell proliferation. How these two pathways interact in detail is however beyond the scope of this thesis.

Results

4. Identification of Notch target genes in stem cells

As previously mentioned, Notch signaling maintains *C.elegans* germline stem cells. Screens based on temperature sensitve *loss* and *gain-of-function* alleles have yielded genes genetically interacting with Notch signaling (Maine and Kimble 1989; Maine and Kimble 1993; Sundaram and Greenwald 1993; Qiao, Lissemore et al. 1995). Many of them were found to modulate pathway component activity and availability, such as the endocytic and proteolytic machinery. Others are still not characterized because they would not have a phenotype on their own (Lissemore, personal communication). However, these screens yielded no hints about events downstream of Notch signaling. How the information provided by Notch signaling is translated into cell behaviour therefore remains a black box. Since Notch signaling regulates transcription (Bray 2006), we wanted to identify genes whose RNA levels change in response to Notch signaling using *C.elegans* tiling arrays and hoped to identify genes directly regulated by Notch signaling and/or of importance for stem cell maintenance.

The phenotypes of Notch *loss* and *gain-of-function* mutations could not be more different. While *gain-of-function* mutations induce stem cell tumors, stem cells are lost already during juvenile stages in the *loss-of-function* (Fig. 6A). mRNA expression patterns of *gain* and *loss of function* mutants can therefore not be compared directly. However, meiotic differentiation in the Notch *loss-of-function* mutant depends on two pro–meiotic factors, GLD–1 and GLD–2 (Kadyk and Kimble 1998). In the absence of GLD–1 and GLD–2, stem cells cannot enter meiosis and continue to proliferate even when they do not receive a Notch signal (Fig. 6A). Accordingly, we compared the expression profiles of germ cells in the presence/absence of Notch signaling in a *gld-1 gld-2* mutant background. Strong Notch *gain-of-function* mutations are dominant and difficult to maintain even at low temperatures and as heterozygotes. The canonical null allele on the other hand is closely linked to a strong unc mutation. We therefore chose temperature–sensitive mutations, *glp-1(ar202tsgf)* and *glp-1(e2141tslf)*. Both are wild type at 15°C and fully penetrant at 25°C. P0 animals of *gld-1 gld-2; glp-1(lf)* and *gld-1 gld-2; glp-1(gf)* animals were raised at the permissive temperature and shifted to the restrictive temperature at the L4–adult transition. Germlines from 50 animals of the F1 generation, which had been raised entirely at the restrictive temperature, were



filename	sample name
ci20081217cet1r_01_gld-1gld-2glp-1(e2141)_266_(Ce25b_MR_v02).CEL	e2141_A_1
ci20081217cet1r_02_gld-1gld-2glp-1(e2141)_266_(Ce25b_MR_v02).CEL	e2141_A_2
ci20081217cet1r_03_gld-1gld-2glp-1(e2141)_266_(Ce25b_MR_v02).CEL	e2141_A_3
ci20081217cet1r_04_gld-1gld-2glp-1(ar202)_266_(Ce25b_MR_v02).CEL	ar202_A_1
ci20081217cet1r_05_gld-1gld-2glp-1(ar202)_266_(Ce25b_MR_v02).CEL	ar202_A_2
ci20081217cet1r_06_gld-1gld-2glp-1(ar202)_266_(Ce25b_MR_v02).CEL	ar202_A_3
ci20090514cet1r_01_gld-1gld-2glp-1(e2141)_303t_(Ce25b_MR_v02).CEL	e2141_B_1
ci20090514cet1r_02_gld-1gld-2glp-1(e2141)_303t_(Ce25b_MR_v02).CEL	e2141_B_2
ci20090514cet1r_03_gld-1gld-2glp-1(e2141)_303t_(Ce25b_MR_v02).CEL	e2141_B_3
ci20090514cet1r_04_gld-1gld-2glp-1(ar202)_303t_(Ce25b_MR_v02).CEL	ar202_B_1
ci20090514cet1r_05_gld-1gld-2glp-1(ar202)_303t_(Ce25b_MR_v02).CEL	ar202_B_2
ci20090514cet1r_06_gld-1gld-2glp-1(ar202)_303t_(Ce25b_MR_v02).CEL	ar202_B_3

Figure 6. **Experimental setup to identify Notch-responsive genes in *C.elegans* germline stem cells.** (A) Schematic drawings of a wild type gonad (top) and the effect of *loss (lf)* and *gain (gf) of function* mutations in the Notch receptor on germline stem cell proliferation (below) in the presence or absence of *gld-1* and *gld-2*. Removing the pro-meiotic proteins GLD-1 and GLD-2 prevents stem cell loss even in the absence of Notch signaling. Red labels, genotypes used for expression analysis. (B) Gonad dissection. Gonads are released by a cut with an injection needle behind the pharynx, dissected away from the soma and collected in sample buffer by mouth pipette. 50 gonads were collected for each technical replicate. (C) Samples hybridized to Affimetrix C.elegans tiling arrays. A, B, biological replicates. 1, 2, 3, technical replicates. *e2141*, loss of function allele of *glp-1*/Notch. *ar202*, gain of function allele of *glp-1*/Notch.

dissected in triplicate and handed over to Eric Cabuy from the Genomics facility at FMI for amplification, array hybridisation and scanning (Figs. 6B and 6C).

Initial analysis performed by Dimos Gaidatzis from the Bioinformatics Facility at FMI revealed high correlation across all arrays (Fig. 7A). This indicates that we successfully reduced background noise, and that only few genes are differentially expressed. Expression values averaged from all arrays display a bipartite distribution (Fig. 7B). The left peak represents transcripts that are not expressed above background noise, the right peak contains expressed transcripts. To identify genes differentially expressed in the presence or absence of Notch, a fold–change value was calculated for each biological repeat seperately. Correlating and averaging changes observed in first and second biological repeat revealed that a small group of genes increases in expression in response to Notch signaling (Figs. 7C and D). 114 genes / 130 isoforms are upregulated more than 2 fold, 10 genes / 11 isoforms go down more than 2 fold (Table 4).

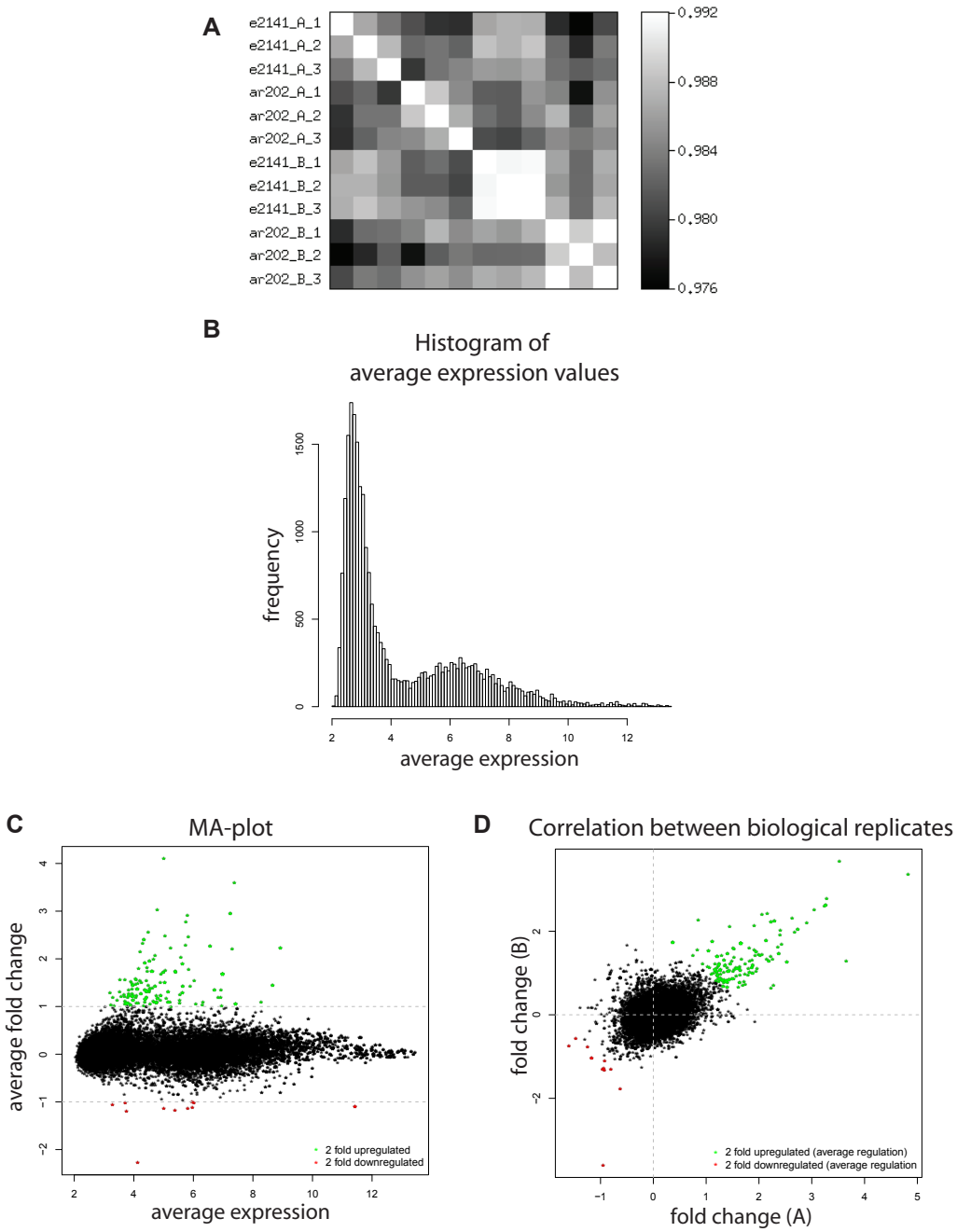


Figure 7. **Quality control and identification of differentially expressed genes.**
(A) Correlation plot of all arrays. Light color indicates high correlation, dark color indicates lower correlation. As expected, correlation is higher within than between genotypes both between technical (1,2,3) and biological (A,B) replicates. (B) Histogram of average gene expression. Left peak, background / noise; right peak, expressed genes. Average Expression Values were obtained by averaging expression across all arrays. (C) MA plot (M, magnitude of change; A, average expression). Green/red dots, genes up/downregulated by Notch signaling 2 fold or more. Most upregulated genes have an average expression value (X-axis) just above background, indicating that they may be not just be upregulated, but switched on by Notch signaling. (D) Correlation of fold changes between first and second biological replicate. Green/red dots, as in (B). Upregulation correlates well between biological replicates. Values in B, C, and D are given in log2.

A

fc_bothrepeats	fc_repeat1	fc_repeat2	avgExpr	WBid	public_name	gene_name	chromosome
-1.005993144	-1.242046996	-0.769939292	5.960300112	WBGene00012480	clec-104	Y18D10A.10	I
-1.011456238	-0.921372628	-1.101539849	6.020179762	WBGene00004707	Y57A10A.21	Y57A10A.21	II
-1.02002354	-1.464998259	-0.575048821	3.718076798	WBGene00013391	Y62H9A.3	Y62H9A.3	X
-1.05570865	-0.799598011	-1.31181929	3.283069976	WBGene00007363	stdh-1	C06B3.4	V
-1.096264901	-1.164454431	-1.028075372	11.42937954	WBGene00004798	sip-1	F43D9.4	III
-1.11374864	-0.937592131	-1.289905148	5.971264839	WBGene00020819	T26A5.2	T26A5.2	III
-1.128651676	-0.962710162	-1.294593191	5.809696603	WBGene00020819	T26A5.2	T26A5.2	III
-1.129364895	-0.927302093	-1.331427696	5.009454034	WBGene00007499	C09H10.5	C09H10.5	II
-1.168910307	-1.598208618	-0.739611997	5.390383483	WBGene00045394	ZK813.7	ZK813.7	X
-1.200684635	-0.627086202	-1.774283067	3.758756572	WBGene00012176	W01C9.2	W01C9.2	II
-2.27539107	-0.947879686	-3.602902454	4.134352568	WBGene00017673	F21F3.3	F21F3.3	I

B

fc_bothrepeats	fc_repeat1	fc_repeat2	avgExpr	WBid	public_name	gene_name	chromosc
1.002833359	1.326869437	0.678797282	5.25064274	WBGene00044644	B0205.13	B0205.13	I
1.005937845	1.318566608	0.693309082	3.342190959	WBGene00019980	R09B5.12	R09B5.12	V
1.006783983	1.186469946	0.82709802	4.327526561	WBGene00013136	Y53C10A.5	Y53C10A.5	I
1.022171088	1.242290251	0.802051925	6.946969349	WBGene00011054	R06B9.3	R06B9.3	II
1.026880007	1.398419163	0.655340852	3.895026822	WBGene00020812	T25G12.5	T25G12.5	X
1.038079286	1.09751127	0.978647302	6.302120119	WBGene00022590	ZC317.7	ZC317.7	V
1.041498224	1.226522801	0.856473647	3.239180236	WBGene00013630	Y102A5D.1	Y102A5D.1	V
1.051644977	0.368651641	1.734638312	7.417835052	WBGene00015803	C15H9.9	C15H9.9	X
1.051926535	1.247044949	0.856808121	6.081398754	WBGene00000735	col-162	Y2H9A.3	V
1.053320445	1.336914513	0.769726376	4.549250266	WBGene00019513	K08A2.4	K08A2.4	II
1.055492139	1.267862577	0.843121702	3.762897524	WBGene00010417	H25P06.4	H25P06.4	I
1.062088864	1.309860055	0.814317673	3.700053318	WBGene00017583	F19B10.1	F19B10.1	II
1.065160468	1.138037555	0.99228338	3.745882656	WBGene00019959	R08E3.3	R08E3.3	X
1.068136352	1.306583565	0.829689138	5.544745747	WBGene00012564	fbxa-107	Y37H2A.4	V
1.069571823	1.216043648	0.923099997	3.821255825	WBGene00001663	gpa-1	T19C4.6	V
1.071896062	0.933067916	1.210724208	4.154214536	WBGene00045050	ZC15.10	ZC15.10	V
1.073380233	1.269066173	0.877694292	3.777976166	WBGene00010433	H40L08.1	H40L08.1	X
1.079285682	1.371472228	0.787099136	4.762501142	WBGene00017419	F13B9.1	F13B9.1	X
1.08055388	1.203705219	0.95740254	4.087210755	WBGene00011679	ucr-2.2	T10B10.2	X
1.085295458	1.408040995	0.762549921	5.147540129	WBGene00007955	C35C5.3	C35C5.3	X
1.086108604	0.751058786	1.421158421	6.321528356	WBGene00002274	lec-11	F38A5.3	IV
1.089936299	1.338511708	0.841360891	4.37757229	WBGene00045048	D1007.19	D1007.19	I
1.093167091	1.379393356	0.806940826	4.763938052	WBGene00017419	F13B9.1	F13B9.1	X
1.093380239	1.028640443	1.158120035	4.686367556	WBGene00009945	bath-38	F52H3.3	II
1.096188352	1.063721359	1.128655345	8.274144442	WBGene00008681	scrm-4	F11A6.2	I
1.098369936	1.385002081	0.811737791	4.777730957	WBGene00017419	F13B9.1	F13B9.1	X
1.101316896	1.221944061	0.980689732	5.163643396	WBGene00016489	C36E6.1	C36E6.1	X
1.101696651	1.393336726	0.810056576	5.218195703	WBGene00007955	C35C5.3	C35C5.3	X
1.103182415	1.186497542	1.019867287	3.702174249	WBGene00016954	C55C3.4	C55C3.4	IV
1.114464188	1.167512627	1.061415749	4.65768823	WBGene00016678	C45G9.7	C45G9.7	III
1.127321475	1.207229956	1.047412993	5.544436771	WBGene00022060	Y67D8A.3	Y67D8A.3	IV
1.128260778	1.170797929	1.085723627	3.980434756	WBGene00015344	C02E11.1	C02E11.1	V
1.129548987	1.2784435	0.980654474	3.447481701	WBGene00007956	C35C5.6	C35C5.6	X
1.134833646	1.614236816	0.655430476	4.895766037	WBGene00007489	C09G5.7	C09G5.7	II
1.143991594	1.436534424	0.851448765	4.383663251	WBGene00003911	pak-1	C09B8.7	X
1.165360954	1.262967434	1.067754474	4.803615109	WBGene00022245	Y73B6BL.24	Y73B6BL.24	IV
1.165522073	1.210621345	1.120422801	5.095412153	WBGene00008007	C38D4.7	C38D4.7	III
1.181235795	1.275830076	1.086641513	4.071545031	WBGene00004056	pmk-2	F42G8.3	IV
1.18266493	1.338031785	1.027298075	3.799517397	WBGene00000591	coh-1	K08A8.3	X
1.18349253	1.495272914	0.871712146	4.421206365	WBGene00003911	pak-1	C09B8.7	X
1.190912756	1.521042748	0.860782764	3.303043784	WBGene00016336	C33C12.4	C33C12.4	II
1.194820467	0.836862516	1.552778417	6.840952497	WBGene00022663	glrx-21	ZK121.1	III
1.195665546	1.169676147	1.221654945	6.918297937	WBGene00009576	F40F8.3	F40F8.3	II
1.201351441	1.635519129	0.767183753	3.95835525	WBGene00020366	T08G2.3	T08G2.3	X
1.20459973	1.232824797	1.176374663	3.581606299	WBGene00018065	F35F11.2	F35F11.2	IV
1.205829241	1.69425205	0.717406433	3.693223713	WBGene00018223	bath-13	F40B1.1	II
1.222588933	1.241760352	1.203417515	3.987366584	WBGene00010339	acl-1	F59F4.4	X
1.228680484	1.409694089	1.047666879	3.83771021	WBGene00000161	apa-2	T20B5.1	X
1.232853356	1.740703688	0.725003023	4.759369457	WBGene00011398	qdpr-1	T03F6.1	III
1.234554238	1.428719042	1.040389434	4.096498207	WBGene00020408	T10D4.6	T10D4.6	II
1.237549303	1.248229962	1.226868643	4.902230789	WBGene00001685	gpd-3	K10B3.7	X
1.247267416	1.569287176	0.925247657	5.548193544	WBGene00019663	K11H12.7	K11H12.7	IV
1.251589056	1.441988621	1.06118949	3.672387659	WBGene00004377	rme-6	F49E7.1	X
1.2623177	1.719285147	0.805350254	4.241146635	WBGene00019546	W07G9.2	W07G9.2	IV
1.26920573	1.397921876	1.140489583	4.244825197	WBGene00004056	pmk-2	F42G8.3	IV
1.270773584	1.355147176	1.186399993	3.950387731	WBGene00017046	utx-1	D2021.1	X
1.27215973	1.510998919	1.033320541	3.833606033	WBGene00004148	pqn-65	T13H2.4	X
1.280148177	1.365586961	1.194709392	3.200825172	WBGene00013917	ZC504.3	ZC504.3	X
1.288972401	1.045888144	1.532056658	5.632423076	WBGene00004788	sft-4	C54H2.5	X
1.299939033	1.207543368	1.392334698	5.15425113	WBGene00012158	ucr-2.1	VW06B3R.1	X
1.301320534	1.641131102	0.961509966	4.429267724	WBGene00006561	tcl-2	Y38C1AA.4	IV
1.305837568	1.219769543	1.391905594	4.515217121	WBGene00015492	lnp-1	C05E11.1	X
1.30634441	1.536867815	1.075821004	3.528134629	WBGene00013032	wht-9	Y49E10.9	III
1.325731492	1.339273793	1.312189191	4.439851917	WBGene00004057	pmk-3	F42G8.4	IV
1.341252045	1.45787193	1.22463216	6.706558895	WBGene00000207	asb-2	F02E8.1	X
1.345454957	1.588999253	1.101910661	4.377535994	WBGene00001684	gpd-2	K10B3.8	X
1.351800845	1.564531994	1.139069697	4.531646566	WBGene00023498	lin-15A	ZK678.1	X
1.352621696	1.734537593	0.970705799	3.82847243	WBGene00021858	Y54F10BM.3	Y54F10BM.3	III
1.354445507	1.291094058	1.417796955	6.933183033	WBGene00021340	Y34F4.5	Y34F4.5	III
1.359181689	1.645552294	1.072811083	4.115500044	WBGene00008110	C46C2.2	C46C2.2	IV
1.366135085	1.630268537	1.102001633	4.137879338	WBGene00013180	Y53H1B.1	Y53H1B.1	I
1.371711919	1.792626494	0.950797344	3.94556286	WBGene00020256	T05C3.6	T05C3.6	V
1.387878876	1.490356936	1.285400815	4.53896225	WBGene00018851	F55A3.6	F55A3.6	I

Table 4. **Notch target genes.**

Genes identified as down- (A) or upregulated (B) at least 2 fold.

B (continued)

1.395281514	1.632597507	1.157965521	4.621601143	WBGene00004245	puf-9	W06B11.2	X
1.42230933	1.824409599	1.02020906	4.72209941	WBGene00023497	lin-15B	ZK662.4	X
1.432216984	1.403997099	1.460436868	5.64330046	WBGene00000294	cas-1	F41G4.2	X
1.433552378	2.227859597	0.639245159	5.895961407	WBGene00043998	T21C12.8	T21C12.8	III
1.44071762	1.114736919	1.76669832	3.962090509	WBGene00015320	C02B8.1	C02B8.1	X
1.442278482	1.881643761	1.002913204	8.667403892	WBGene00001607	gln-6	C28D4.3	IV
1.448596049	1.528144643	1.369047454	4.4901078	WBGene00009075	F23B2.7	F23B2.7	IV
1.456812369	1.798565964	1.115058774	4.065289379	WBGene00002060	ife-2	R04A9.4	X
1.476833324	1.740953726	1.212712922	4.131520759	WBGene00003638	nhr-48	ZK662.3	X
1.485558267	2.275702257	0.695414276	3.944596123	WBGene00009469	F36D3.1	F36D3.1	V
1.497475227	1.947518422	1.047432033	4.900315067	WBGene00003087	lsy-2	F49H12.1	X
1.500425141	1.866510889	1.134339394	5.03712765	WBGene00003087	lsy-2	F49H12.1	X
1.505339607	1.671035817	1.339643397	4.259405978	WBGene00010066	F54F7.6	F54F7.6	X
1.531432527	1.880675812	1.182189242	3.904593982	WBGene00020256	T05C3.6	T05C3.6	V
1.534475142	1.965604177	1.103346107	4.428900009	WBGene00020496	spat-3	T13H2.5	X
1.543380398	1.884632178	1.202128618	4.210216125	WBGene00009302	F31F6.3	F31F6.3	X
1.549895519	1.530486078	1.56930496	6.038195129	WBGene00003083	lst-1	T22A3.3	I
1.55460025	1.8226129	1.2865876	4.082526234	WBGene00003638	nhr-48	ZK662.3	X
1.557107079	0.851778574	2.262435584	3.542421118	WBGene00020967	W03A5.2	W03A5.2	III
1.557680217	1.509381423	1.60597901	3.708297938	WBGene00004055	pmk-1	B0218.3	IV
1.588711618	1.873219176	1.30420406	4.064712689	WBGene00010332	F59D12.5	F59D12.5	X
1.67269042	1.931539053	1.413841786	4.920763594	WBGene00014259	ZK1320.11	ZK1320.11	II
1.68369506	1.659474591	1.707915529	6.986695078	WBGene00003083	lst-1	T22A3.3	I
1.711989379	1.819954945	1.604023814	5.409638735	WBGene00008975	F20D1.3	F20D1.3	X
1.719393439	1.991992924	1.446793954	4.998046678	WBGene00021370	Y37E11AR.3	Y37E11AR.3	IV
1.723690972	2.025006803	1.422375141	4.986500547	WBGene00021370	Y37E11AR.3	Y37E11AR.3	IV
1.731808728	1.94782604	1.515791416	4.5984696	WBGene00011474	T05D4.1	T05D4.1	III
1.732918793	1.910329554	1.555508033	4.461431539	WBGene00006472	tag-123	F08F1.7	X
1.741827581	2.148765432	1.334889731	4.633323605	WBGene00008974	F20D1.2	F20D1.2	X
1.742493696	2.189026373	1.295961019	5.397758066	WBGene00003911	pak-1	C09B8.7	X
1.756294644	2.060699824	1.451889465	5.067286537	WBGene00021370	Y37E11AR.3	Y37E11AR.3	IV
1.775725402	1.445267893	2.106182911	5.842599326	WBGene00016953	C55C3.3	C55C3.3	IV
1.799076469	2.259589629	1.33856331	4.161526901	WBGene00003149	mbk-1	T04C10.1	X
1.809823024	2.226292829	1.39335322	4.444710393	WBGene00020496	spat-3	T13H2.5	X
1.904099197	2.53226382	1.275934575	5.632516404	WBGene00001329	epn-1	T04C10.2	X
1.923546122	2.375265345	1.4718269	4.36947145	WBGene00011680	T10B10.3	T10B10.3	X
1.961359101	2.136814084	1.785904117	4.531185562	WBGene00001082	dpy-23	R160.1	X
1.992578457	2.360304073	1.624852841	4.670604044	WBGene00003185	mek-1	K08A8.1	X
2.022716196	1.914799064	2.130633328	5.585503935	WBGene00016956	C55C3.6	C55C3.6	IV
2.062089869	2.42785191	1.696327828	4.720459056	WBGene00003185	mek-1	K08A8.1	X
2.157499613	2.400646576	1.914352649	4.212693702	WBGene00000164	apm-3	F53H8.1	X
2.214753543	2.404980551	2.024526535	7.311206537	WBGene00001520	gas-1	K09A9.5	X
2.224788303	2.227554396	2.22202221	8.927390728	WBGene00004423	rpl-11.2	F07D10.1	X
2.228599108	2.060630217	2.396567999	5.244110587	WBGene00008586	F08G12.2	F08G12.2	X
2.266154228	2.294205798	2.238102659	6.567057698	WBGene00009812	F47B10.1	F47B10.1	X
2.288326787	2.160091074	2.4165625	5.745405359	WBGene00043065	C55C3.7	C55C3.7	IV
2.329275641	2.678608947	1.979942335	4.301810259	WBGene00010984	nkat-3	R03A10.4	X
2.396152587	2.738333748	2.053971425	4.341870559	WBGene00010984	nkat-3	R03A10.4	X
2.469443424	3.652487879	1.286398968	5.831316392	WBGene00009572	F40D4.13	F40D4.13	V
2.477339384	2.630271818	2.324406951	5.053931736	WBGene00000443	ceh-20	F31E3.1	III
2.551328978	2.908999386	2.19365857	4.487850575	WBGene00013847	ZC84.3	ZC84.3	III
2.776297452	3.047714325	2.504880579	5.749954167	WBGene00015181	B0416.5	B0416.5	X
2.919074928	3.240447489	2.597702367	5.807497407	WBGene00015181	B0416.5	B0416.5	X
2.944427244	3.265464174	2.623390314	7.249195759	WBGene00008973	F20D1.1	F20D1.1	X
3.02851077	3.282259121	2.77476242	4.791074069	WBGene00015572	C07G1.6	C07G1.6	IV
3.601548139	3.526008997	3.677087281	7.382269268	WBGene00012104	T27F6.4	T27F6.4	I
4.100890675	4.829046186	3.372735165	5.013056193	WBGene00015573	C07G1.7	C07G1.7	IV

Table 4. **Notch target genes.**

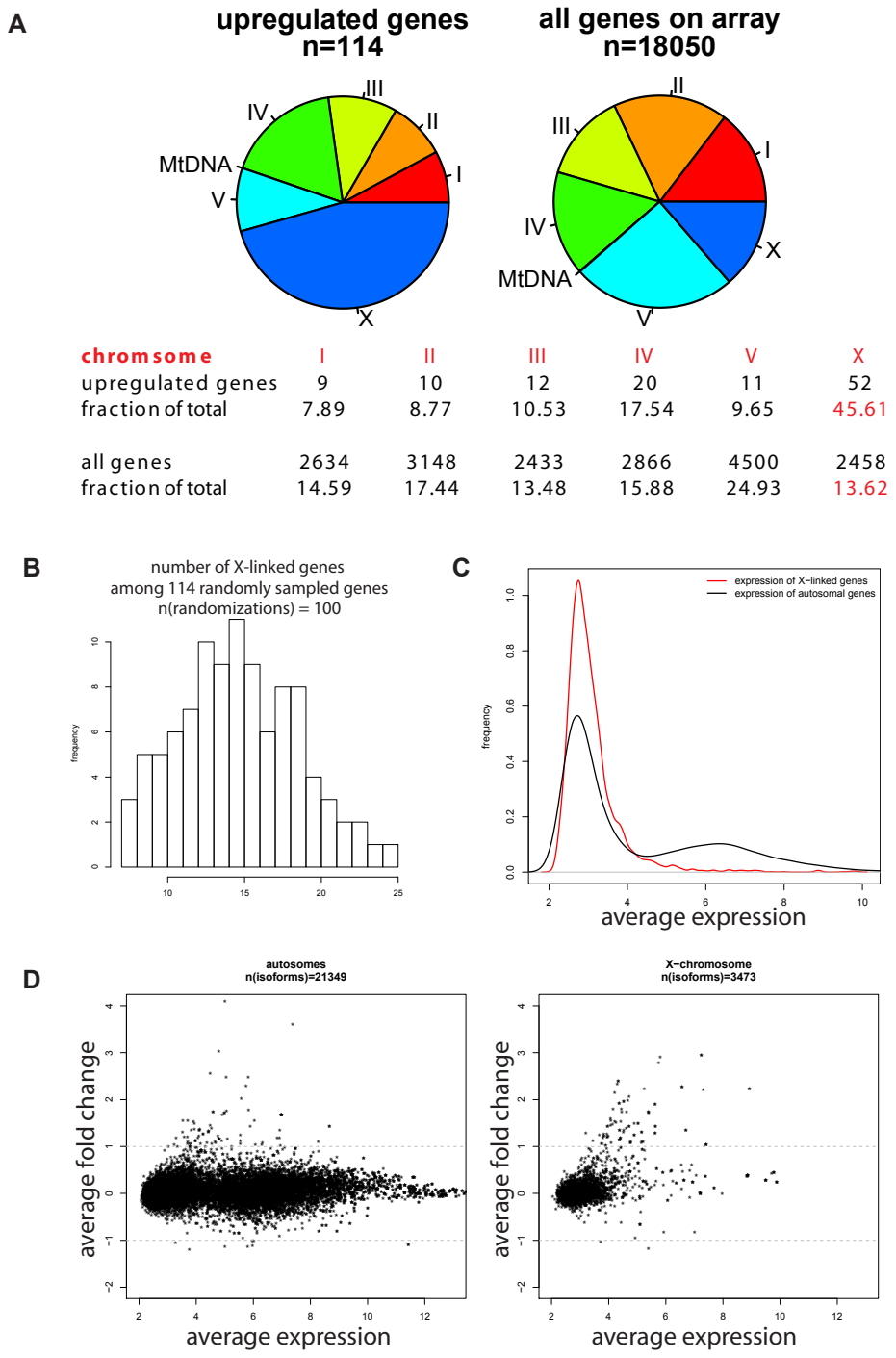
Genes identified as down- (A) or upregulated (B) at least 2 fold.

4.1 Notch signaling crosstalks to the *C.elegans* Polycomb proteins

Using the online gene–ontology analysis tool DAVID, components of the core proteasome or genes linked to mitochondrial metabolism appeared mildly enriched among Notch regulated genes (data not shown). We were however struck by another property of coregulated genes: Among 114 genes upregulated 2fold, 52 reside on the X–chromosome, which is almost 4 fold more than expected (Fig. 8A). This enrichment is significant (Fig. 8B). The X–chromosome is largely silent in the *C.elegans* germline. We confirmed that this is also true in the germline tumors used for analysis (Fig. 8C and 8D).

The machinery responsible for X–chromosome silencing in the germline consists of a group of four proteins: MES–2, MES–3, MES–4 and MES–6 (Pirrotta 2002). The MES proteins were discovered in screens for Maternal Effect Sterile mutations (hence the name; Paulsen, Capowski et al. 1995). Homozygous F1 animals develop normally. However, germ cells arrest proliferation and degenerate during larval stages iin the F2 generation (Paulsen, Capowski et al. 1995; Holdeman, Nehrt et al. 1998). MES proteins were found to belong to two distinct complexes with different chromatin modifying activities (Xu, Fong et al. 2001). MES–2, MES–3 and MES–6 are considered the C.elegans equivalent of Polycomb repressive complex 2 (PRC2). MES–2 is orthologous to Enhancer of Zeste, MES–6 to Extra Sex Combs, while MES–3 is a novel protein. MES–2, MES–3 and MES–6 methylate H3K27 via the SET domain of the EZH protein MES–2 (Bender, Cao et al. 2004). MES–4 is not part of the complex and deposits H3K36 methylation marks (Bender, Suh et al. 2006). The two complexes localize to and modify histones on autosomes (MES–4) and heterosomes (MES–2, MES–3, MES–6), respectively, and prevent association of the other with their territory (Fong, Bender et al. 2002; Fig. 8A). Deletion of MES–4 causes relocalisation of MES–2,3,6/PRC2 from the X–chromosome to autosomes. In the following, H3K27 methylation levels and repression cannot be maintained on the X–chromosome in *mes-4* mutants, similar to the situation in *mes-2/3/6* mutants.

Genes deregulated in the F1 generation of MES–4 mutants have been identified by microarray analysis (Bender, Cao et al. 2004). Many of these genes are X–linked. Since we found a bias towards X–linkage among Notch target genes, we wondered whether Notch signaling and the MES proteins would co–regulate genes. Indeed we



chromsome	I	II	III	IV	V	X
upregulated genes	9	10	12	20	11	52
fraction of total	7.89	8.77	10.53	17.54	9.65	45.61
all genes	2634	3148	2433	2866	4500	2458
fraction of total	14.59	17.44	13.48	15.88	24.93	13.62

Figure 8. **Notch-responsive genes are disproportionally X-linked.**

(A) 52 of 114 upregulated (>2 fold) genes reside on the X chromsome (dark blue; 45.6%). This is almost four fold more than the expected fraction (13.6%). (B) Among 100 independent random samplings of 114 genes, the maximum number of X-linked genes obtained was 25. Therefore, the probability to obsere 52 X-linked genes among 114 genes is less than 1% ($p < 0.01$). (C) Density plot of average expression values. Red line, distribution of average expression of X-linked genes. Black line, distribution of average expression of autosomal genes. Most X-linked genes are not or only lowly expressed. (D) MA-plot similar to Figure 7B, but separated by chromosomal location. Few X-linked genes are expressed above background; most upregulated genes are X-linked.

found that Notch target genes overlapped with 71 genes described as significantly deregulated in MES–4 F1 mutants (data not shown and Bender, Suh et al. 2006). Susan Strome (University of California) in the following kindly shared her unpublished data on gene regulaiton in *mes-2*, *mes-3*, *mes-4*, and *mes-6* mutants obtained from two different microarray platforms, "oligo" and "amplicon". Fascinatingly, genes deregulated in these mutants overlap very well with genes that respond to Notch signaling (Fig. 9B). While according on the "oligo" data, the correlation is true for *mes-4* but not *mes-2* or *mes-3*, the "amplicon" data suggests that genes deregulated in all *mes* mutants correlate with Notch target genes. Interestingly, the correlation is restricted to the X–chromosome (Fig. 9B). We figured that correlation may be coincidental if both Notch signaling and MES–2,3,6/PRC2 were involved in X–silencing in the germline. However, correlation dissappeared when MES–4 dependent fold changes of X–linked genes were randomly attributed to X–linked genes, demonstrating that Notch signaling and the MES–2,3,6/PRC2 converge on a specific subset of genes (Fig. 9C).

Assuming that MES–2,3,6/PRC2 and Notch signaling regulate common targets, one would expect them to interact genetically. To this end, a weak *gain-of-function* allele of Notch, *glp-1(ar202)gf*, was crossed with *mes-2* and *mes-4* null mutants. Strikingly, 100% of *glp-1(gf) mes-4* double mutant germlines (n=42) displayed germline tumors already at the permissive temperature of 15°C, while all *glp-1(ar202)* single mutant germlines (n=31) have wild type morphology at this temperature (Fig. 10A). In line with our observation that Notch–responsive genes correlate better with genes deregulated in *mes-4* than in *mes-2*, *mes-3*, or *mes-6* mutant animals, *glp-1(gf)* and *mes-2* did interact genetically, but the phenotype was less penetrant than the *glp-1(gf) mes-4* phenotype (Figs. 10B and 10C).

Based on these results, we postulate that Notch signaling in *C.elegans* germ cells and the MES–proteins, particularly MES–4, antagonistically regulate a subset of genes residing on the X–chromosome. Also, based on the genetic interaction phenotype, coregulated genes are involved in the mitosis–meiosis decision of germ cells in *C.elegans*. Since germ cells of *glp-1(gf) mes-4* double mutants are able to differentiate into sperm (Fig. 10A, proximal gonad end), female and male germ cells are inherently different in their response to Notch signaling or in their requirement for MES proteins. Future analysis of the interaction of Notch signaling with the Polycomb system will be carried out by Balazs Hargitai, a Post Doc in the lab.

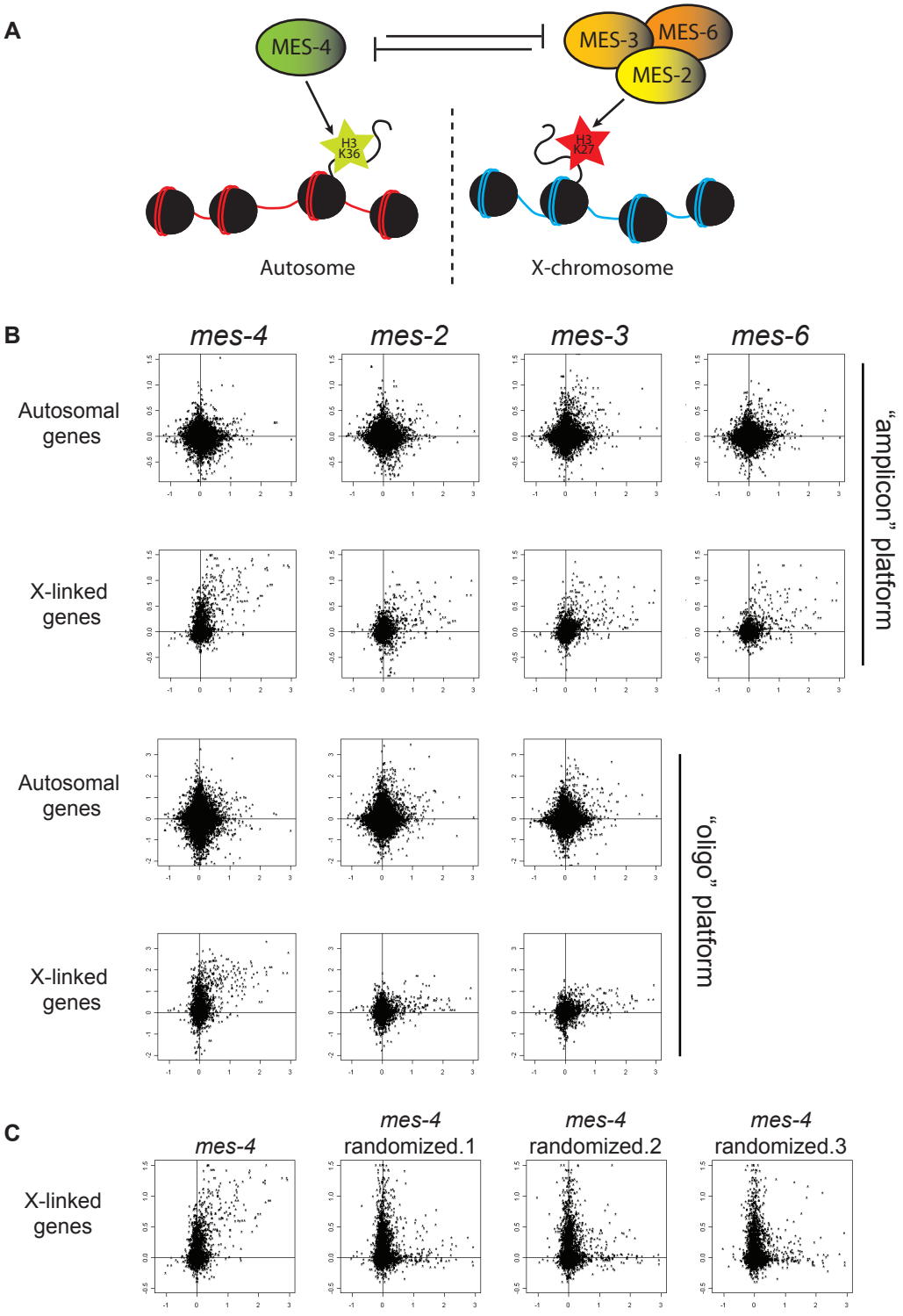


Figure 9. **Notch signaling and MES/Polycomb proteins regulate the same genes.**
(A) Schematic drawing of chromatin-modifying activity and mutual regulation of MES proteins as described in the text. (B) Correlation of fold change in response to Notch signaling (X-axis) with fold change in the indicated *mes* mutant obtained with amplicon oligo microarray platforms (Y-axis). X-linked genes and autosomal genes are shown separately. Correlation is restricted to X-linked genes. (C) Randomly assorting X-linked genes with their respective fold changes in *mes-4* mutants (panels #2-4) disrupts correlation (panel #1), indicating that Notch signaling and MES proteins co-regulate specific genes rather than X-linked genes in general.

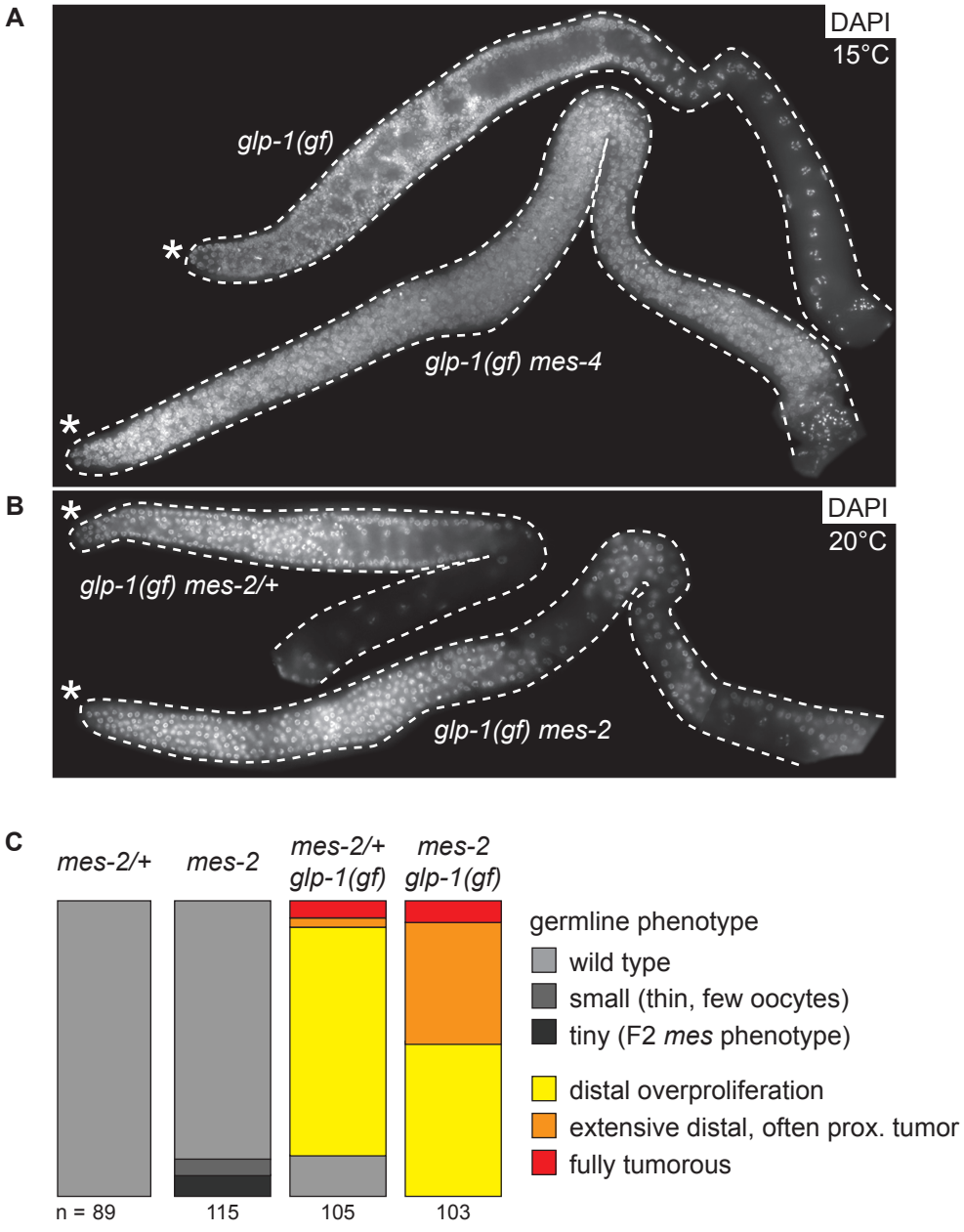


Figure 10. **Genetic interaction of *mes-4* and *mes-2* with *glp-1(gf)***
(A) DAPI staining of dissected *glp-1(ar202gf)* and *glp-1(ar202gf) mes-4(bn85)* gonads (outlined). Animals were raised at the temperature permissive for the *glp-1(gf)* mutation, 15°C. Nonetheless, the *glp-1(gf) mes-4* double mutant forms a germline tumor. (B) DAPI staining of *glp-1(gf)* gonads heterozygous (top) or homozygous (bottom) for *mes-2(bn11)*. The double mutant displays a compound tumorous phenotype at the semipermissive temperature of 20°C. (C) Quantification of the *glp-1(gf) mes-2* compound phenotype as observed in (B). n, total number of gonads. In (A) and (B), an asterisk marks the distal gonad.

4.2 CKI-2 repression promotes self-renewal of C.elegans germline stem cells

4.2.1 Cip/Kip CKI-2 mRNA levels are affected by Notch signaling

Several lines of evidence suggest that the decision of stem cells to self–renew or differentiate is closely connected to the cell cycle status. Terminal differentiation and cell cycle exit usually go hand in hand, but also partial restriction of progenitor potential is accompagnied with cell cycle changes. While pluripotent cells typically display rapid G1–to–S phase transitions, differentiation correlates with slower divisions, longer gap phases, and the introduction of cell cycle checkpoints (Richard–Parpaillon, Cosgrove et al. 2004; Lange, Huttner et al. 2009). This has prompted the idea that cell cycle changes may not only be a consequence of differentiation, but substantially contribute to the decision of a cell to differentiate and, in some cases, may be sufficient to direct the fate decision. Forced expression of factors promoting cell divisions, such as Cyclin D1 and Cdk4, impede differentiation (Skapek, Rhee et al. 1995; Lange, Huttner et al. 2009). Inversely, the cell cycle inhibitor p21 acts as a barrier to reprogramming (Hong, Takahashi et al. 2009).

Despite ample evidence for the importance of cell cycle regulation in self–renewal and differentiation, the connection between the cell cycle and critical stem cell factors is not well understood. We therefore analysed whether any cell–cycle related genes would respond to Notch signaling based on our expression data. The expression of most cell cycle components was not affected. This is expected, since both tissues are composed of proliferating cells with stem–cell like morphology. One cell cycle mRNA, however, is less abundant in the presence of Notch signaling: the mRNA encoding the Cip/Kip family cell cycle inhibitor CKI–2 (Fig. 11A and B). This was confirmed by quantitative PCR on dissected gonads (Fig. 11C).

CKI–2 is a member of the Cip/Kip family of cell cycle inhibitors. These small proteins bind to Cyclin E/CDK2 complexes and prevent their activation. Active Cyclin E/CDK2 promotes transition through the restriction point preceeding S phase. Transition beyond this point of the cell cycle is irreversible. Thus, Cip/Kip cell cycle inhibitors act as the last possible break when all components are assembled and ready for another cell cycle. Given previous work on the significance of G1–S regulation in the stem cell self–renewal versus differentiation decision in other systems (Lange and

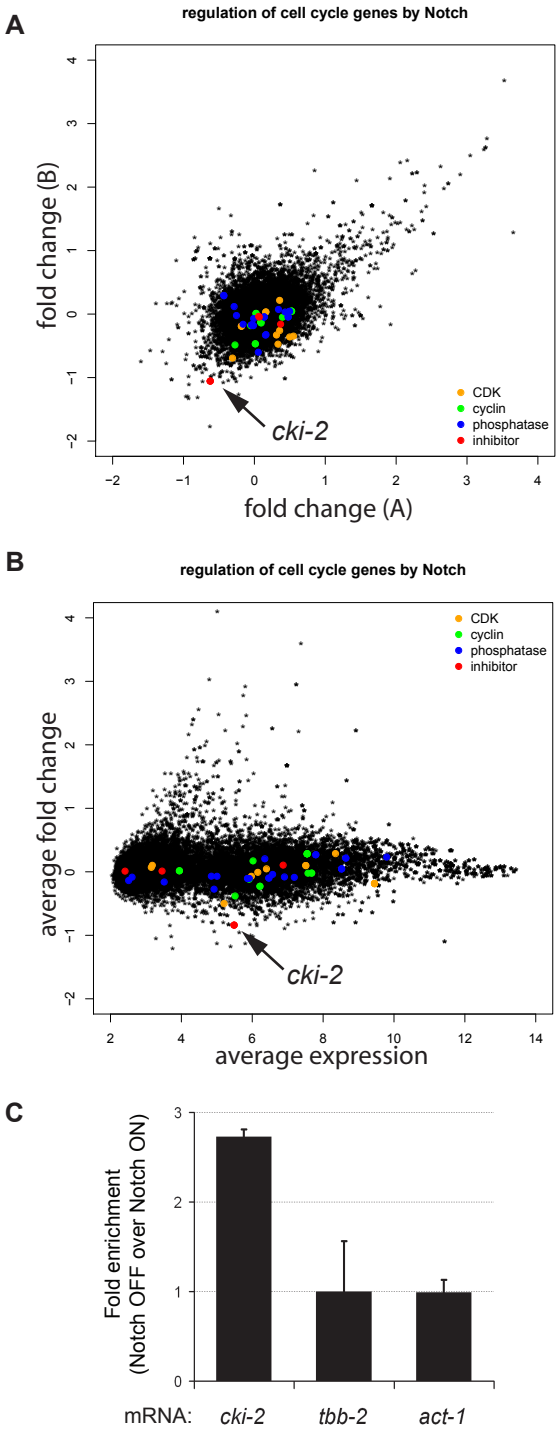


Figure 11. **Notch signaling downregulates *cki-2* mRNA levels.**
(A) Correlation plot as in Figure 8C. Coloured dots, cell cycle genes of indicated category. *cki-2* is downregulated in both biological repeats, A and B. (B) MA plot as in Figure 8B. Most cell cycle genes are expressed above background. *cki-2* is downregulated by Notch signaling. (C) Verification of (A) and (B) by qPCR. Relative expression of *cki-2*, *tbb-2* (tubulin) and *act-1* (actin) mRNAs in *gld-2 gld-1; glp-1(lf)* (Notch OFF) and *gld-2 gld-1; glp-1(gf)* (Notch ON) as determined by quantitative RT-PCR on dissected gonads. *cki-2* mRNA levels are three fold higher in the absence of Notch signaling.

Calegari 2010) and our own findings on the importance of CYE–1/CDK–2 regulation by GLD–1 during meiotic progression (Biedermann, Wright et al. 2009), we decided to have a closer look at the role of CKI–2 in the germline.

4.2.2 The Cip/Kip protein CKI-2 is repressed in the germline stem cells

The *C. elegans* genome encodes two members of the Cip/Kip family: CKI–1 and CKI–2 (Fig. 12A). They are separated by less than five kilobases and therefore likely arose from a gene duplication event. However, CKI–1 and CKI–2 have diverged substantially since both in sequence and function. CKI–1 is required in somatic blast cells for the proper timing of cell cycle withdrawal in the embryo as well as in juvenile seam cell lineages (Hong, Roy et al. 1998; Fukuyama, Gendreau et al. 2003). CKI–2 on the other hand is not essential and plays a minor role during vulval development (Buck, Chiu et al. 2009). By semi–quantitative RT–PCR and immunofluorescent detection, we found that CKI–1 and CKI–2 differ, in accordance with their mutant phenotypes, in their expression patterns. CKI–1 is expressed in specific cells in the embryo, but not in the germline (Fig. 12B). CKI–2 on the other hand is the predominant CKI in the adult germ line (Fig. 12C and 12E).

During 3'RACE experiments to determine the length of the *cki-2* 3'UTR (3'UTRs are usually badly annotated in *C.elegans*), we found that the *cki-2* gene encodes two mRNAs (*cki-2 L* and *cki-2 S*, 1316 and 951nt long, encoding a 259 and 175 amino acid–long protein, respectively; Fig. 12D). The larger form was cloned at a much higher frequency. Northern blots not only confirmed that the long isoform was far more abundant, but also showed that *cki-2* is a germline specific transcript (Fig. 12E). According to in situ hybridisations, *cki-2* mRNA is less abundant in the stem cell region, which has been observed previously (Kim and Roy 2006; Fig. 12F). This confirms our initial observation that Notch signaling negatively affects *cki-2* mRNA levels.

The protein expression pattern suggests a role for CKI–2 in entry or progression through meiosis. However, the *cki-2* null mutant is viable and fertile (Buck, Chiu et al. 2009), and CKI–2 is not essential for either process. However, the *absence* of Cip/Kip proteins is a hallmark of pluripotent cells (Ramalho–Santos, Yoon et al. 2002;

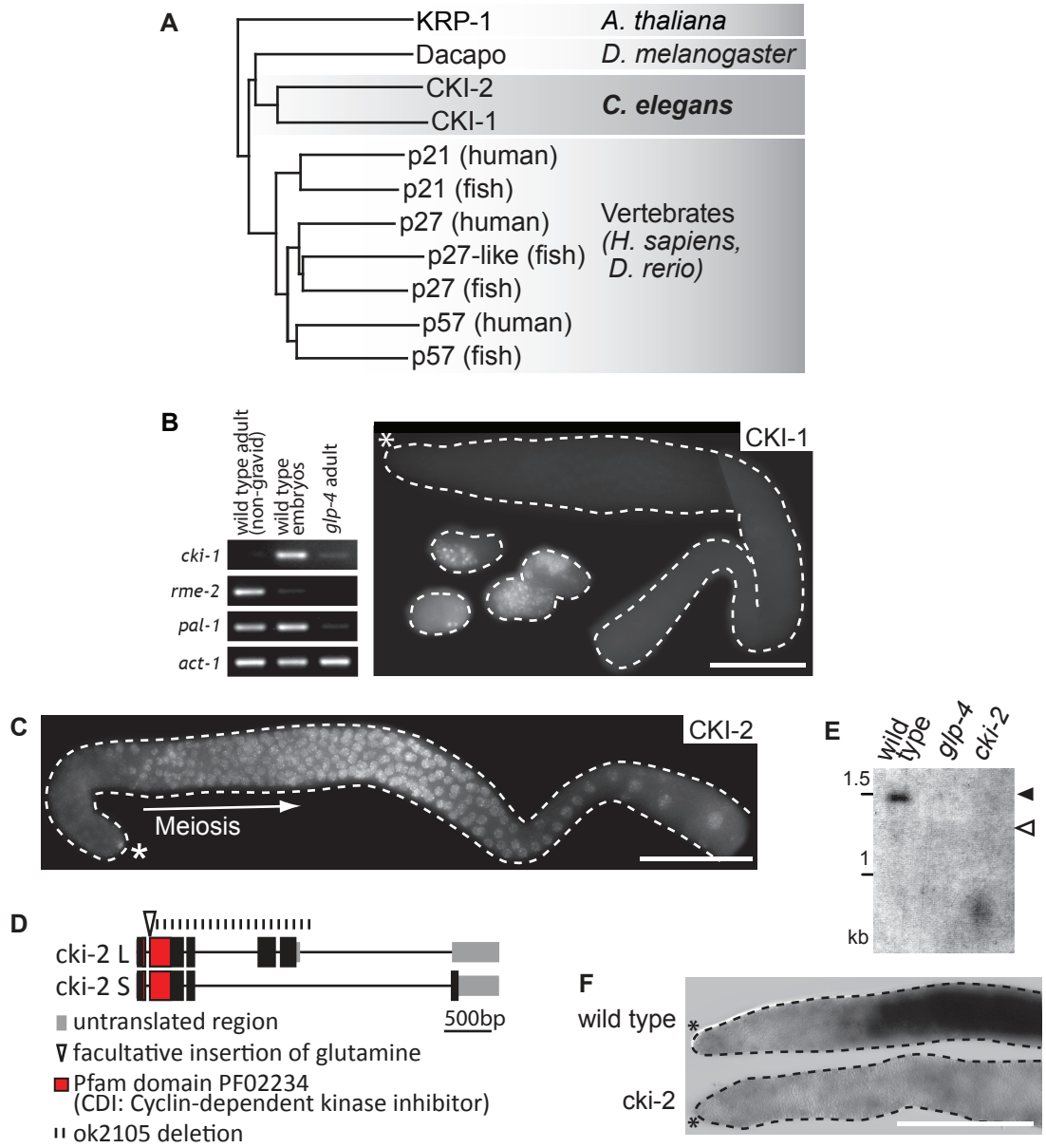


Figure 12. **The Cip/Kip protein CKI-2 is expressed in meiotic germ cells.**
(A) Phylogenetic tree of Cip/Kip proteins obtained with ClustalW (default settings). Protein sequences were retrieved from Uniprot. (B) Semi-quantitative RT-PCR of indicated mRNAs on indicated RNA samples (left); immuno-staining for CKI-1 protein on dissected gonad and embryos (outlined; right). Both *cki-1* mRNA and CKI-1 protein are exclusively detected in embryos. *rme-2*, germline-specific transcript. *pal-1*, germline and embryonic transcript. *act-1*, actin. *glp-4*, germlineless animals. (C) Immunodetection of CKI-2 in a dissected gonad (outlined). CKI-2 is expressed upon meiotic entry but absent from the distal-most stem cell compartment. (D) *cki-2* gene structure. By 3'RACE analysis, *cki-2* encodes two alternatively spliced mRNA isoforms, *cki-2* L and S. (E) Northern blot of *cki-2* mRNA isolated from non-gravid wild type, *glp-4* and *cki-2(ok2105)* mutants using a probe against both isoforms. *cki-2* L (black arrowhead, 1316nt plus poly-A tail) predominates, while *cki-2* S (951nt + poly-A tail, empty arrowhead) is hardly detectable. *cki-2* mRNA is absent from germlineless *glp-4* animals. (F) In situ hybridisation for *cki-2* on dissected gonads (outlined; distal region) of the indicated genotypes. *cki-2* mRNA is absent from the stem cell compartment. Scale bars: 50µm. .

Ginis, Luo et al. 2004; Barta, Vinarsky et al. 2010), which made us wonder whether repression of CKI–2 in GSCs is important for their maintenance.

4.2.3 MEX-3 regulates expression of CKI-2 in certain genetic backgrounds

Although *cki-2* mRNA is present in Notch ON as well as in Notch OFF tumors (Fig. 11), we could not observe CKI–2 protein by immunofluorescence in either case (data not shown). Thus, an additional regulatory mechanism preventing CKI–2 protein expression must exist. Posttranscriptional regulation is common in germ cells and is often mediated by the 3'UTR of mRNAs (Merritt, Rasoloson et al. 2008). A prime candidate for regulation of CKI–2 expression in Notch OFF tumors, where *cki-2* mRNA, but not the protein, is expressed, is the KH domain RNA binding protein MEX–3. Previous experiments by Björn Biedermann, a former PhD student in the lab, had demonstrated that MEX–3 was required for proliferation and the maintenance of stem cell identity in Notch OFF, but not Notch ON tumors (Fig. 13A). If MEX–3 would promote proliferation through repression of *cki-2,* this may explain why Notch OFF tumors, which express *cki-2* mRNA, but not Notch ON tumors, where the mRNA is less abundant, depend on MEX–3. Indeed, CKI–2 protein is upregulated in *mex-3, gld-1, gld-2; glp-1(lf)* (Notch OFF *mex-3(-)*) germ cells, indicating that MEX–3 prevents CKI–2 expression in Notch OFF tumors (Fig. 13B).

We therefore tested a direct association of MEX–3 with the *cki-2* mRNA. To this end, we expressed a GFP–tagged MEX–3 in Notch OFF *mex-3(-)* animals. The transgene was originally created in the laboratory of Jennifer Schisa (Jud, Razelun et al. 2007) and curiously, although it does not rescue the embryonic lethality of *mex-3* mutants, rescues the proliferation defect of Notch OFF *mex-3(-)* germ cells. *cki-2* mRNA can indeed by co–immunoprecipitated with MEX–3:GFP to the same extent as the established targets *rme-2* and *pal-1* (Fig. 13C).

Next, we tested whether CKI–2 expression in Notch OFF *mex-3(-)* germ cells was causal to germ cell proliferation arrest and differention. To this end, we constructed *mex-3 gld-1 gld-2; cki-2; glp-1* (Notch OFF *mex-3(-) cki-2(-)*) mutant animals. To our satisfaction, germcells in Notch OFF *mex-3(-) cki-2(-)* animals proliferated just like in Notch OFF animals (Table 5). Since removal of CKI–2 suppresses the proliferation defect caused by the *mex-3* mutation, we assume that CKI–2 is sufficient to arrest

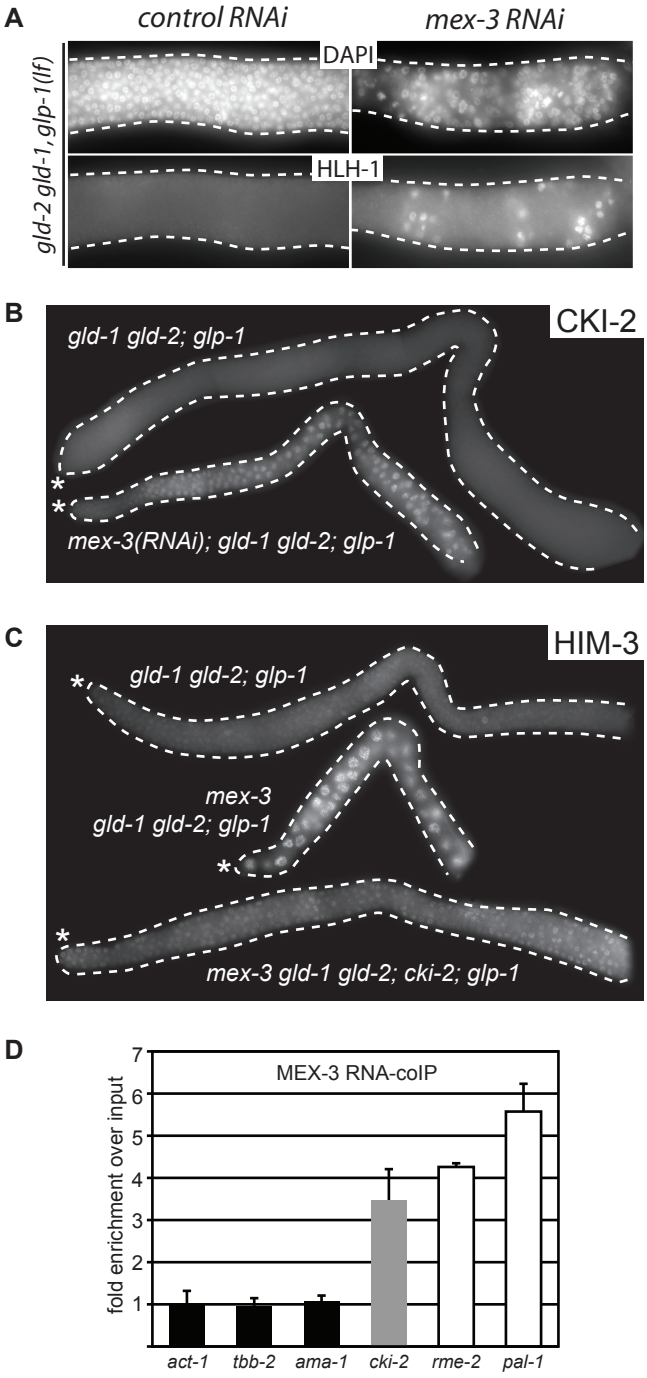


Figure 13. **MEX-3 maintains stem cell identity by repressing CKI-2**

(A) Proximal region of dissected gonads (outlined) of the indicated genotype stained for DAPI and HLH-1. In the absence of Notch signaling, germ cells initiate somatic differentiation (eg, express the muscle-specific transcription factor HLH-1) upon depletion of MEX-3, indicating that MEX-3 in this background prevents differentiation of germ cells. (B) CKI-2 staining on dissected gonads (outlined) of the indicated genotypes. MEX-3 prevents accumulation of CKI-2 and promotes proliferation of germ cells (compare gonad size). (C) HIM-3 staining on dissected gonads (outlined) of the indicated genotypes. Removal of CKI-2 not only rescues the proliferation defect (compare gonad size), but also prevents accumulation of the meiotic marker HIM-3, an early step in germ cell differentiation. (D) *cki-2* mRNA co-immunoprecipitates with MEX-3. Fold enrichment over input of the indicated mRNAs in a MEX-3:GFP RNA-co-IP. *rme-2*, *pal-1*, known targets of MEX-3. *act-1*, actin; *tbb-2*, tubulin; *ama-1*, RNA Pol II.

germ cell proliferation, to induce differentiation, and that it can associate with and be regulated by MEX–3.

Table 5. *Contributions of MEX-3 and Notch signaling to CKI-2 regulation and stem cell maintenance.*

genotype			**phenotype**	***cki-2* mRNA**	**CKI-2 protein**
gld–1 gld–2; glp–1(–)			stem cell tumor	high	–
gld–1 gld–2; glp–1(–)	– MEX–3		differentiation	n.d	+
gld–1 gld–2; glp–1(–)	– MEX–3	– CKI–2	stem cell tumor		
gld–1 gld–2; glp–1(++)			stem cell tumor	low	–
gld–1 gld–2; glp–1(++)	– MEX–3		stem cell tumor	n.d	–

The fact that the proliferation defect of Notch OFF *mex-3(-)* germ cells could be rescued by depleting *cki-2* allowed us to test potential differential requirements of *cki-2 S* and *cki-2 L*. An RNAi clone targeting exons 4 and 5, which are present in *cki-2 L* but not *cki-2 S*, rescued the proliferation defect of Notch OFF *mex-3(-)* germ cells as efficiently as an RNAi clone directed against the entire *cki-2* coding sequence (data not shown). At least in the Notch OFF background, *cki-2 S* therefore does not contribute to cell cycle regulation.

Immunostainings for meiotic markers in the germline stem cell tumors Notch OFF and Notch OFF *mex-3(-) cki-2(-)* indicated that, although germ cells proliferate in both cases to a similar extent, they may be inherently different in their meiotic disposition. HIM–3 and HTP–3, components of the synaptonemal complex, are absent from stem cells and are deposited on chromosomes in early meiosis. Both are however expressed in mitotically proliferating Notch OFF *mex-3(-) cki-2(-)* germ cells and, at lower levels, in Notch OFF germ cells (Fig. 14). Thus, proliferating germ cells in those tumors may represent some kind of transit amplifying stem cell population, which in the wild type is short lived and therefore has not yet been described.

However, although MEX–3 regulates CKI–2 protein expression and associates with the *cki-2* mRNA in sensitized genetic backgrounds, CKI–2 is not overexpressed in the stem cell compartment of the *mex-3* single mutant (Fig. 15). This left us with the task to identify additional regulators of CKI–2 expression.

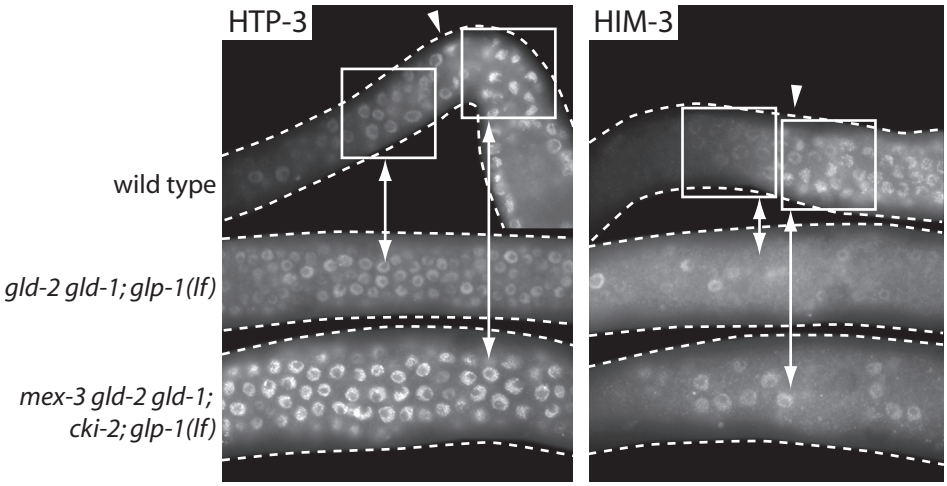


Figure 14. **Germ cell tumors may represent a transit amplifying population.**
Meiotic entry region of dissected gonads (outlined) of indicated genotype stained for the meiotic markers HTP-3 and HIM-3. Arrowhead, meiotic entry zone as indicated by the appearance of half-moon shaped nuclei. HIM-3 or HTP-3 are not expressed in the wild type distal stem cell compartment. Levels increase prior to and during meiotic entry (top gonad). In terms of HIM-3/HTP-3 expression, *gld-2 gld-1; glp-1* germ cell tumors resemble wild type germ cells prior to transition zone, while *mex-3 gld-2 gld-1; cki-2; glp-1* germ cell tumors resemble germ cells after meiotic entry (boxes/arrows).

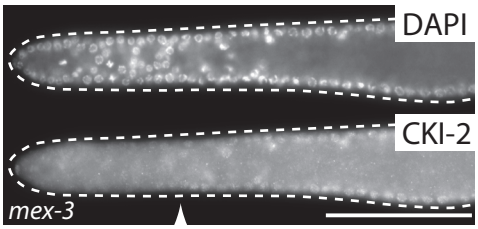


Figure 15. **MEX-3 is not required for CKI-2 repression in the wild type stem cell compartment.**
Distal end of dissected *mex-3* mutant gonads (outlined) stained for DAPI (top) and CKI-2 (bottom). CKI-2 is not expressed in stem cells in the absence of MEX-3.

4.2.4 The *cki-2* 3'UTR mediates repression of a reporter in germline stem cells

To test if CKI–2 expression may be regulated via its 3'UTR, we produced transgenic lines expressing a reporter gene (Histone 2B coupled to GFP) from a defined locus on chromosome two (Frokjaer–Jensen, Davis et al. 2008; Merritt, Rasoloson et al. 2008). The expression pattern of this reporter is entirely controlled by the 3'UTR and regulatory elements therein (Merritt, Rasoloson et al. 2008). All germ cells expressed H2B–GFP when it was fused to the 3'UTR of the housekeeping gene *tbb-2* (tubulin). When coupled to *cki-2* 3'UTR, the reporter was repressed in the stem cell compartment, just like endogenous CKI–2 (Fig. 16A). The *cki-2* 3'UTR is therefore sufficient to reproduce the expression pattern of endogenous CKI–2, and accordingly must recruit regulators that prevent translation of the reporter in stem cells.

4.2.5 The *cki-2* 3'UTR recruits two conserved stem cell factors: MEX-3 and FBF

Regulation conferred by the *cki-2* 3'UTR may depend on miRNAs. According to available online prediction tools MirWip, PicTar, MirZ, and TargetScan (Lall, Grun et al. 2006; Ruby, Jan et al. 2006; Hammell, Long et al. 2008; Hausser, Berninger et al. 2009), *cki-2* is however likely not a miRNA target – in contrast to its sibling gene *cki-1*, which harbours several conserved miRNA binding sites in its 3'UTR. We figured – based on our observation that CKI–2 overexpression in compound backgrounds drives stem cells into meiosis – that proteins required to maintain stem cells in the *C.elegans* germline may be candidates to regulate *cki-2*. RNA binding stem cell factors in *C.elegans* include FBF–1 and FBF–2 (collectively referred to as FBF), two very similar and largely redundant proteins of the Pumilio family, as well as the Pumilio family member PUF–8 and the conserved KH–domain protein MEX–3, which function redundantly to maintain stem cell proliferation. *mex-3 puf-8* double mutants germ cells arrest proliferation during juvenile stages (Ariz, Mainpal et al. 2009). *fbf-1 fbf-2* double mutants set up a germline during juvenile stages, but after the initiation of spermatogenesis in late L4, stem cells are lost by differentiation into sperm (Crittenden, Bernstein et al. 2002).

Sequences required for association of MEX–3 and Pumilio proteins with their targets have been identified *in vitro* and *in vivo* (Table 1 and Pagano, Farley et al. 2009).

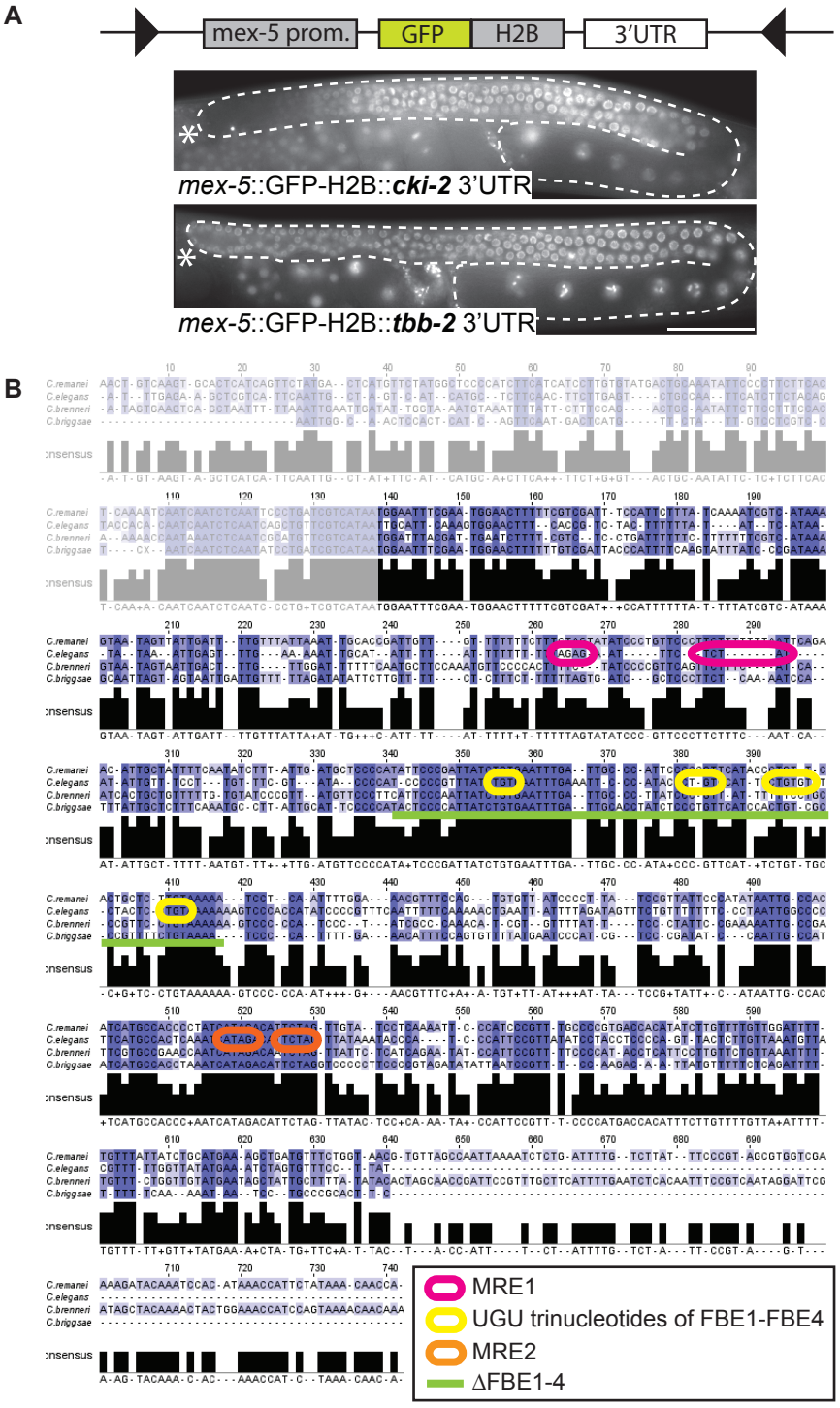


Figure 16. **The *cki-2* 3'UTR confers repression in stem cells and contains conserved elements.**
(A) Gonads (outlined) from live worms expressing reporters (depicted schematically; black triangles: recombination sites) under the control of the indicated 3'UTR. The distal gonad is marked by an asterisk. The *cki-2* 3'UTR prevents expression in stem cells. Scale bar, 50µm. (B) Alignment of the *C.elegans cki-2* 3'UTR and equivalent sequences from *C.briggsae, C.brenneri,* and *C.remanei.* Several regions are conserved, indicating functionality. MRE1, MRE2, FBE1-4 and ΔFBE1-4 are indicated by colours. Pale region, exon 4 of *cki-2* S (see Figure 12).

In both cases, recognition sites are fairly redundant and therefore likely to occur by chance every few hundred base pairs. Relevant sites in sequences such as UTRs, which have to fit functional constraints locally and are otherwise free to evolve, can be identified based on conservation. For example, miRNA target prediction algorythms use conservation to determine the probability of a site being true. Hans Rudolf Hotz from the Bioinformatics facility at FMI therefore extracted the putative *cki-2* 3'UTR sequences from *C.briggsae*, *C.brenneri*, and *C.remanei*. In alignments using ClustalW and VectorNTI, several regions were conserved, indicating functionality (Fig. 16B). To our satisfaction, one conserved block contained the MEX–3 binding motif DKAG(N0–8)UHUA (MRE; D = A, G, or T; K = G or T; H = A, C, or T; Pagano, Farley et al. 2009). Other conserved clusters contained variations of the UGU – AU motif, hallmark of Puf protein binding sites (Wickens, Bernstein et al. 2002) Table 1, and Fig. 16B).

The assay normally used in the lab to test protein–RNA interactions includes cell–free *in vitro* translation of the protein and coprecipitation with a biotin–labelled RNA. This assay has been used successfully to confirm GLD–1 binding to its targets (Lee and Schedl 2001; Biedermann, Wright et al. 2009; Wright, Gaidatzis et al. 2010). However, the same experiment with FBFs, PUF–8 and MEX–3 proved technically challenging. While MEX–3 would require the addition of crude worm extract to associate with its putative cognate sequence – rendering our attempts to show direct association pointless – Pumilio proteins would not associate with the positive control *gld-1* mRNA (Crittenden, Bernstein et al. 2002) at all. We therefore initiated a collaboration with the lab of Sean Ryder at Massachusettes Medical School, who have expertise in determining RNA–protein interactions with fluorescence electrophoretic mobility shift (F–EMSA) and fluorescence polarization (FP) assays and have previously worked with Pumilio and MEX–3 (Pagano, Farley et al. 2009; Pagano, Clingman et al. 2011). The putative MEX–3 recognition element occurs twice in the *cki-2* 3'UTR. The 5' MRE (MRE2; ATAGacatTCTA) caught our attention because of its conservation. The 3' MRE (MRE1; AGAGaauuUCUC) was identified only by targeted search because is not conserved. MRE1 cannot be tested by *in vitro* methods because it folds into a stable hairpin, and is not expected to associate with MEX–3 *in vivo*. In contrast, the 5' MRE (MRE2; AUAGacauUCUA) is highly conserved and associated with MEX–3 tightly ($K_{d, app}$ = 9 ± 1 nM F–EMSA, 15 ± 1 nM FP). Mutant variants that disrupt MRE2 bound MEX–3 with reduced affinity, confirming the specificity of the interaction (Fig. 17A; Table 6).

Interestingly, the MEX–3 motif was originally reported to consist of two half sites (A and B) separated by maximally eight bases. However, in the case of the *cki-2* 3'UTR, two B sites seem to contribute to binding, with more than eight bases intervening between the A site and the second B site (Table 6).

Table 6. Affinities of MEX–3 and FBF–2 for predicted binding sites in the *cki-2* 3'UTR.

Identifier	Sequence	Kd,app (GS)	Kd,app (FP)
cki–2 MRE2 WT	ACUCAAAUCAUAGACAUUCUAGUUAUAAAU	9 ± 1 nM	15 ± 1 nM
cki–2 MRE2 mut1	ACUCAAAUC**CCCC**ACAU**CCCC**GUUAUAAAU	80 ± 20 nM	76 ± 4 nM
cki–2 MRE2 mut2	ACUCAAAUC**CCCC**ACAU**CCCC**GU**CCCC**AAU	> 500 nM	> 500 nM
gld–1 FBEa	AUAGAAUCAUGUGCCAUACAUCAUGUUG	32 ± 1 nM	11 ± 2 nM
cki–2 FBE1 WT	UUUAUCUGUGAAUUUGAAAU	26 ± 12 nM	5 ± 1 nM
cki–2 FBE1 mut	UUUAUC**ACA**GAAUUUGAAAU	> 500 nM	> 500 nM
cki–2 FBE2 WT	CAUACCCUGUCCAUUUCUGU	> 500 nM	> 500 nM
cki–2 FBE2 mut	CAUACCC**ACA**CCAUUUCUGU	> 500 nM	> 500 nM
cki–2 FBE3 WT	CAUUUCUGUGUUCUACUCCU	240 ± 8 nM	114 ± 2 nM
cki–2 FBE3 mut	CAUUUC**ACA**GUUCUACUCCU	NB	> 500 nM
cki–2 FBE4 WT	CUACUCCUGUAAAAAAAGUC	300 ± 40 nM	95 ± 3 nM
cki–2 FBE4 mut	CUACUCC**ACA**AAAAAAAGUC	NB	>> 2 µM

reported values are the average of three replicates ± one standard deviation

Results obtained in the meantime with reporters *in vivo* suggested that PUF–8 may not be crucial for regulation via the *cki-2* 3'UTR, while FBF proteins were definitely involved in regulating the reporter (see next section). Within a highly conserved region required for reporter regulation *in vivo* (next section), we observed five UGU motifs in the context of four possible FBF binding elements, FBE1 through 4 (Fig. 16B). FBE1 and FBE4 are conserved, while FBE2 and FBE3 are not. FBF–1 and FBF–2 act redundantly to promote stem cell divisions (Bernstein, Hook et al. 2005) and bind identical sequences *in vitro* (Wang, Opperman et al. 2009). Because FBF–2 is easier to handle biochemically, it was used for interaction assays. FBF–2 was found to bind with high affinity to FBE1 ($K_{d,\ app}$ = 26 ± 12 nM, F–EMSA, 5 ± 1 nM, FP), which is comparable to the previously described FBF affinity to a site in the *gld-1* 3'UTR ($K_{d,\ app}$ = 32 ± 1 nM, F–EMSA, 11 ± 2 nM, FP; (Merritt and Seydoux 2010). We also found that FBF–2 bound with modest affinity to FBE3 and FBE4 elements (FBE3: $K_{d,\ app}$ = 240 ± 8 nM F–EMSA, 114 ± 2 nM FP; FBE4: $K_{d,\ app}$ = 300 ± 40 nM F–EMSA, 95 ± 3 nM FP), and weakly to FBE2, which is less

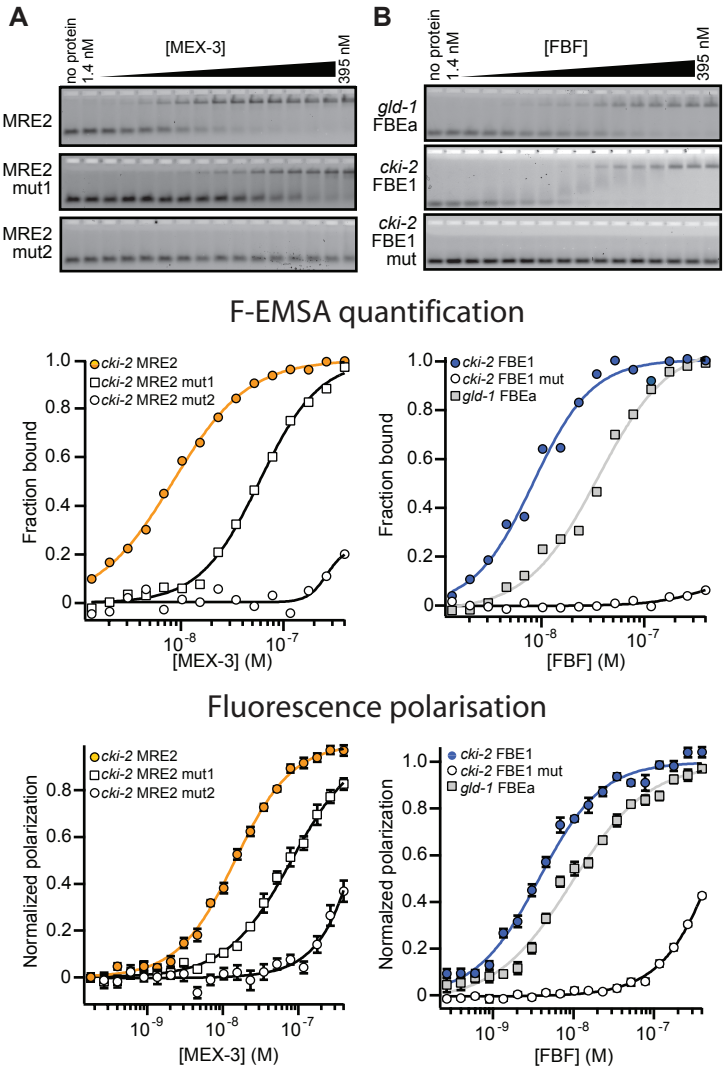


Figure 17. **Affinities of MEX-3 and FBF-2 for binding elements in the *cki-2* 3'UTR.**
(A) Representative F-EMSA gel pictures demonstrating association of MEX-3 with wild type and mutated elements (top). Quantification of F-EMSA gel pictures (center). Quantification of affinity as determined by fluorescence polarization assays (bottom). Fraction of bound RNA (center) and polarization (bottom) were plotted as a function of protein concentration and fit to the Hill equation to determine the apparent equilibrium dissociation constant (Kd,app). (B) Association of FBF-2 to cognate elements in the *cki-2* and *gld-1* 3'UTRs as in (B). See Experimental Procedures or Table 6 for oligonucleotide sequences used.

well conserved ($K_{d,\ app}$ > 500 nM). Mutating the UGU sequence to ACA eliminated FBF binding to all elements (Fig. 17B; Table 6).

These experiments demonstrate that both MEX–3 and FBF–2 tightly associate with specific conserved elements in the *cki-2* 3'UTR and may therefore not only be relevant for regulation in vivo, but actually promote stem cell identity and proliferation through the repression of the cell cycle inhibitor and differentiation–promoting factor CKI–2.

4.2.6 A region associating with FBF-2 *in vitro* is required for regulation *in vivo*

In parallel to above *in vitro* experiments, we took a more unbiased approach to identify regulatory regions in the *cki-2* 3'UTR. We generated reporter lines with overlapping deletions in the *cki-2* 3'UTR (Fig. 18A). Deletion of the putative MEX–3 binding site, MRE2, did not affect distal repression of the reporter (ΔM2). Among five deletions removing both MRE2 and a substantial portion of the 3'UTR (Δ1ΔM2, Δ2ΔM2, Δ3ΔM2, Δ4, ΔM2Δ5), only Δ3ΔM2 caused distal expression of the reporter (Fig. 18A). Intriguingly, the Δ3ΔM2 deletion covers several partially conserved putative Pumilio/FBF binding elements. Removing only those elements (ΔFBE1–4) caused distal de–repression to the same extent as Δ3ΔM2 (Fig. 18A). In contrast, removing only the high–affinity site ΔFBE1 did not cause substantial derepression, although extremely faint GFP–H2B expression could be observed, as if the ΔFBE1 reporter was just barely repressed (data not shown). We therefore conclude that FBE1, although the highest affinity site as determined in vitro, needs to cooperate with other sites in the ΔFBE1–4 region to achieve repression.

4.2.7 Removing CKI-2 rescues the stem cell loss of FBF mutants

To test whether FBF proteins not only associate with FBE1–4 *in vitro*, but really mediate reporter regulation *in vivo*, we introduced the wild–type *cki-2* 3'UTR into the *fbf-1 fbf-2* mutant. Since *fbf-1 fbf-2* adults do not maintain a stem cell compartment, we assayed L4 larvae, in which stem cells do not yet depend on FBF and look superficially normal (Merritt and Seydoux 2010). Indeed, the reporter was derepressed in the distal germline in *fbf-1 fbf-2* animals (Fig. 18B). However, expression was still comparably

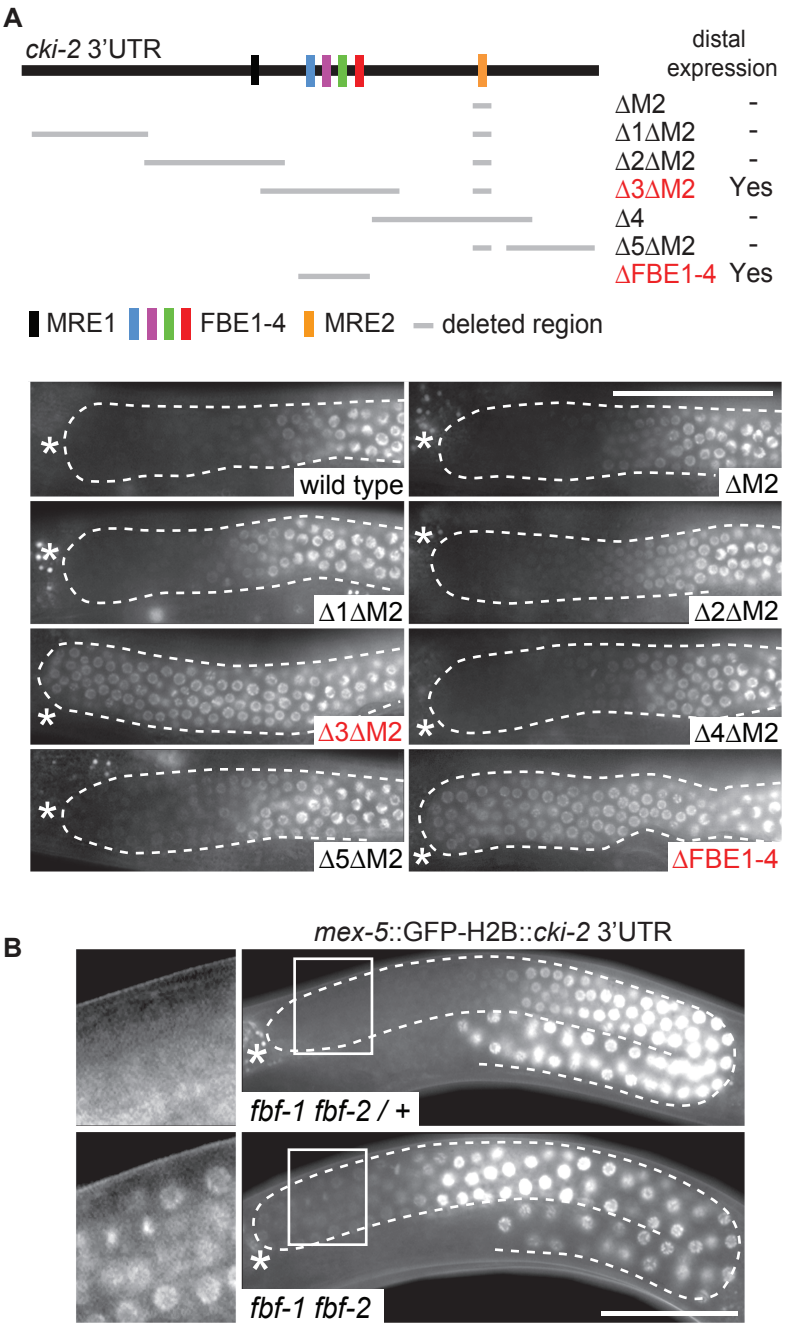


Figure 18. **FBE1-4 and FBF-1/2 are required for *cki-2* 3'UTR repression.**
(A) FBE1-4 are required for distal reporter repression mediated by the *cki-2* 3'UTR. Schematic representation of deletions introduced into the *cki-2* 3'UTR reporter transgene (top). Images of the stem cell compartment of live worms (outlined) expressing the indicated reporter transgenes. A region containing FBE1-4 is required for repression. (B) *cki-2* 3'UTR reporter expression in wild type (top) or *fbf-1 fbf-2* mutant (bottom) gonads (outlined). Boxed regions are displayed magnified and contrast-enhanced on the left. Scale bars, 50µm.

weak. To test which sequences are required for this residual repression, we also recombined the ΔFBE1–4ΔMRE2 and ΔFBE1–4 reporters with *fbf-1 fbf-2*. Unfortunately, the only *fbf-1 fbf-2* ΔFBE1–4 reporter line obtained from 700 singled balanced worms would not stably express the reporter even after repeated outcrossing (see Experimental Procedures). Since both a weakly expressing *fbf-1 fbf-2* ΔFBE1–4 and the stably expressing *fbf-1 fbf-2* ΔFBE1–4ΔMRE2 animals express GFP–H2B uniformly along the germline, we think that FBE1–4 recruits other repressors in *fbf-1 fbf-2* mutants. RNAi mediated depletion of candidate proteins – MEX–3, PUF–8, and all other Puf proteins of *C.elegans* for which RNAi clones were available in the lab (PUF–3, PUF–11, PUF–7, PUF–5, PUF–6) – did not enhance distal expression of the *cki-2* 3'UTR reporter in *fbf-1 fbf-2* mutants.

Our results demonstrate that FBF represses *cki-2* in germline stem cells, and suggests that an additional factor we could not identify contributes to repression of *cki-2* during larval development, when FBF is not critical. However, the effect is mediated through the region encompassing FBE1–FBE4, because this region is required for the residual repression observed in *fbf-1 fbf-2* mutant animals.

To test whether deregulation of CKI–2 was involved in the stem cell loss of *fbf-1 fbf-2* animals, we constructed *fbf-1 fbf-1 cki-2* triple mutants. As expected, most *fbf-1 fbf-2* mutant animals lost GSCs by day one of adulthood (Figs. 19A and B). In contrast, 90% of the *fbf-1 fbf-2 cki-2* adult animals retained GSCs (Fig. 19B). The reduced proliferation phenotype seen in *puf-8 (RNAi)* (Fig. 20A) or *puf-8 (RNAi); mex-3* animals (Ariz, Mainpal et al. 2009) could not be suppressed by removing CKI–2 (Fig. 20B), and the *cki-2* 3'UTR reporter was not derepressed by *puf-8(RNA*i). Together, these results demonstrate that CKI–2 is repressed in GSCs by FBF proteins, but not by PUF–8, and suggest that this repression is critical for the maintenance of GSCs.

4.2.8 Induced overexpression of CKI-2 in stem cells

Previously described experiments in sensitized genetic backgrounds demonstrated that ectopic expression of CKI–2 is sufficient to induce proliferation arrest and differentiation. For full proof of concept, we wanted to test a potential effect of CKI–2 overexpression on stem cell proliferation or stem cell identity. However, there are currently no tools available for inducible expression in the *C.elegans* germline.

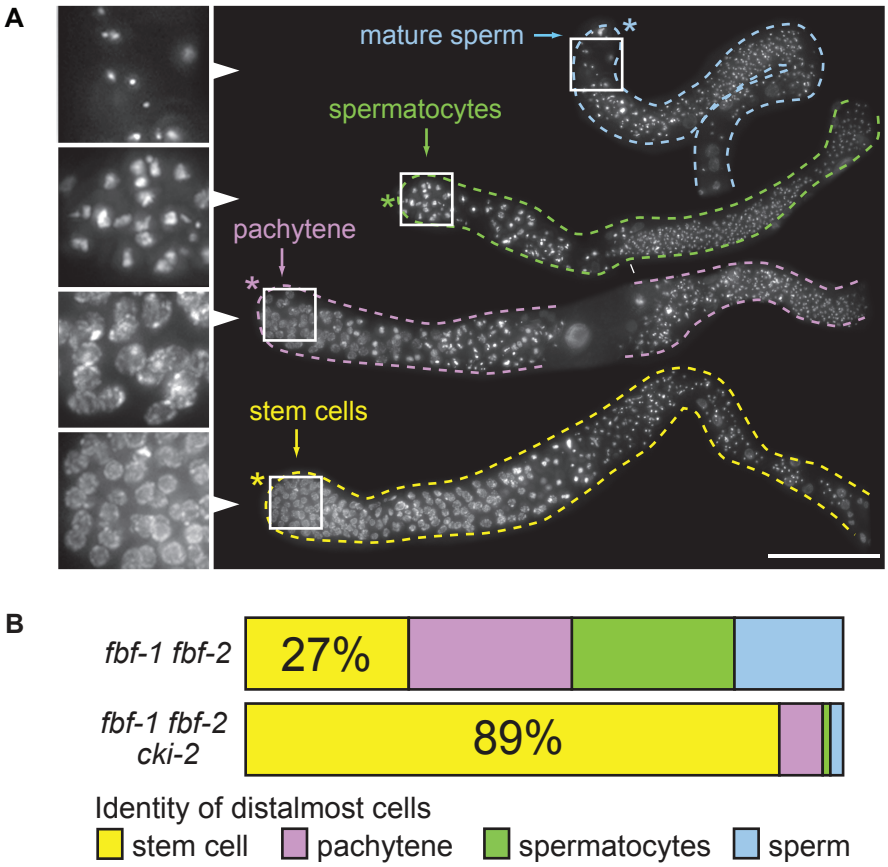


Figure 19. **CKI-2 contributes to stem cell loss in *fbf-1 fbf-2* mutants.**
(A) Dissected DAPI-stained gonads (outlined) from 1 day old *fbf-1 fbf-2* animals. Stem cells in *fbf-1 fbf-2* mutants are lost by differentiation via pachytene and spermatocyte stages into mature sperm. Representative images for four stages of progressive stem cell loss are shown. Boxed regions are magnified to the left. (B) Quantification of stages described above in *fbf-1 fbf-2* and *fbf-1 fbf-2 cki-2* mutant animals on day one of adulthood. While stem cells have been mostly lost from *fbf-1 fbf-2* double mutant gonads, they are retained in 90% of *fbf-1 fbf-2 cki-2* triple mutant gonads.

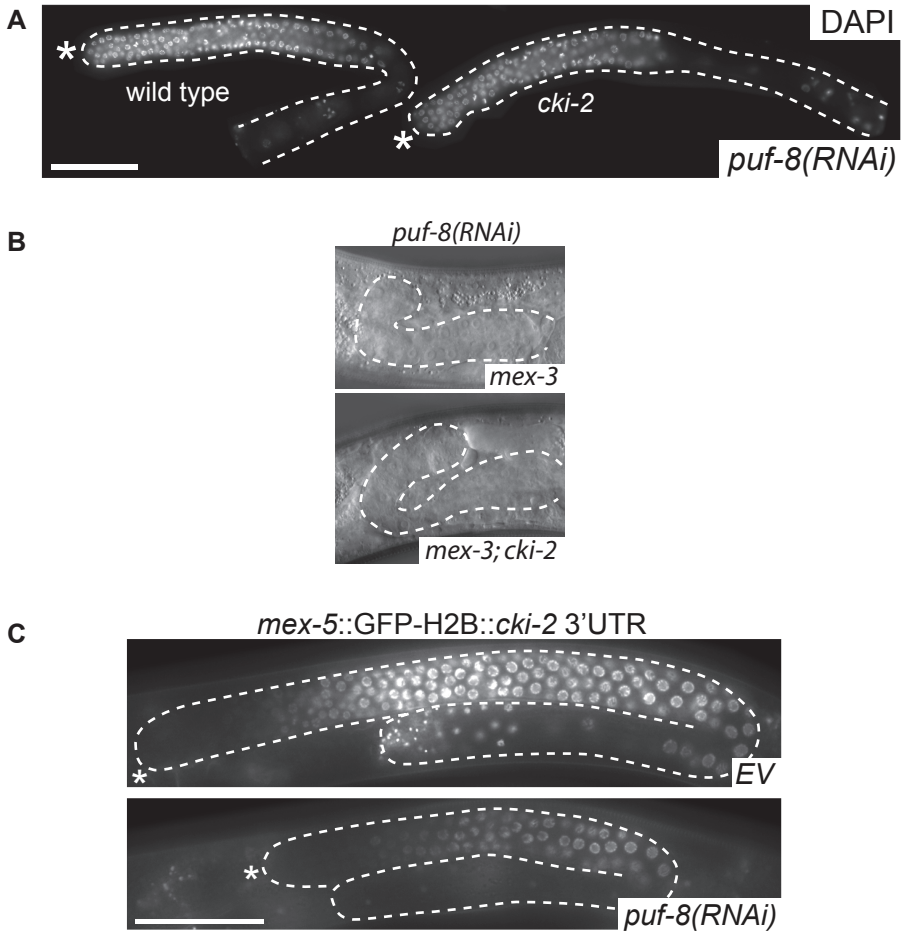


Figure 20. **PUF-8 does not regulate CKI-2.**

(A) DAPI stainings of gonads (outlined) dissected from *puf-8*(RNAi)-treated worms of the indicated genotypes. *puf-8*(RNAi) induces a "dwarf" gonad in wild type and *cki-2* mutants alike, indicating that the *puf-8*(RNAi) phenotype is not caused by CKI-2 expression (B) DIC images of gonads (outlined) from *puf-8*(RNAi)-treated worms of the indicated genotypes. Depletion of PUF-8 in a *mex-3* mutant background prevents germ cell proliferation irrespective of a *cki-2* mutation, indicating that the germ cell proliferation defect of *puf-8*(RNAi) *mex-3* animals is not caused by CKI-2 expression. (C) Live gonads (outlined) expressing the *cki-2* 3'UTR reporter treated with control (top) or *puf-8* (bottom) RNAi. The reporter is not derepressed in the stem cell compartment upon depletion of PUF-8.

Heatshock promotors work, if at all, only from the bend region onwards. We therefore decided to use a constitutive promotor to express a CKI–2 transgene in stem cells, and to confer inducible derepression using the *rme-2* 3'UTR. The mRNA encoding the yolk receptor *rme-2* is translationally repressed in stem cells by MEX–3 (Ciosk, DePalma et al. 2004). Depleting MEX–3 from the germline derepresses *rme-2*, but does not have any adverse effects on stem cells. By thethering the *cki-2* coding sequence to the *rme-2* 3'UTR, we hoped to induce CKI–2 expression in the distal stem cell compartment upon depletion of MEX–3. Since tagging CKI–2 with GFP may interfere with its function, we used the operon technique (Merritt, Rasoloson et al. 2008) to monitor expression of the transgene. With this tool, two genes separated by intergenic sequence from the endogenous *gpd-2/gpd-3* operon are expressed from the same promotor, but are individually controlled posttranscriptionally by their repective 3'UTRs (Fig. 21A). As 3' gene in the operon, we cloned GFP–H2B under the control of the *tbb-2* 3'UTR. GFP–H2B expression would therefore confirm that the mRNA encoding both genes was expressed throughout the germline. The CKI–2 coding sequence under the control of the *rme-2* 3'UTR was cloned as 5' gene in the operon (Fig. 21A). Transgenic lines were crossed into the *mex-3(or20)* mutant – and, surprisingly, developed normally. Antibody stainings for CKI–2 confirmed that we could not properly overexpress CKI–2 in the stem cell compartment with this approach. Although we observed overexpression of CKI–2 from transition zone onwards, distal levels remained low (Fig. 21B). Either the *rme-2* 3'UTR does not permit substantial distal expression, or CKI–2 protein is produced but subsequently degraded. An experiment demonstrating sufficiency of CKI–2 in promoting differentiation/meiotic entry therefore still needs to be performed.

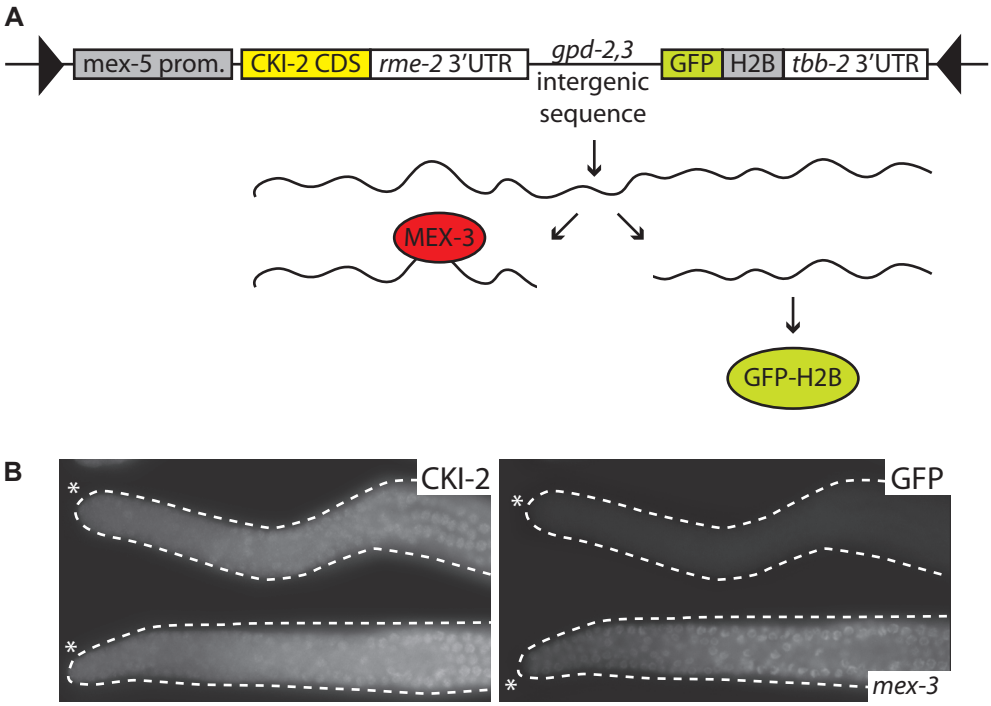


Figure 21. **CKI-2 cannot be overexpressed in the stem cell compartment.**
(A) Schematic drawing of a transgene designed for inducible overexpression of CKI-2 in the stem cell compartment. The operon concept from Merritt et al, *3' UTRs are the primary regulators of gene expression in the C. elegans germline*, 2008, was adapted to allow expression of untagged CKI-2 and GFP-H2B from the same promotor. CKI-2 was placed under the control of the *rme-2* 3'UTR, which is repressed in stem cells by MEX-3 (Ciosk et al, *ATX-2, the C. elegans ortholog of ataxin 2, functions in translational regulation in the germline*, 2004), while GFP-H2B under the control of the *tbb-2* 3'UTR is ubiquitously expressed (see Figure 16A). (B) Distal region of dissected *mex-3* mutant gonads (outlined) carrying the transgene described in (A), stained for CKI-2 and GFP, respectively. Top, sibling strain that did not inherit the transgene. Bottom, sibling strain carrying the transgene. CKI-2 is not overexpressed in the distal germline, and accordingly, stem cells are not affected.

Discussion

5. Notch target genes and stem cell identity

We did our best to design the microarray experiment in a way that would exclude most unwanted disturbing effects. Still, several features of the worm strains used may impact the result in an unwanted way. To be able to assay mRNA expression in the absence of Notch signaling, we had to use a genetic background devoid of the differentiation–promoting RNA regulators GLD–1 and GLD–2. These proteins are not expressed in germline stem cells (Jones, Francis et al. 1996; Wang, Eckmann et al. 2002), but since both GLD–1 (as a repressor of translation) and GLD–2 (as a poly(A)polymerase) may affect the abundance of mRNAs, we cannot exclude unwanted effects caused by both background mutations. It would be necessary to compare expression profiles of Notch *gain-of-function* tumors in the presence and absence of GLD–1 and GLD–2 to determine the influence of those background mutations. Also, the two Notch receptor mutations – *ar202tsgf* and *e2141tslf* – are temperature sensitive, which may affect our results. The *e2141tslf* allele was chosen because the null allele usually used in the lab, *q175*, is linked to a marker mutation (*unc-32(e189)*), which cannot easily be introduced into the Notch *gain-of-function* strain. Firstly, it is highly desirable in expression studies to keep the inherent genetic variations to a minimum; secondly, *unc-32(e189)* animals are severely uncoordinated and have difficulties feeding, which may indirectly affect germline proliferation. The temperature–sensitive nature of both alleles implies that we cannot exclude residual Notch signaling in *e2141tslf*, nor partial or inhomogenous activation of Notch signaling in *ar202tsgf*. Finally, parental animals were raised at the permissive temperature and then moved to the restrictive temperture for egglay. Slight variations in the onset of the temperature sensitive phenotype may have cause heterogeneity in the samples. Also, temperature shifts present a stressor for worms, which also may induce off–effects.

Nonetheless, we have reason to believe that the observed mRNA fold changes in response to Notch signaling reflect the actual relative abundance of transcripts and are not affected by *gld-1* and *gld-2* or *glp-1* alleles. Quantitative RT–PCR experiments by Balazs Hargitai confirmed the regulation and the fold change range for several putative targets, both using temperature sensitive and null alleles. Also, a strong Notch *gain-of-*

function mutant in which GLD–1 and GLD–2 were intact compared well to the temperature sensitive Notch mutant in the *gld-1 gld-2* background.

Whether putative Notch target genes as determined by our approach are bona fide Notch targets in the distal stem cell compartment remains to be determined. Unlike wild type germline stem cells, cells in tumorous germ lines are in close contact with sheath cells that outline the proximal gonad. Proximal sheat cells communicate to germ cells, possibly also via Notch signaling (McCarter, Bartlett et al. 1997; Hall, Winfrey et al. 1999; Killian and Hubbard 2005; McGovern, Voutev et al. 2009). Genes that we identified as Notch targets may have responded to a signal from proximal sheath cells and may not react to Notch signaling distally. Also, we dissected gonads from adult animals that had been exposed to constitutive Notch signaling for their entire life span. We therefore observed a steady state after prolonged signaling, including secondary effects, not the initial transcriptional reaction of germ cells to Notch signaling. Unequivocal determination of Notch signaling targets would require chromatin immunoprecipitation of either NICD or LAG–3 followed by microarray detection or deep sequencing from wild type animals. When designing these experiments, it is important to bear in mind that Notch signaling can also bypass the nuclear component LAG–3/CSL. CSL–independent activation of target gene transcription has been suggested (Sanalkumar, Dhanesh et al. 2010). In addition, Notch signaling has been reported to directly affect protein stability without the need for transcription (Sriuranpong, Borges et al. 2002) and to crosstalk to other signaling pathways (Wnt (Hing, Sun et al. 1994; Couso, Knust et al. 1995; Diaz–Benjumea and Cohen 1995; Rulifson and Blair 1995; Axelrod, Matsuno et al. 1996; Blair 1996; Neumann and Cohen 1996; Brennan, Tateson et al. 1997; Brennan, Klein et al. 1999; Wesley 1999; Lawrence, Klein et al. 2000; Strutt, Johnson et al. 2002; Hayward, Brennan et al. 2005; Sanalkumar, Dhanesh et al. 2010), GSK3beta (Foltz, Santiago et al. 2002; Saint Just Ribeiro, Hansson et al. 2009), BMP (Dahlqvist, Blokzijl et al. 2003; Takizawa, Ochiai et al. 2003), TGF–beta (Blokzijl, Dahlqvist et al. 2003; Rao and Kadesch 2003), EGFR (Tsuda, Nagaraj et al. 2002), MAP–kinase (Ordentlich, Lin et al. 1998; Zecchini, Brennan et al. 1999; Shaye and Greenwald 2002; Weijzen, Rizzo et al. 2002), NFkappaB (Nickoloff, Qin et al. 2002; Osipo, Golde et al. 2008), PKB/Akt (Rangarajan, Syal et al. 2001)) and thus may regulate target genes indirectly.

Whether or not genes that respond to Notch signaling in the tumorous settings used in our experiments are really expressed in stem cells, are really involved in their maintenance, and really react to Notch signaling therefore remains to be determined.

5.1 Crosstalk to MES-2/3/6 proteins/PRC2

The bias towards X–linked genes among genes upregulated in response to Notch signaling is striking, as well as their correlation with genes deregulated in Polycomb mutants *mes-2*, *mes-3*, *mes-4*, and *mes-6*. However, only a subset of X–linked genes and a handful of autosomal genes are affected, if we assume that both platforms grasp the full extent of regulation and report few false negatives. Correlation is lost upon randomization, which demostrates that X–linkage alone is not sufficient for correlation. Also, immunofluorescence experiments performed by Balazs Hargitai show that, despite the upregulation of a number of X–linked genes, the X–chromosome is still globally painted with repressive H3K27me3 marks in the Notch *gain-of-function* germline tumor.

Notch *gain-of-function* mutants and *mes* mutants interact genetically. If MES–2,3,6/PRC2 usually repress genes that are upregulated by Notch signaling, removing MES–2,3,6/PRC2 from mild Notch *gain-of-function* mutants should aggravate the phenotype, which is indeed the case. This confirms that the observed correlation is not an artefact, and indirectly supports the idea that we really identified Notch responsive genes, and that those genes we identified really contribute to germ cell proliferation (and possibly, stem cell identity). We are therefore confident that Notch signaling and *C.elegans* Polycomb regulatoin converge on a common set of genes. How they mechanistically do so remains to be determined. Notch target gene regulation requires chromatin remodeling (see also introduction). In the absence of Notch signaling, target genes are thought to be constitutively repressed by a transcription–hostile chromatin environment. Many remodelers and modifying complexes have been shown to affect the constitutive repression of target genes, the degree of transcriptional activation, and the shutdown of transcription; the precise composition and requirements of repressive and activating complexes however seemingly is strongly contextdependent. Based on our results, the MES proteins may be involved in repression of target genes before Notch signaling or in their shutdown after Notch signaling.

Polycomb was first discovered in Drosophila as a repressor of homeotic gene expression and body patterning. In vertebrates, repressive H3K27 methylation deposited by PRC2 in stem cells is commonly associated with genes encoding developmental regulators. PRC2 is supposed to mediate cellular memory of developmental cues (Margueron and Reinberg 2011). Similarly, MES–4, by association with germline expressed genes, has been suggested to memorize "germ cell identity" over generations (Rechtsteiner, Ercan et al. 2010). A possible scenario would be that Notch signaling as the key cue maintaining *C.elegans* germline stem cells provides information on germ cell identity that is remembered by the MES proteins, and thus passed on in the absence of Notch signaling to the next generation.

A link between Notch signaling and Polycomb has been previously suggested in *Drosophila*. Mutations in the fly protein Tantalus, which interacts with the Polycomb protein ASX (additional sex combs), elicits phenotypes reminiscent of Notch mutations and interacts with Notch genetically (Dietrich, Yang et al. 2005). Deregulation of the Polycomb proteins Pipsqueak and Lola cooperate with Delta overexpression in the formation of tumors in *Drosophila* (Ferres–Marco, Gutierrez–Garcia et al. 2006). However, Polycomb has been suggested to affect the availablity of core pathway components in flies and thus to act upstream of Notch signaling (Martinez, Schuettengruber et al. 2009; Herz, Madden et al. 2010). To our knowledge, we therefore show for the first time that Polycomb regulation and Notch signaling converge on a defined set of genes, which should also enable us to dissect the specifics of the coregulatory events. Do NICD and MES–2,3,6/PRC2 actually physically associate with the genes that are deregulated in their absence/hyperactivity? Do they co–occupy the same promotors? Does NICD (or other components of the signaling cascade) guide MES–2,3,6/PRC2 proteins to target genes, or inversely? Do the MES proteins affect repression, activation, maintenance, or shutdown of transcription of Notch targets? Do the two pathways also target genes independently of each other? Does the MES system somehow affect Notch signaling upstream of target gene activation, for example by modulating availability of core pathway components, as has been observed for the CoREST complex and Notch signaling pathway components in *C.elegans* vulval development (Bender, Kirienko et al. 2007)? All these experiments require the establishment of tissue–specific chromatin–IP procedures and reagents, which are not yet available for the worm. During the design of such experiments, it is important to

keep in mind that the strongest correlation as well as genetic interaction of Notch signaling was actually observed for MES–4. Although mutations in MES–4 cause identical defects as mutation of the *C.elegans* PRC2 complex components MES–2, MES–3, and MES–6, MES–4 is not actually part of the complex and deposits a different mark, H3K36me. Therefore, Notch may actually crosstalk to H3K36 methylation rather than to the MES–2,3,6/PRC2 mark H3K27me, and the weak interaction with MES–2, MES–3, and MES–6 may be a secondary effect. In contrast to H3K27me, H3K36me correlates with high transcription levels and is thought to be deposited during transcription in the gene body (Buratowski and Kim 2011). Interestingly, H3K36 methylation has also been associated with post–transcriptional deacetylation and re–silencing of genes (Lee and Shilatifard 2007) and is thought to be mutually exclusive with the H3K27 methylation mark deposited by PRC2 (Yuan, Xu et al. 2011). The hypothesis that Notch target genes cannot not be properly silenced in *mes* mutants might explain why genes that react to Notch signaling and genes that are upregulated in *mes* mutants overlap. However, the F1 *mes* mutant phenotype does not suggest hyperactive Notch signaling, since these germlines are – if anything – underproliferated, while Notch *gain-of-function* germlines are tumorous.

5.2 CKI-2

5.2.1 Role and regulation of CKI-2 in the *C.elegans* germline

In genomewide expression analyses, we found that mRNA encoding the Cip/Kip cell cycle inhibitor CKI–2 was less abundant in the presence of Notch signaling. The effect of Notch signaling on *cki-2* mRNA levels was verified by quantitative PCR. Nonetheless, the observed regulation could very well be indirect. We find that *cki-2* is targeted by FBF proteins, which have a record of destabilizing their targets (Goldstrohm, Hook et al. 2006; Goldstrohm, Seay et al. 2007). FBF proteins may just be more abundant or more active in Notch ON germ cells than in Notch OFF germ cells (although *fbf-1* and *fbf-2* mRNA levels are not affected by Notch signaling, Fig. 22). Promotor studies and chromatin–IP experiments would be required to determine if Notch signaling also regulates *cki-2* transcription directly. The putative *cki-2* promotor, 4.5kb of intergenic sequence separating *cki-1* and *cki-2*, was not able to drive expression of a GFP reporter

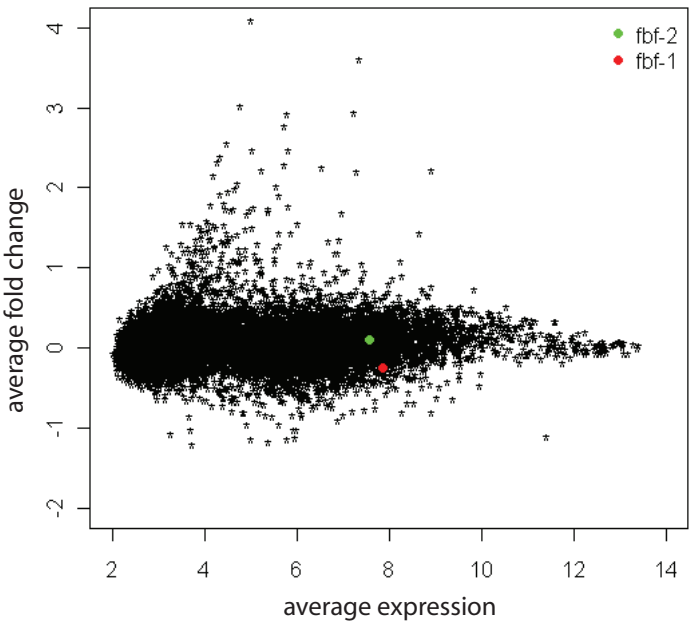


Figure 22. ***fbf* transcription does not depend on Notch signaling.**

MA-plot (X-axis, average expression; Y-axis, fold change) of transcript levels in response to Notch signaling. *fbf-1* and *fbf-2* mRNAs (red and green dot, respectively) are expressed above background and do not change in abundance in response to Notch signaling, indicating that Notch signaling is not required for *fbf-1 / fbf-2* transcription. Values are given in log2.

in the germline upon microparticle bombardment. However, since microparticle bombardment notoriously produces multicopy arrays prone to heterochromatisation in the germline, we cannot exclude that the used promotor fragment would have been competent to drive expression of a single–copy integrated transgene. Alternatively, the *cki-2* promotor may extend beyond the 5' intergenic sequence of *cki-2*. Indeed, regions upstream of *cki-1* are required for *cki-2* expression (Buck, Chiu et al. 2009). Fosmid engineering (Dolphin and Hope 2006; Sarov, Schneider et al. 2006; Zhang, Nash et al. 2008; Tursun, Cochella et al. 2009) would be the method of choice to determine whether *cki-2* expression is regulated transcriptionally, through which elements, and whether Notch signaling is involved.

Based on our immunofluorescence and semi–quantitative PCR assays, CKI–2 is expressed in the germline and also in the somatic gonad (data not shown), while CKI–1 is specific to embryos. This conflicts with earlier evidence of CKI–1 expression in the germline and regulation by E3 ubiquitin ligase CUL–2 (Feng, Zhong et al. 1999). We confirmed that CKI–2, but not CKI–1 staining disappears in *cki-2* mutants. Since both antibodies do not perform very well, different staining protocols may provide an explanation why Feng et al. could not notice CKI–2 expression in the germline.

We observe a division of labor between CKI–1 and CKI–2, CKI–1 acting in the soma, and CKI–2 in the germline. CKI–1 and CKI–2 likely arose from a gene duplication event, since they are separated by only 4.5 kilobases. Since CKI–1 in contrast to CKI–2 is essential, CKI–1 most likely retained the function of the ancestral gene to mediate withdrawal of cells from the cell cycle during development. Somatic expression of CKI–2, which we and others have observed with transgenes containing genomic *cki-2* sequence (Fukuyama, Gendreau et al. 2003), is likely due to incomplete inclusion of regulatory sequences in those transgenes, since we cannot detect *cki-2* in the soma by Northern blot or by immunofluorescence. Interestingly, according to new annotations, the intergenic region between *cki-1* and *cki-2* as well as sequences upstream of *cki-1* are rich in non–coding RNA loci, which may contribute to regulation of the locus.

Generally, we think that *cki-2* is expressed at low levels. Immunofluorescent stainings are of low intensity, in situ hybridisation requires a long (eg, label–rich) probe and, just like Northern blots, extended development times. In particular, the alternative isoform *cki-2 S* was hardly detectable by Northern blot. The exclusion of exons 4 and 5 in *cki-2 S* generates alternative C–termini, but does not affect the CKI

domain. We therefore assume that both *cki-2 S* and *cki-2 L* retain inhibitory activity towards CyclinE/CDK2 complexes. They may however differ in cell cycle independent functions, as has been proposed for vertebrate Cip/Kip proteins. Regions outside the inhibitory domain of vertebrate p27 and p57 have been reported to promote differentiation independently of the proteins' effect on cell cycle progression (Ohnuma, Philpott et al. 1999; Gui, Li et al. 2007). *cki-2 S* and l may also be differentially expressed between germline and somatic gonad, where CKI–2 staining has also been observed (data not shown).

Our experiments indicate that repression of CKI–2 in stem cells is important to promote stem cell proliferation and stem cell identity. But why would CKI–2 be expressed in more proximal gonad regions? CKI–2 is upregulated in the transition zone concomitantly with meiotic entry, but the protein is not essential for entry or progression through meiosis, since *cki-2* null mutants set up a wild type germline and are fertile. One clue may lie in the observation that most germline events in *C.elegans* are regulated redundantly. For example, MEX–3 and PUF–8 are required redundantly for mitotic proliferation, and GLD proteins together promote meiotic entry and progression. Similarly, CKI–2 may act redundantly with promeiotic proteins. CKI–2 has been proposed to be required for centrosome elimination, a finding which we and others could not confirm (Kim and Roy 2006; Buck, Chiu et al. 2009). However, CKI–2 may be required for other cell cycle related processes during meiotic progression, such as distinguishing pre–meiotic S–phase from mitotic S–phases, recombination, apoptosis, DNA damage repair, or oocyte maturation. The Drosophila Cip/Kip protein Dacapo is specifically required for genome stability during pre–meiotic S–phase (Narbonne–Reveau and Lilly 2009). In *C.elegans*, RAD–51, a marker of double strand breaks induced during meiosis, displays a wild type localization pattern in *cki-2* mutants, and the *cki-2* mutant strain does not diplay abnormally high mutation rates (as indicated by the spontaneous occurrence of locomotion mutants in the population) or defects in meiotic segregation (as indicated by high spontaneous incidence of males in the population; data not shown). We did however notice that the meiotic marker phospho–SUN–1, a linker protein anchored in the nuclear envelope (Penkner, Fridkin et al. 2009), labels a slightly expanded zone in *cki-2* mutants although it is eventually downregulated in late pachytene. A more detailed characterization of meiotic processes and their timing in the *cki-2* mutant, or an RNAi screen for genes affecting

cki-2 mutants but not wild type worms, may help to determine a role of CKI–2 in the germline.

Despite the fact that CKI–2 is still in search of a function in the wild type germline, it is expressed in a regulated pattern. The 3'UTR of *cki-2* is sufficient to confer this expression pattern to a reporter, indicating that the *cki-2* 3'UTR may be the prime source of expression regulation also for endogenous CKI–2. However, the expression pattern of the reporter and the expression pattern of endogenous CKI–2 as observed by immunofluorescence are not entirely identical. While the reporter gets upregulated gradually and uniformly in all nuclei in early transition zone, endogenous CKI–2 is specifically expressed in crescent–shaped transition–zone nuclei but not in neighbouring cells if they have not yet adopted the typical transition zone morphology. Additional mechanism such as protein degradation are therefore likely regulating CKI–2 prior to meiotic entry.

To identify regions involved in 3'UTR directed regulation, we looked for sequences conserved between the nematode species *C.elegans, C.briggsae, C.brenneri*, and *C.remanei*. This approach was partly successful – FBE1, which associates tightly with FBF–2 *in vitro*, and FBE4, which associates weakly, are indeed conserved, and FBF–1/FBF–2 regulate a reporter *in vivo*. However, one of the nonconserved elements (FBE3) also associates with FBF–2 *in vitro*. Inversely, the conserved MRE2 element associates tightly with MEX–3 in vitro, but in wild type adults, MEX–3 does not appear to regulate CKI–2 expression. MEX–3 restricts CKI–2 expression only in the compound mutant background *gld-1 gld-2; glp-1*. Although considering conservation was thereful helpful in our approach, the unbiased approach using reporter deletions was definitely crucial. Also, recently evolved regulatory elements would have escaped our attention with the conservatory approach. For example, miRNA binding sites are not necessary conserved. Most miRNA prediction programs do not consider the *cki-2* 3'UTR as a miRNA target; however, the TargetScan software identified the seed region of miRNAs *44, 45, 247*, and *61*, CUAGUCA, in the *cki-2* 3'UTR. The Δ1 deletion removes this site, but does not affect distal repression, indicating that these miRNAs do not contribute to CKI–2 regulation in the germline.

One potential MEX–3 binding element, MRE1, supposedly forms a stable stem loop structure and therefore is not predicted to associate with MEX–3. However, a recent report suggests that protein binding to the 3'UTR can unfold secondary

structures occluding regulatory sites (Kedde, van Kouwenhove et al. 2010). We cannot exclude a similar mechanism for MRE1, although we would expect some sequence conservation in that case. MRE2, the second, conserved MEX–3 binding element, tightly associates with MEX–3. Mutation experiments revealed that each halfsite of MRE2 is sufficient to direct association, at least *in vitro*. Also, they identified a second halfsite that does not conform to the in vitro defined criteria for MREs (Pagano, Farley et al. 2009) because it is separated by more than eight nucleotides from the first halfsite. This kind of binding element structure would likely not have been determined by oligonucleotide selection experiments *in vitro*, and it will be interesting to see whether other MEX–3 targets contain similar arrangements. The second halfsite may increase the affinity of MEX–3 for the region, enhancing its chances to recognize its bona fide binding site. Alternatively, an intermediate association/dissociation step involving the second halfsite may render MEX–3 binding and dissociation energetically more favourable or regulatable. It is also possible that the second half site is not relevant in vivo, and is an artefact provoqued by the use of short oligos in the interaction assays that lack the context of the entire 3'UTR.

Based on our observations that the region containing FBE1–4 is required for reporter regulation, that FBFs are required to fully repress a wild type reporter, and that the FBF mutant phenotype, but not the *puf-8(RNAi)* phenotype can be rescued by depletion of *cki-2*, we focused our *in vitro* association studies on FBF. FBF–1 and FBF–2 redundantly remain germline stem cells, implying that both associate with key targets. Therefore, only affinity to FBF–2 was determined. However, FBF–1 and FBF–2 do differ in a few amino acids in the first Pumilio repeat and may therefore also differ in their affinities towards targets. Comparing affinity and association dynamics of FBF–1 and FBF–2 with various cognate sequences would therefore be an interesting experiment. PUF–8 was not tested in vitro because mutating *cki-2* does not rescue the phenotype of *puf-8(RNAi)* animals, nor is the reporter derepressed distally upon depletion of PUF–8. However, both experiments rely on RNAi, because *cki-2* as well as the reporter integration site are too close to *puf-8* to attempt recombination. Also, for unknown reasons, the *cki-2* 3'UTR reporter expresses very poorly in PUF–8 depleted animals, hampering attempts to detect – possibly faint – reporter derepression in the distal stem cell compartment. Since additional factors that require the region between FBE1

and FBE4 are obviously required for reporter regulation, PUF–8 remains a candidate regulator of CKI–2 expression.

The sequences of FBE1–4 and their consensus correspond to the FBF–1 binding element determined by yeast–3–hybrid based selection and mutagenesis of short sequences (Table 8; Bernstein, Hook et al. 2005). The high–affinity element FBE1 and the non–binding element FBE2 both conform to the UGU(N2–4)AU consensus and are flanked by similar nucleotides. However, the FBE1 spacer contains a Purine following the UGU trinucleotide (which is according to the consensus strongly favoured but not obligatory in this position). FBE3 and FBE4 lack the 3' AU dinucleotide, but do contain AU rich 3' sequences. Interestingly, FBFs were thought to require three nucleotides between UGU trinucleotide and AU element (as opposed to two nucleotides required by PUF–8; Opperman, Hook et al. 2005). We find that FBE1, which has a two–nucleotide spacer, associates with FBF tightly.

Table 8. FBE1–4 correspond to the FBF consensus motif.

element	**sequence**	***in vitro* affinity for FBF-2**
FBE1	UUUAUCUGUGAAUUUGAAAU	++++
FBE2	CAUACCCUGUCCAUUUCUGU	–
FBE3	CAUUUCUGUGUUCUACUCCU	+
FBE4	CUACUCCUGUAAAAAAAGUC	+
consensus	YWYWYCUGURWWHWW	
FBF–1 binding site	NVNDNNHNUKHDHNDDN	

R = A,G; K = G,U; Y = C,U; W = A,U; V = A,C,G; D = A,G,U ; H = A,C,U ; N = any base

Curiously, the *cki-2* 3'UTR of *cki-2 L* (not of *cki-2 S*) contains, similar to its mammalian counterparts, an intron of 1.5kb only 54 bases downstream of the stop codon. The presence of exon junction complexes downstream of the stop codon usually triggers nonsense mediated decay (NMD; Chang, Imam et al. 2007). Possibly, NMD contributes to degradation of *cki-2* mRNA in proliferating cells both in worms and vertebrates. NMD components have also been implied in translational regulation (Isken, Kim et al. 2008). Since *cki-2 S* does not contain an intron, alternative splicing in this case may determine whether *cki-2* is subject to NMD regulation or not.

Interestingly, the 3'UTR of vertebrate p27 and p57 is also spliced. Certainly, the conservation of Cip/Kip 3'UTR splicing indicates the presence of a conserved regulatory mechanism acting on those mRNAs which is yet to be discovered.

5.2.2 The role of MEX-3 in stem cell regulation

MEX–3, as indicated in the introduction, is a curious protein. We find that MEX–3 tightly associates with the MRE2 site in the *cki-2* 3'UTR, which is also extraordinarily conserved – yet, in the wild type, neither MRE2 nor MEX–3 are required for reporter regulation. Additionally, in a genetic background where we know MEX–3 is essential for germ cell proliferation (*gld-2 gld-1; glp-1*), deleting the element from a reporter does not cause the expected effect (derepression), but the opposite (mild repression; data not shown), an observation we cannot explain to date. The requirement of crude worm extracts for association of MEX–3 with the *cki-2* mRNA in biotin assays implies the need of cofactors or protein modifications for association. During monoclonal antibody testing, we observed two closely spaced bands on western blots (data not shown), as if part of the protein pool carried a modification. Human MEX–3 proteins are phosphorylated (Buchet–Poyau, Courchet et al. 2007); phosphorylation is also predicted for *C.elegans* MEX–3. Also, *C.elegans* MEX–3 has been shown to associate with several proteins and, since it is the only MEX–3 family member without a C–terminal RING finger domain, may require protein partners that contain a RING finger. Our observation that an ectopic MRE site implanted into the *tbb-2* 3'UTR cannot confer regulation to a reporter agrees with the idea that MEX–3 may be unable to recognize its targets unless modified or bound by a cofactor. Sensitized backgrounds where MEX–3 is required for proliferation, *puf-8* or *gld-1 gld-2; glp-1(lf),* may be useful to screen for cofactors required for MEX–3 function in stem cells.

In the *C.elegans* germline, MEX–3 is required during early development, since germ cells never proliferate in *puf-8 mex-3* double mutant larvae. The early requirement for MEX–3 may indicate a role for MEX–3 in the proliferation arrest of L1 germ cells upon starvation. In our hands, *mex-3* mutant animals were able to enter and exit the dauer stage as assayed by body morphology. They were also able to enter and exit adult reproductive diapause, but neither entry or exit rates, nor the number of germ cells over time and their ability to reconstitute a germline after prolonged

starvation were quantified, experiments that might finally reveal a role for MEX–3 in germline stem cells.

5.2.3 FBF/Pumilio proteins and stem cell maintenance

FBF/Pumilio proteins notoriously pop up in germ cell and stem cell contexts (Wickens, Bernstein et al. 2002), an observation substantiated by the expression of DjPum in planarian stem cells, which have extraordinary regenerative capabilities (Salvetti, Rossi et al. 2005). RNA–coIP expermiments have suggested that FBF/Pumilio proteins govern entire pathways and functional units of the transcriptome. Indeed, *C.elegans* FBF associates with many factors of the meiotic entry program, such as the structural components of meiotic chromosomes HIM–3, HTP–1 and –2, SYP–1 and SYP–2. All these proteins are ectopically expressed in *fbf-1 fbf-2* mutants, but their depletion does not restore stem cells in *fbf-1 fbf-2* mutants (Merritt and Seydoux 2010). This suggests that among the many targets of FBF/Pumilio proteins, few may be actually relevant, and also indicates that the *cki-2* mRNA is a key target of FBF in germline stem cell maintenance in *C.elegans*.

Cip/Kip mRNAs have previously been co–immunoprecipitated with FBF/Pumilio proteins in vertebrates and *C.elegans* (Morris, Mukherjee et al. 2008; Kershner and Kimble 2010). Human Pum1 has recently been shown to modulate the accessibility of the p27 3'UTR for miRNAs, and thus to regulate the decision of cultured cells to remain quiescent or divide (Kedde, van Kouwenhove et al. 2010). Sterical hindrance of miRNA mediated regulation by RNA binding proteins has been previously demonstrated for DND in zebrafish germ cells (Kedde, Strasser et al. 2007) and may be of yet unrecognized importance in miRNA mediated regulation. Indeed, FBF/Pumilio targets contain disproportionally many miRNA binding sites (Galgano, Forrer et al. 2008; Leibovich, Mandel–Gutfreund et al. 2010), but *cki-2* is a weak candidate for miRNA–mediated regulation based on the available prediction tools MirWip, TargetScan, PicTar, MirZ (our observation; Lall, Grun et al. 2006; Ruby, Jan et al. 2006; Hammell, Long et al. 2008; Hausser, Berninger et al. 2009).

While *cki-2* is likely not co–targeted by miRNAs and FBF/Pumilio proteins, it may be destabilized by FBF–2. In yeast, Puf proteins have been shown to recruit the CCR4–NOT–POP deadenylase complex, resulting in deadenylation and de–stabilization

of mRNA (summarized in (Chritton and Wickens 2010). At least the *gld-1* mRNA appears to be repressed by *C.elegans* FBF in a similar manner (Suh, Crittenden et al. 2009), suggesting a possible mechanism for *cki-2* regulation. Consistently with this possibility, the *cki-2* message is less abundant in germline stem cells (Fig. 12F and Kim and Roy 2006). However, since mRNAs associating with FBF proteins can be recovered (Kershner and Kimble 2010), FBF likely does not induce immediate degradation of all associated mRNAs.

During our experiments, we observed that another FBF/Pumilio protein redundantly required for germline stem cell proliferation in *C.elegans*, PUF–8, was required more generally to maintain reporter expression (Fig. 20C). Also, *puf-8* germlines are generally smaller than wild type, but mostly correctly patterned, suggesting that PUF–8 may be globally required to maintain mRNA expression levels in the germline, a working model that may be worth following up.

FBF/Pumilio proteins are also essential for neuronal functions (reviewed by Baines 2005; Weston and Baines 2007). Interestingly, neuronal fate has been postulated as "default" differentiation pathway of ESCs (Tropepe, Hitoshi et al. 2001; Smukler, Runciman et al. 2006), and *C.elegans* germ cells can be transformed into neurons by the removal of just one histone chaperone (Tursun, Patel et al. 2011). RNA regulators originally described in germ cells, Nanos and Brat, regulate core neuronal processes such as excitability and synapse growth (Muraro, Weston et al. 2008; Menon, Andrews et al. 2009). In this context, it is worth mentioning that *Drosophila* neurons express MEX–3, though a neuronal function of MEX–3 remains to be determined. Among the few messages enriched in previous MEX–3 RNA co–IP experiments in the lab (unpublished data) were vacuolar ATPases, proteins that set up proton gradients across membranes. Also, Notch responsive genes are enriched for messages involved in mitochondrial function. Mitochondria, which control the availability of protons in a cell, have been implied in stem cell maintenance previously (Rehman 2010). The electrochemical properties of C.elegans germline stem cells and stem cells more generally may therefore impact their decision to self–renew and differentiate.

5.2.4 Regulation of the cell cycle in stem cell self-renewal and differentiation

Though *cki-2* deletion has no obvious consequences for germ cell development, our findings suggest that CKI–2 *repression* is critical for stem cell maintenance. This agrees with observations that cell cycle inhibitors, including Cip/Kip family members, are usually depleted from stem cells (Stead, White et al. 2002; Ginis, Luo et al. 2004; Barta, Vinarsky et al. 2010), and that differentiation correlates with upregulation of Cip/Kip cell cycle inhibitors in cell culture and *in vivo* (Lee, Reynisdottir et al. 1995; Matsuoka, Edwards et al. 1995; Parker, Eichele et al. 1995). Phenotypes of p27 and p57 knockout mice suggest that Cip/Kip cell cycle inhibitors are required for timely cell cycle exit and differentiation of developmental progenitor cells in many tissues, for example in the lens (Fero, Rivkin et al. 1996; Kiyokawa, Kineman et al. 1996; Nakayama, Ishida et al. 1996; Yan, Frisen et al. 1997; Zhang, Liegeois et al. 1997; Zhang, Wong et al. 1998; Zhang, Wong et al. 1999). p21 knockout mice are superficially wild type, but display impressive regenerative abilities and have therefore been termed “healer strains” (Bedelbaeva, Snyder et al. 2010). p21 has also been shown in vitro to act as a barrier to reprogramming (Hong, Takahashi et al. 2009), suggesting that pluripotency and p21 expression are not compatible. However, reports on the sufficiency of Cip/Kip cell cycle inhibitors in the induction of differentiation are controversial. While overexpression of p27 in oligodendrocyte precursors arrests proliferation but does not induce expression of terminal differentiation markers (Tikoo, Osterhout et al. 1998), p27 and p21 seem both necessary and sufficient for terminal marker expression in human intestinal epithelial cells (Quaroni, Tian et al. 2000; Deschenes, Vezina et al. 2001).

Our results suggest that ectopic expression of CKI–2 contributes to stem cell differentiation in *fbf-1 fbf-2* mutants, and is sufficient to drive germ cells into differentiation in *gld-2 gld-1; glp-1(lf)* mutants. Cip/Kip proteins are well–established inhibitors of Cyclin E/Cdk2 activity, which promotes G1–S progression in the cell cycle. We therefore think that CKI–2 promotes differentiation via inhibition of Cyclin E/CDK2. *C.elegans* germline stem cells require CyclinE, since they fail to proliferate in *cye-1* mutants or upon depletion of CYE–1 (Seydoux, Savage et al. 1993; Fay and Han 2000; Jeong, Verheyden et al. 2011), and regulation of Cyclin E by GLD–1 is essential for meiotic progression (Biedermann, Wright et al. 2009). We therefore propose that a

high–low gradient of CYE–1/CDK–2 in the germ line, produced and controlled by conserved stem cell factors and pro–meiotic proteins, mediates the self–renewal versus differentiation decision (Fig. 23). Nonetheless, we cannot exclude cell cycle independent functions of CKI–2 during differentiation. Regions outside the CDK–inhibiting domain of vertebrate p27 and p57 have been reported to promote differentiation independently of the proteins' effect on cell cycle progression (Ohnuma, Philpott et al. 1999; Gui, Li et al. 2007), for example by stabilizing neurogenin (Vernon, Devine et al. 2003). Stem cell loss in *fbf-1 fbf-2* mutants is not completely prevented by depleting CKI–2; however, Cyclin E/CDK2 may not be sufficiently present or active in this mutant in the first place to sustain stem cell proliferation throughout adulthood.

Cyclin E and CDK2 have critical functions also in the mouse germ line (Geng, Yu et al. 2003; Ortega, Prieto et al. 2003), and mitotic arrest and differentiation of germ cells in the fetal testes correlates with suppression of Cyclin E and activation of several CKIs, including $p27^{Kip1}$ (Western, Miles et al. 2008). Accumulation of p27, achieved by protection of p27 mRNA from miRNA–mediated repression through the RBP DND1 (Kedde, Strasser et al. 2007), is thought to facilitate cell cycle arrest and differentiation of spermatogenic cells (Cook, Munger et al. 2011). Thus, Cyclin E/Cdk2 emerges as a conserved player in germ cell development. This stands to reason, since the decision to embark on the meiotic program is thought to preceed S–phase (Forsburg and Hodson 2000; Watanabe, Yokobayashi et al. 2001), and thus happens when the cell cycle is controlled by Cyclin E/CDK2.

Although a direct role of Cyclin E/Cdk2 in the self–renewal versus differentiation decision in the germ line remains to be tested, it is intriguing that a similar role for Cdk2 has been reported in embryonic stem cells (Neganova, Zhang et al. 2009). This might reflect an intimate connection between Cyclin E/Cdk2 and self–renewal in general, or a shared mechanism operating in germ cells and early embryonic cells (from which ES cells are derived).

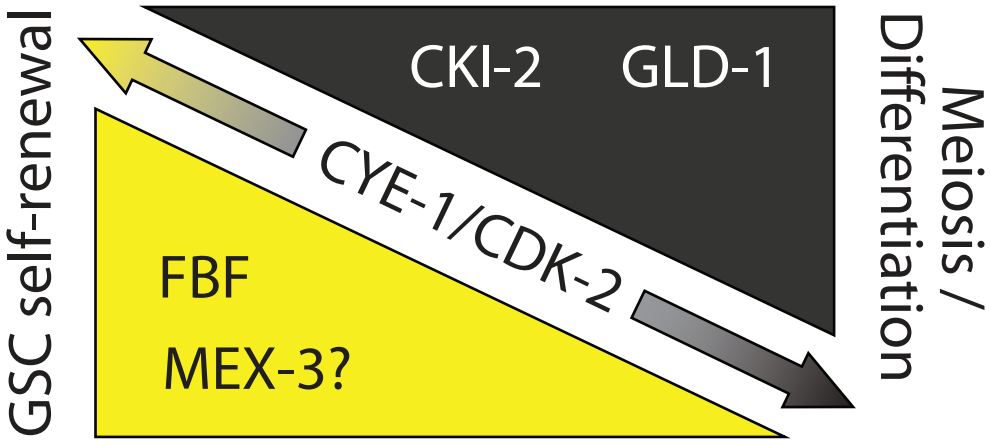


Figure 23.

Master regulators of the self-renewal versus differentiation decision converge on Cyclin E/CDK2.

This work together with Biedermann et al, *Translational repression of cyclin E prevents precocious mitosis and embryonic gene activation during C. elegans meiosis*, 2008 suggests that many, if not all, regulators of the self-renewal versus differentiation decision in the C.elegans germline converge on CYE-1/CDK-2, by regulating the activity (FBF and MEX-3 via the CYE-1/CDK-2 inhibitor CKI-2) or availability (GLD-1) of the complex.

General conclusions

General conclusions

How do insights gained during this work integrate with previous findings? In what respect have we contributed to the fields of *C.elegans* stem cell biology, Notch signaling, and stem cell biology in general?

Several of the described results should contribute substantially to each area. For example, we are not aware of any previous successful attempts to identify Notch target genes in *C.elegans* stem cells, or in pure stem cell populations in a genetically controlled way in any other organism. In addition, we describe for the first time convergence of Notch signaling and Polycomb–mediated regulation on the same genes. This is exciting, since both pathways are of paramount importance during developmental processes and cell fate decisions. Notch signaling presents short and pulsed cues during development; Polycomb proteins mediate cellular memory by maintaining the expression states of genes. In *C.elegans*, MES proteins mediate transgenerational memory of germline stem cell identity – possibly "remembering" the information provided by Notch signaling in the previous generation.

In addition to the link between Polycomb and Notch signaling, we newly characterized the role of a previously unknown player in the selfrenewal–versus–differentiation decision of *C.elegans* germline stem cells, CKI–2. CKI–2 was previously not known to be expressed in the germline. We also find that the *cki-2* mRNA is targeted by two conserved stem cell factors, the RNA binding proteins MEX–3 and FBF/Pumilio. Both had been previously implicated in stem cell maintenance in *C.elegans* and, in the case of FBF/Pumilio, other organisms as well, but it was not clear how they would control stem cell fate. We can now for the first time link both proteins directly to the machinery executing the decision between stem cell selfrenewal and differentiation, the cell cycle. In the case of FBF/Pumilio, this link has been described; however, we show for the first time that it is actually relevant for stem cell populations *in vivo*. MEX–3 in vertebrates has not yet been implicated in stem cell biology; it is however highly expressed in proliferating cell lines, and it will be very interesting to follow research on MEX–3 in vertebrates. In addition, our findings strengthen the cell cycle hypothesis. This concept has been around for many years and states that cell cycle and cell fate decisions are not only tightly linked, but that cell cycle regulation, particularly of the G1–S phase transition, may actually be upstream of cell fate choice in some cases. The fact that stem cells are maintained longer when the cell cycle inhibitor CKI–2 is absent supports that view.

Experimental Procedures

Nematode culture, mutants, constructs, and transgenic lines

Standard procedures were used to maintain animals and perform RNAi by feeding. Worms were grown at 25°C unless stated otherwise. Alleles used are *mex-3(or20), gld-2(q492), gld-1(q485), glp-4(bn2)* (I); *fbf-1(ok91), fbf-2(q704), cki-2(ok2105), mes-2(bn11)* (II), *glp-1(ar202), glp-1(e2141)* (III), *mes-4(bn85)* (V).

Previously described strains used are JA1522 (*unc-119(ed3)* III; *weSi6*(mex–5 promotor::GFP–H2B::tbb–2 3'UTR), courtesy of Eva Zeiser, laboratory of Julie Ahringer) and *pie-1::GFP::mex-3* from (Jud, Czerwinski et al. 2008), courtesy of Jen Schisa. Transgenic lines expressing reporter constructs were generated using the Gateway Reporter Cloning System (Merritt, Rasoloson et al. 2008) and the MosSCI direct insertion protocol (Frokjaer–Jensen, Davis et al. 2008).

We observed that GFP–containing reporters got silenced in trans to the GFP–marked *mIn1[mIs14]* balancer. The balancer contains pharyngeal GFP integrated as multicopy transgene; these are usually silenced in the germline and are known to elicit small–RNA dependent silencing of similar sequences. Derepression required outcrossing to wild type for several generations and the use of the unmarked *mIn1* balancer.

RNAi was performed by feeding with RNAi clones from the Ahringer and Vidal RNAi libraries, except for *cki-2 L* RNAi generated in this study and *mex-3* available from the lab stocks.

Primers / cloning:

The RNAi clone specifically targeting *cki-2 L* was generated with primers IK252 and IK253, and cloned into pMD3. Small deletions were introduced into the *cki-2* 3'UTR entry clone (generated by Mathias Senften) by site directed mutagenesis using primers IK298 and IK299 for M1, IK300 and IK301 for FBE2, IK302 and IK303 for M2, IK314 and IK315 for M2exp, and IK312 and IK313 for FBE1–4. The *cki-2* coding sequence for the overexpression construct was cloned into pDONR221 using primers IK278 and IK279 on cDNA. The operon entry vector was generated using primers IK280 and IK281 to amplify the gpd–2,3 intergenic region; IK282 and IK283 to amplify GFP–H2B; IK284 and IK285 to amplify the tbb–2 3'UTR. Fragments were then joined by fusion PCR and cloned into pDONRP2R–P3. The ectopic M2 site was inserted into the *tbb-2* 3'UTR by site directed mutagenesis on the entry vector containing *tbb-2* 3'UTR using the primers IK323 and IK324.

Worm strains used/generated

Notch project	
118	glp-1(ar202);
522	glp-1(ar202); + / DnTIG
853	unc-32(e189) glp-1(175) / hT2[qIs48]
397	unc-32(e189) glp-1(oz112) / unc-36(e251) glp-1(q175)
318, 319	gld-2(q492) gld-1(q485); glp-1(e2141) / hT2[qIs48]
90, 324, 344	gld-2(q497) gld-1(q485); glp-1(ar202) / hT2[qIs48]
93	gld-2(q497) gld-1(q485); glp-1(ar202) / hT2[qIs48] muscle-YFP
91, 95	mex-3(or20) gld-2(q497) gld-1(q485); glp-1(ar202) / hT2[qIs48]
88, 92	mex-3(or20) gld-2(q497) gld-1(q485); glp-1(ar202) / hT2[qIs48]; muscle-YFP
213	mex-3(or20) gld-2(q497) gld-1(q485), unc-32(e189) glp-1(q175) / hT2 [qIs48] [unc-119::GFP, rol-6 (su1006)]
210	mex-3(or20) gld-2(q497) gld-1(q485); unc-32(e189) glp-1(q175) / hT2 [qIs48]; muscle-YFP
520	mes-4(bn85) / DnTIG
521	glp-1(ar202); mes-4(bn85) / DnTIG
433	mes-2(bn11) unc-4(e120)/mnC1 dpy-10e128() unc-52(e444)
526	mes-2(bn11) unc-4(e120) / mnC1 dpy-10(e128) unc-52(e444); glp-1(ar202)
CKI-2 project	
diverse strains	
357	cki-2(oK2105)
371, 358	cki-1(gk132) / mIn1[dpy-10(e128) mIs14]
107	MR258 (N2; rrEx258 [fem-1::cki-2C; elt-2::GFP])
527	fbf-1(ok91)
574	fbf-2(q738)
842, 848	fbf-1(ok91) fbf-2(q704) / mIn1[mIs14 dpy-10(e128)]
858	fbf-1(ok91) fbf-2(q704) / + ; mex-3 / hT2[qIs48]
359, 370	puf-8(q725)
367	puf-8(ok302)
860	mIn1[dpy-10(e128)] / unc-4(e120)
372	cul-2(ek1) / unc-64(e246)
407	axIs1471[pie-1 prom:GFP:FBF-1 ORF:fbf-1 3'UTR, unc-119 (+)]
410	axIs1702[pie-1 prom:GFP:FBF-2 ORF:fbf-2 3'UTR, unc-119 (+)]

64	nmy-2::PGL-1::GFP
strains carrying cki-2(ok2105)	
412, 413	cki-2(ok2105); cul-2(ek1) / unc-64(e246)
398	cki-2(ok2105); unc-32(e189) glp-1(175) / hT2[qIs48]
744	cye-1(eh10) / dpy-14(e188) I; cki-2(oK2105)
841, 845, 856	fbf-1(ok91) fbf-2(q704) cki-2(ok2105) / mIn1[mIs14 dpy-10(e128)]
400	gld-2(q497) gld-1(q485) / hT2[qIs48]; cki-2(ok2105)
399	mex-3(or20) / hT2[qIs48]; cki-2(ok2105)
401	mex-3(or20) gld-2(q497) gld-1(q485) / hT2[qIs48]; cki-2(ok2105)
396	mex-3(or20) gld-2(q497) gld-1(q485); cki-2(ok2105); unc-32(e189) glp-1(175) / hT2[qIs48]
to be frozen	fbf-1(ok91) fbf-2(q704) cki-2(ok2105) / mIn1[mIs14 dpy-10(e128)]; fem-1(hc17)
to be frozen	fbf-1(ok91) fbf-2(q704) / mIn1[mIs14 dpy-10(e128)]; fem-1(hc17)
to be frozen	cki-2(ok2105); nmy-2::PGL-1::GFP
to be frozen	gld-1(q485) / hT2[qIs48]; cki-2(ok2105)
to be frozen	mex-3(or20) gld-1(q485) / hT2[qIs48]; cki-2(ok2105)
MEX-3 coIP	
343	mex-3(or20) gld-2(q497) gld-1(q485); unc-32(e189) glp-1(q175) / hT2 [qIs48]; GFP::MEX-3
reporter transgenes	
467	axIs[cki-2 promoter(5kb)::GFP-H2B::tbb-2 3'UTR]
461, 462	axIs[mex-5::CKI-2(start to end of 3'UTR)::gpd-2-3 GFP-H2B tbb-2 3'UTR]
482, 470, 476	axIs[mex-5::GFP-H2B::tbb-2 3'UTR]
418	axIs[pie-1::GFP-H2B::cki-2 3'UTR (short isoform)]
420, 421, 436	axIs[pie-1::GFP-H2B::cki-2 3'UTR]
528, 530, 539	axIs[mex-5::GFP-H2B::cki-2 3'UTR wt]
474	axIs[mex-5::GFP-H2B::cki-2 3'UTR ΔM2]
531	axIs[mex-5::GFP-H2B::cki-2 3'UTR ΔM2exp]
533	axIs[mex-5::GFP-H2B::cki-2 3'UTR ΔM2exp]
534	axIs[mex-5::GFP-H2B::cki-2 3'UTR ΔM2exp]
471, 472, 473, 477, 478, 483	axIs[mex-5::GFP-H2B::cki-2 3'UTR ΔM1 ΔM2]
466, 468, 469, 475, 480, 481	axIs[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE2]
484	axIs[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE2 ΔM2]
532, 535	axIs[mex-5::GFP-H2B:cki-2 3'UTR ΔFBE1-4]
529, 536	axIs[mex-5::GFP-H2B:cki-2 3'UTR ΔFBE1-4 ΔM2exp]

538	axIs[mex-5::GFP-H2B:cki-2 3'UTR Δ3 ΔM2]
537	axIs[mex-5::GFP-H2B:cki-2 3'UTR Δ5 ΔM2]
524	axIs[mex-5::GFP-H2B:tbb-2 3'UTR + MEX-3 site]
525	axIs[mex-5::GFP-H2B:tbb-2 3'UTR + MEX-3 site]
548	rrrSi9[mex-5::GFP-H2B::cki-2 3'UTR wt]
550	rrrSi11[mex-5::GFP-H2B::cki-2 3'UTR wt]
555	rrrSi12[mex-5::GFP-H2B::cki-2 3'UTR ΔM2]
558	rrrSi13[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE1]
559	rrrSi14[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE1]
560	rrrSi15[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE1]
727	rrrSi153[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE2]
728	rrrSi154[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE2 ΔM2]
731	rrrSi156[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE2 ΔM2]
732	rrrSi157[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE1 ΔM2]
733	rrrSi158[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE1 ΔM2]
857	rrrSi196[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE1-4]
859	rrrSi195[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE1-4 ΔM2]
551	rrrSi16[mex-5::GFP-H2B::cki-2 3'UTR Δ1 ΔM2]
561	rrrSi1[mex-5::GFP-H2B::cki-2 3'UTR Δ2 ΔM2]
557	rrrSi17[mex-5::GFP-H2B::cki-2 3'UTR Δ2 ΔM2]
562	rrrSi19[mex-5::GFP-H2B::cki-2 3'UTR Δ2 ΔM2]
737	rrrSi160[mex-5::GFP-H2B::cki-2 3'UTR Δ3 ΔM2]
556	rrrSi20[mex-5::GFP-H2B::cki-2 3'UTR Δ3 ΔM2]
563	rrrSi21[mex-5::GFP-H2B::cki-2 3'UTR Δ4]
564	rrrSi22[mex-5::GFP-H2B::cki-2 3'UTR Δ5 ΔM2]
reporters in div.backgrounds	
746, 747, 748	fbf-1(ok91) fbf-2(q704) / mIn1[mIs14 dpy-10(e128)]; rrrSi9, rrrSi10 or rrrSi11[mex-5::GFP-H2B::cki-2 wt 3'UTR]
854	fbf-1(ok91) fbf-2(q704) / mIn1[mIs14 dpy-10(e128)]; rrrSi10[mex-5::GFP-H2B::cki-2 wt 3'UTR]
749, 750, 751, 752, 754	fbf-1(ok91) fbf-2(q704) / mIn1[mIs14 dpy-10(e128)]; rrrSi12[mex-5::GFP-H2B::cki-2 ΔM2 3'UTR]
847	fbf-1(ok91) fbf-2(q704) / mIn1[mIs14 dpy-10(e128)]; rrrSi195[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE1-4 ΔM2]
to be frozen	fbf-1(ok91) fbf-2(q704) / mIn1[dpy-10(e128)]; rrrSi10[mex-5::GFP-H2B::cki-2 wt 3'UTR]
to be frozen	fbf-1(ok91) fbf-2(q704) / mIn1[dpy-10(e128)]; rrrSi12[mex-5::GFP-H2B::cki-2 ΔM2 3'UTR]
to be frozen	fbf-1(ok91) fbf-2(q704) / mIn1[dpy-10(e128)]; rrrSi195[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE1-4 ΔM2]

872	gld-2(q497) gld-1(q485) / hT2[qIs48]; rrrSi10[mex-5::GFP-H2B::cki-2 wt 3'UTR]
742	gld-2(q497) gld-1(485); unc-32(e189) glp-1(q175) / hT2[qIs48]; rrrSi12[mex-5::GFP-H2B::cki-2 3'UTR ΔM2]
to be frozen	gld-2(q497) gld-1(485); unc-32(e189) glp-1(q175) / hT2[qIs48]; rrrSi11[mex-5::GFP-H2B::cki-2 3'UTR wt]
to be frozen	gld-2(q497) gld-1(485); glp-1(ar202) / hT2[qIs48]; rrrSi12[mex-5::GFP-H2B::cki-2 3'UTR ΔM2]
to be frozen	gld-2(q497) gld-1(485); glp-1(ar202) / hT2[qIs48]; rrrSi11[mex-5::GFP-H2B::cki-2 3'UTR wt]
703	glp-1(ar202); rrrSi9, rrrSi10 or rrrSi11[mex-5::GFP-H2B::cki-2 wt 3'UTR]
855	glp-1(ar202); rrrSi10[mex-5::GFP-H2B::cki-2 wt 3'UTR]
to be frozen	unc-32(e189) glp-1(q175) / hT2[qIs48]; rrrSi10[mex-5::GFP-H2B::cki-2 wt 3'UTR]
743	glp-1(ar202); rrrSi12[mex-5::GFP-H2B::cki-2 ΔM2 3'UTR]
851	mex-3(or20) gld-1(q485) gld-2(q497); unc-32(e189), glp-1(175) / hT2[qIs48]; rrrSi12[mex-5::GFP-H2B::cki-2 ΔM2 3'UTR]
to be frozen	fbf-1(ok91) fbf-2(q704) / mIn1[mIs14 dpy-10(e128)]; axIs1702[pie-1 prom:GFP:FBF-2 ORF:fbf-2 3'UTR, unc-119 (+)]
to be frozen	axIs1702[pie-1 prom:GFP:FBF-2 ORF:fbf-2 3'UTR, unc-119 (+)]; rrrSi10[mex-5::GFP-H2B::cki-2 wt 3'UTR]
to be frozen	axIs1702[pie-1 prom:GFP:FBF-2 ORF:fbf-2 3'UTR, unc-119 (+)]; rrrSi196[mex-5::GFP-H2B::cki-2 3'UTR ΔFBE1-4]
CKI-2 overexpression	
453	rrrSi159[mex-5::CKI-2(start to end of 3'UTR)::gpd-2-3_GFP-H2B_tbb-2 3'UTR]
852	cye-1(eh10) / dpy-14(e188); rrrSi159[mex-5::CKI-2(start to end of 3'UTR)::gpd-2-3 GFP-H2B tbb-2 3'UTR]
745	mex-3(or20) / hT2[qIs48]; rrrSi159[mex-5::CKI-2(start to end of 3'UTR)::gpd-2-3 GFP-H2B tbb-2 3'UTR]
735	mex-3(or20) gld-1(q485) / hT2[qIs48]; rrrSi159[mex-5::CKI-2(start to end of 3'UTR)::gpd-2-3 GFP-H2B tbb-2 3'UTR]

Gonad dissection for C.elegans tiling array analysis

Parental animals were raised at 15°C and shifted to 25°C for egglay. 50 gonads per sample were dissected from the F1 generation in M9 containing Levamisole, transferred to PicoPure sample buffer obtained from Eric Cabuy, Genomics Facility, FMI by mouth pipette, and frozen at –80° for further processing by the Genomics Facility.

First biological replicate (A):

e2141_A_1, e2141_A_2, e2141_A_3 (technical replicates of *gld-2(q497) gld-1(485); glp-1(e2141tslf)*), ar202_A_1, ar202_A_2, ar202_A_3 (technical replicates of *gld-2(q497) gld-1(485); glp-1(ar202tsgf)*)

Second biological replicate (B):

e2141_B_1, e2141_B_2, e2141_B_3 (technical replicates of *gld-2(q497) gld-1(485); glp-1(e2141tslf)*), ar202_B_1, ar202_B_2, ar202_B_3 (technical replicates of *gld-2(q497) gld-1(485); glp-1(ar202tsgf)*)

Microarray

200 ng total RNA were amplified using the Affymetrix GeneChip WT Amplified Double Stranded cDNA Synthesis Kit according to the manufacturer‘s instructions. The Affymetrix GeneChip WT Double–Stranded cDNA Terminal Labeling Kit was used for fragmentation and labeling of 7.5 ug cDNA. The labeled material was loaded on Affymetrix GeneChip C. elegans Tiling 1.0R Arrays and hybridized at 65□ C for 16h. The arrays were washed in an Affymetrix Fluidics stations with the protocol FS450_0001 and scanned in an Affymetrix GeneChip Scanner 3000 with autoloader using Affymetrix GCC Scan Control v. 3.0.0.1214 software.

Probesets were summarized and probeset–level values normalized with the justRMA() function from R (version 2.10.0) / Bioconductor (version 2.5) package affy using the CDF environment MoGene–1_0–st–v1.r3.cdf (as provided by Bioconductor) and annotation from Netaffx (www.netaffx.com).

Data analysis

Initial analysis of the raw data was performed by Dimos Gaidatzis of the Genomics Facility at the FMI. All later analysis was performed in R by IK and is documented in the Appendix.

Validation by qPCR

qPCR validations were performed (in contrast to the array) on Notch null mutants. *gld-2(q497) gld-1(485); unc-32(e189) glp-1(q175)/hT2[qIs48]* and *gld-2(q497) gld-1(485); glp-1(ar202)tsgf / ht2(qIs48)* animals were grown at 20°C. Embryos were harvested by hypochlorite treatment, transferred to OP50 plates and incubated at 25°C until d0.5 of adulthood. For each genotype, 3 x 50 gonads were dissected in M9 containing Levamisole, transferred into 100µl ice–cold Trizol by mouth pipette, and frozen in liquid nitrogen. Afterwards, samples were thawed and 1µg mouse RNA was added to each sample.

RNA was isolated by adding 20µl Chloroform, incubating 10 minutes at RT, 10 minutes centrifugation at 13.000 rpm in a table centrifuge. The water phase was transferred, 50µl Isopropanol were added, followed by 15 minutes incubation at RT and 10 minutes centrifugation at 13.000 rpm. The supernatant was removed, and the pellet was washed with 600µl 70% EtOH, followed by 10 minutes centrifugation at 13.000 rpm. The samples were dried at RT for 10 minutes, resuspended in 20µl DEPC, and incubated for 10 minutes at 60°C, followed by concentration measurement by NanoDrop. cDNA was synthesized with oligoT primer using the ImProm II Reverse transcription system from Promega according to manufacturers instructions from 400ng RNA. 1/7 of the cDNA reaction was used for qPCR using Absolute QPCR SYBR green ROX mix (AbGene) on an ABI PRISM 7700 system (Applied Biosystems). PCR reactions were performed with an initial activation step of 15 min at 95°C, then 40 cycles of 20 s at 95°C and 60 s at 60°C. Standard curves for quantification were generated from a serial dilution of input cDNA for each primer pair.

The amount of target present in each replicate was derived from the standard curve; an average was calculated for the triplicates and fold enrichment was normalized to tubulin.

Primers used were IK259 and IK260 for cki–2, MS–act–1–p1 and MS–act–1–p2 for actin, and tbb–2 f 495 and tbb–2 r 696 for tubulin.

Northern Blot

RNA was extracted with Trizol from young adults prior to embryo production, from *glp-4* animals grown at restrictive temperature, and from embryos. 40µg RNA were loaded per lane. The probe corresponds to SL1, 5'UTR and CDS until nucleotide 462

(exons 1–3) and was generated using primers IK254 and IK261. The DIG Northern Starter Kit from Roche was used to prepare the probe and perform hybridization according to the manufacturer's instruction, except that the blocking solution was used at 10x concentration.

Biotin-RNA Pull-down

Biotin–RNA pull–downs were performed as in (Biedermann, Wright et al. 2009).

Immunofluorescence

Dilutions were 1:15 for CKI–1 antibody and 1:20 for CKI–2 antibody (Feng, Zhong et al. 1999). Secondary antibodies were goat anti–mouse alexa–568, anti–rabbit alexa–568, and anti–rabbit alexa–488 (Molecular Probes). In most experiments, gonads were prepared essentially as described previously (Lin, Hill et al. 1998). For HIM–3 staining, gonads were dissected in M9 with Levamisole, frozen on dry ice, fixed first in 100% methanol (–20°C) for 5 min, and then in 3.7% paraformaldehyde, PBS, 0.08 M HEPES, 1.6 mM MgSO4, 0.8 mM EGTA (pH 6.9) at room temperature (RT) for 5 min. Fluorescence and DIC images were captured with a Zeiss ImagerZ1 microscope equipped with an AxiocamMRm (Zeiss). Images were acquired with the same exposure and processed in Adobe Photoshop CS2 in an identical manner.

RNA-co-IP

Worm extracts were performed as previously described (Biedermann, Wright et al. 2009) in duplicate from N2 and from *mex-3 gld-1 gld-2; glp-1(lf);* MEX–3::GFP animals (previously separated from balanced animals with a COPASTM Biosort from Union Biometrica), diluted to 2µg/µl and preincubated with 10µl Protein A Sepharose Cl–4B beads per 700µg total protein at 4°C for 30 minutes. 20µl Protein A Sepharose beads that had been incubated with mouse anti–GFP antibody from Roche (11814460001) overnight at 4°C were added to 700µg total protein in a volume of 350µl and incubated for 5h at 4°C. IPs were then washed three times with extraction buffer. RNA was isolated with Trizol and processed as described before for real–time quantitative PCR. All incubations were done on the rotating wheel.

Primers used were IK259 and IK260 for cki–2, MS–act–1–p1 and MS–act–1–p2 for actin, tbb–2 f 495 and tbb–2 r 696 for tubulin, MS–rme2–p1 and MS–rme2–p2 for rme–2, Jane's ama–1 primers for ama–1, and MS–pal–1–p1 and MS–pal–1–p2 for pal–1.

RNA In situ hybridisation

RNA hybridizations were performed as described by Broitman–Maduro and Maduro (http://www.faculty.ucr.edu/_mmaduro/resources.htm). The probe was generated using primers AM015 and IK249. A Zeiss AxioImager Z1 microscope equipped with AxioncamMRm REV 2 CCD camera was used to capture images. All images were processed with Adobe Photoshop 7.0 or CS2 in an identical manner.

3'RACE

The *cki-2* cDNA was determined by 3'RACE (Scotto–Lavino, Du et al. 2006). cDNA was generated with IK001, 3' and 5' ends were determined using IK2 with internal forward primers, and IK91 with internal reverse primers and was cloned with the TOPO® TA Cloning® Kit from Invitrogen.

Protein purification for *in vitro* interaction assays

The PUF domain (amino acids 121–632) of FBF–2 was cloned into pGEX–6P–1 (GE Healthcare) and transformed into BL21(DE3) codon plus cells. Cultures were grown at 37 ºC to an OD_{600} of 0.4, shifted to 4 ºC for 15 minutes, induced with 0.1 mM IPTG, then incubated at 16 ºC overnight. Cell pellets were resuspended in lysis buffer (50 mM Tris pH 7.5, 500 mM KCl, and 2 mM DTT, supplemented with one Complete Mini EDTA–free protease tablet (Roche) per 50 mL of buffer) and disrupted using a microfluidizer. Following clarification, the protein was purified by affinity chromatography using a glutiathione sepharose 4B (GE Healthcare) column. To remove co–purifying nucleic acids, the column was washed with lysis buffer supplemented with 1 M KCl then exchanged into 50 mM Tris pH 7.5, 150 mM KCl, 1 mM EDTA, 2 mM DTT. The protein was eluted by incubation with Precission protease (GE Healthcare) at 4 ºC overnight which cleaves in between FBF and the N–terminal GST tag. Additional contaminants were removed using a HiTrap Q HP (GE Healthcare) column where purified FBF was recovered in the flow through. MEX–3 was purified as previously described (Pagano, Farley et al. 2009).

RNA binding assays

RNA oligonucleotides (IDT) were labeled as described by (Pagano, Clingman et al. 2011), and MEX-3 binding assays were performed as described in (Pagano, Farley et al. 2009). FBF binding reactions were perfomed as described in (Koh, Wang et al. 2011) and (Wang, Opperman et al. 2009), with the exception that F-EMSA and FP data were collected as described in (Pagano, Clingman et al. 2011).

Oligonucleotide sequences used to determine affinities:

cki-2 MRE2 WT	ACUCAAAUCAUAGACAUUCUAGUUAUAAAU
cki-2 MRE2 mut1	ACUCAAAUC**CCCC**ACAU**CCCC**GUUAUAAAU
cki-2 MRE2 mut2	ACUCAAAUC**CCCC**ACAU**CCCC**GU**CCCC**AAU
gld-1 FBEa	AUAGAAUCAUGUGCCAUACAUCAUGUUG
cki-2 FBE1 WT	UUUAUCUGUGAAUUUGAAAU
cki-2 FBE1 mut	UUUAUC**ACA**GAAUUUGAAAU
cki-2 FBE2 WT	CAUACCCUGUCCAUUUCUGU
cki-2 FBE2 mut	CAUACCC**ACA**CCAUUUCUGU
cki-2 FBE3 WT	CAUUUCUGUGUUCUACUCCU
cki-2 FBE3 mut	CAUUUC**ACA**GUUCUACUCCU
cki-2 FBE4 WT	CUACUCCUGUAAAAAAAGUC
cki-2 FBE4 mut	CUACUCC**ACA**AAAAAAAGUC

Appendix

urodele amphibian	amphibian retaining a tail after metamorphosis, eg. Salamander
anuran amphibian	amphibian losing their tail during metamorphosis, such as frogs
Ctenophores	marine combed jellyfish
Diptera	order of insects characterized by one wing pair
Epimorphis	regeneration of bodyparts by the formation of new tissue (as opposed to morphallaxis]
Ecdysozoa	animals shedding their cuticle during growth
Gonochoristic species	individuals are either male or female (as opposed to hermaphroditic species, where individuals are both male and female).
Hydra	radial–symmetrical freshwater polyp
Morphallaxis	regeneration of bodyparts by the rearrangement of cells, not by proliferation. Example: Hydra.
Teratoma	Teratoma are cancerous lesions likely derived from early, undifferentiated germ cells.
Tunicata, Ascidians	order of marine filter feeder
diploblast	organisms which develop from two primary germlayers, endoderm and ectoderm
monotremes	egglaying mammals
HAT	Histone acetyl transferase
TRIM protein	Tripartite Motif protein
HDAC	Histone deacetylase
CIR	Co–repressor interacting with RBP–J
SAGA complex	Spt–Ada–Gcn5–Acetyltransferase complex
SKIP	Ski–interacting protein
TFIID, TFIIA	Transcription factors IID, IIA, components of the transcription initiation complex.
NICD	Notch intracellular domain
MAM	Mastermind
CSL	collective name for CBF1, Su(H), and LAG–3
GCN5	Acetyltransferase
PCAF	P300/CBP–associated factor
SWI/SNF	SWItch/Sucrose NonFermentable, ATPase
H3K4, H3K27	Lysine 4 and 27, respectively, of Histone 3
LID/KDM	Little imaginal discs/Lysine demethylase
ASX	Additional sex combs
Cdk	Cyclin dependent kinase

script_1 (data distribution – avgExpr vs fold change, density plot).r

```
# read in contrast file obtained from Dimosthenis Gaidatzis / Bioinformatics Facility, FMI
	contrast <- read.delim("NotchContrasts.txt")
	head(contrast)
	dim(contrast)    # 24822 isoforms called

# changing column names
	colnames(contrast) <- c("isoform", "fcboth", "fc1", "fc2", "avgExpr")
	summary(contrast)
	dim(contrast)
	# [1] 24822     5

# checking distribution of average expression values with histogram
	pdf("average expression values.pdf")
	hist	(contrast$avgExpr, breaks = 100,
		main = c("average expression values", "n = 24822 isoforms"),
		xlab = "average expression from all arrays"
		)
	dev.off()

# checking correlation of fold changes from first and second repeat

	pdf("fold change correlation from rep1 and rep2.pdf")
	plot	(contrast$fc1, contrast$fc2,
		pch = "*", col = "black",
		main = "repeat 1 vs repeat 2",
		xlab = "fold change (1), log2", ylab = "fold change (2), log2"
		 )
	points	(contrast$fc1[contrast$fcboth>=1], contrast$fc2[contrast$fcboth>=1],
		pch = "*", col="green")
	points	(contrast$fc1[contrast$fcboth<=-1], contrast$fc2[contrast$fcboth<=-1],
		pch = "*", col="red")
	abline	(h = 0, col = "grey", lty = 2)
	abline	(v = 0, col = "grey", lty = 2)
	legend	("bottomright", bty = "n",
		legend=c("2 fold upregulated (average regulation)", "2 fold downregulated
		(average regulation"),
		col=c("green", "red"), pch = "*")
	dev.off()

# checking relation of average expression values with pooled fold changes from both repeats

	pdf("MA plot.pdf")
	plot	(contrast$avgExpr, contrast$fcboth,
		pch = "*", col = "black",
		main = "fold change versus average Expression",
		xlab = "average expression", ylab = "fold change (1+2), log2"
		 )
	points	(contrast$avgExpr[contrast$fcboth>=1], contrast$fcboth[contrast$fcboth>=1],
		pch = "*", col="green")
	points	(contrast$avgExpr[contrast$fcboth<=-1], contrast$fcboth[contrast$fcboth<=-
		1], pch = "*", col="red")
	abline	(h = c(1, -1), col = "grey", lty = 2)
	legend	("bottomright", bty = "n",
		legend=c("2 fold upregulated", "2 fold downregulated"),
		col=c("green", "red"), pch = "*")
	dev.off()
```

Appendix – R scripts

script_2 (reading in annotation, generating workspace).r

```
# reading in Dimos' contrast file
	contrast <- read.delim ( "NotchContrasts.txt")

# changing to workable column names
	colnames(contrast) <- c("isoform", "fcboth", "fc1", "fc2", "avgExpr")

# adding as columns: WBGeneID, Intronerator, etc
	# fetch identifiers
		i <- (contrast$isoform)
		head(i)
		length(i)
		write.table(
					i,
					"isoforms_on_chip.txt",
					col.names=F,
					row.names=F,
					quote=F
					)

# retrieve annotation from wormmart, using build 190. saved as annotation.txt

# read annotation into R
	annotation <- read.delim("annotation.txt")
	dim(annotation)		# all 24822 returned

# merge contrast values with annotation, test, save
	cont.ann <- merge(contrast, annotation, by.x=1, by.y=5)
	dim(cont.ann)
	head(cont.ann)
	cont.ann[cont.ann$isoform =="C55C3.7",]
	contrast[contrast$isoform =="C55C3.7",]
	annotation[annotation$Sequence.Name..Transcript. =="C55C3.7",]
	colnames(cont.ann) <- c("isoform", "fc_bothrepeats", "fc_repeat1", "fc_repeat2",
				"avgExpr", "WBid", "public_name", "gene_name",
				"chromosome")
	write.table	(cont.ann,
			"NotchContrasts_annotated.txt",
			row.names = F, quote = F
			)

 # Workspaced used from now: notchcontrast_ann.RData
```

script_3 (extracting regulated genes, chromosome bias).r

```
# open Workspace notchcontrast_ann.RData

# extract differentially regulated genes (unique list)

	up <- as.factor(as.character(unique(cont.ann$gene_name[cont.ann$fc_bothrepeats >=
		1])))
	down <- as.factor(as.character(unique(cont.ann$gene_name[cont.ann$fc_bothrepeats
		<= -1])))
	 length(up)   # 114 genes
	 length(down) # 10 genes

	write.table	(unique(cont.ann[cont.ann$fc_bothrepeats >= 1,c(2:9)]),
			quote = FALSE, row.names = FALSE, sep = "\t",
			"upregulated genes.txt")
	write.table	(unique(cont.ann[cont.ann$fc_bothrepeats <= -1,c(2:9)]),
			quote = FALSE, row.names = FALSE, sep = "\t",
			"downregulated genes.txt")
```

```
dim(unique(cont.ann[cont.ann$fc_bothrepeats >= 1,c(2:9)]))
dim(unique(cont.ann[cont.ann$fc_bothrepeats <= -1,c(2:9)]))

# compare chromosome distribution

up <-           unique(cont.ann[cont.ann$fc_bothrepeats >= 1,c(6:9)])
down <-         unique(cont.ann[cont.ann$fc_bothrepeats <= -1,c(6:9)])
alle <-         unique(cont.ann[,c(6:9)])
dim(up)    # 114 genes go up
summary         (up$chromosome)
dim(alle)  # 18050 genes
summary         (alle$chromosome)
dim(up[up$chromosome == "X",])  # 52 of up genes are on X

# generate 100 random lists of 114 genes, count genes on X, save results.
test1 <- as.factor(as.character(sample(alle$gene_name, 114)))
test2 <- as.factor(as.character(sample(alle$gene_name, 114)))
test3 <- as.factor(as.character(sample(alle$gene_name, 114)))
test4 <- as.factor(as.character(sample(alle$gene_name, 114)))
test5 <- as.factor(as.character(sample(alle$gene_name, 114)))
test6 <- as.factor(as.character(sample(alle$gene_name, 114)))
test7 <- as.factor(as.character(sample(alle$gene_name, 114)))
test8 <- as.factor(as.character(sample(alle$gene_name, 114)))
test9 <- as.factor(as.character(sample(alle$gene_name, 114)))
test10 <- as.factor(as.character(sample(alle$gene_name, 114)))


t1 <- alle[alle$gene_name %in% test1,]
length(t1$chromosome[t1$chromosome == "X"])
t2 <- alle[alle$gene_name %in% test2,]
length(t1$chromosome[t2$chromosome == "X"])
t3 <- alle[alle$gene_name %in% test3,]
length(t1$chromosome[t3$chromosome == "X"])
t4 <- alle[alle$gene_name %in% test4,]
length(t1$chromosome[t4$chromosome == "X"])
t5 <- alle[alle$gene_name %in% test5,]
length(t1$chromosome[t5$chromosome == "X"])
t6 <- alle[alle$gene_name %in% test6,]
length(t1$chromosome[t6$chromosome == "X"])
t7 <- alle[alle$gene_name %in% test7,]
length(t1$chromosome[t7$chromosome == "X"])
t8 <- alle[alle$gene_name %in% test8,]
length(t1$chromosome[t8$chromosome == "X"])
t9 <- alle[alle$gene_name %in% test9,]
length(t1$chromosome[t9$chromosome == "X"])
t10 <- alle[alle$gene_name %in% test10,]
length(t1$chromosome[t10$chromosome == "X"])

# results saved in .txt

pdf("random 114 genes – X linkage.pdf")
randoms <- read.table("random_samples.txt")
hist    (randoms[,1], breaks = 20, main = c("X linked fraction among 114 randomly
        sampled genes", "n = 100"), ylab = "frequency", xlab = "X–linked genes among
        114 random genes")
dev.off()

pdf("chromsome bias.pdf")
cols <- rainbow(10)
par(mfrow = c(3,2))
pie     (summary(up$chromosome), main = c("upregulated genes", "n=114"), col=cols,
        radius = 1)
pie     (summary(alle$chromosome), main = c("all genes on array", "n=18050"),
```

```
        col=cols, radius = 1)
dev.off()

# compare expression of x and autosomes

x <- unique(cont.ann[cont.ann$chromosome == "X",c(2:9)])
auto <- unique(cont.ann[cont.ann$chromosome != "X",c(2:9)])
pdf("silent X-chromsome.pdf")
plot    (density(x$avgExpr), col = "red", lwd = 2,
        main = "X-linked genes are mostly silent in germ cell tumors",
        ylab = "frequency", xlab = "average expression level")
lines   (density(auto$avgExpr), col = "black", lwd = 2)
legend ("topright", lty = 1, lwd = 2, col = c("red", "black"), legend = c("expression of X-
        linked genes", "expression of autosomal genes"), bty = "n")
dev.off()

# MA plot by auto/heterosomes, respectively.

auto <- cont.ann[cont.ann$chromosome != "X",]
hetero <- cont.ann[cont.ann$chromosome =="X",]
dim(auto) # 21349
dim(hetero) # 3473

pdf("MA plot, auto-hetero.pdf", width = 14)
par(mfrow=c(1,2))
plot    (auto$avgExpr, auto$fc_bothrepeats,
        pch = "*", col = "black",
        main = c("autosomes", "n(isoforms)=21349"), xlim=c(2,13), ylim=c(-2,4),
        xlab = "average expression", ylab = "fold change (1+2), log2"
        )
abline  (h = c(1, -1), col = "grey", lty = 2)
plot    (hetero$avgExpr, hetero$fc_bothrepeats,
        pch = "*", col = "black",
        main = c("X-chromosome","n(isoforms)=3473"), xlim=c(2,13), ylim=c(-2,4),
        xlab = "average expression", ylab = "fold change (1+2), log2"
        )
abline  (h = c(1, -1), col = "grey", lty = 2)
dev.off()
```

script_4 (correlating notch and mes data).r

```
# open Workspace containing Notch data

mes <- read.delim("gene.sets_mes.expr.table_2009.11.13.txt")
colnames(mes)
dim(mes)
summary(mes)

# two platforms: oligo and amplicon.
# oligo: mes2, mes3, mes4 - value of mutant over wildtype
# amplicon: mes3, mes3, mes4, mes6 - value of mutant over wildtype

# How does the data distribute in terms of fold changes and expression values?

hist    (mes$A.mes.2.amplicon,  # exchange colname for other categories
        breaks = 100, ylim=c(0,500), xlim=c(4,16))

# The mes data contains some less genes or isoforms:

dim(mes)        # 22227 rows
dim(cont.ann)   # 24822 rows
```

```
# merge the two tables by Wormbase ID

        colnames(mes)
        colnames(cont.ann)
        m <- merge(mes, cont.ann, by.x=3, by.y=6)
        dim(m)          # 24571 rows

# save as new workspace: notch_and_mes.RData
# separate into X-linked and autosomal genes, test
# correlate notch with each platform, separated by autosome and heterosome.

        auto <- c("I", "II", "III", "IV", "V")
        x <- m[m$chromosome == "X",]
        a <- m[m$chromosome %in% auto,]
        x[c(1:50), c(1,4,69)]
        a[c(1:50), c(1,4,69)]

        xl <- c(-1.2, 3)
        yl.a <- c(-0.8, 1.5)
        yl.o <- c(-2, 3.5)
        pdf     ("notch-mes-correlationplot_amplicon.pdf", height = 8, width = 14)
        par     (mfrow=c(2,4), pch= "*")
        plot    (a$fc_bothrepeats, a$M.mes.4.amplicon, xlim = xl, ylim = yl.a)
                abline  (v=0, h=0)
        plot    (a$fc_bothrepeats, a$M.mes.2.amplicon, xlim = xl, ylim = yl.a)
                abline  (v=0, h=0)
        plot    (a$fc_bothrepeats, a$M.mes.3.amplicon, xlim = xl, ylim = yl.a)
                abline  (v=0, h=0)
        plot    (a$fc_bothrepeats, a$M.mes.6.amplicon, xlim = xl, ylim = yl.a)
                abline  (v=0, h=0)
        plot    (x$fc_bothrepeats, x$M.mes.4.amplicon, xlim = xl, ylim = yl.a)
                abline  (v=0, h=0)
        plot    (x$fc_bothrepeats, x$M.mes.2.amplicon, xlim = xl, ylim = yl.a)
                abline  (v=0, h=0)
        plot    (x$fc_bothrepeats, x$M.mes.3.amplicon, xlim = xl, ylim = yl.a)
                abline  (v=0, h=0)
        plot    (x$fc_bothrepeats, x$M.mes.6.amplicon, xlim = xl, ylim = yl.a)
                abline  (v=0, h=0)
        dev.off ()

        pdf     ("notch-mes-correlationplot_oligo.pdf", height = 8, width = 14*3/4)
        par     (mfrow=c(2,3), pch= "*")
        plot    (a$fc_bothrepeats, a$M.mes.4.oligo, xlim = xl, ylim = yl.o)
                abline  (v=0, h=0)
        plot    (a$fc_bothrepeats, a$M.mes.2.oligo, xlim = xl, ylim = yl.o)
                abline  (v=0, h=0)
        plot    (a$fc_bothrepeats, a$M.mes.3.oligo, xlim = xl, ylim = yl.o)
                abline  (v=0, h=0)
        plot    (x$fc_bothrepeats, x$M.mes.4.oligo, xlim = xl, ylim = yl.o)
                abline  (v=0, h=0)
        plot    (x$fc_bothrepeats, x$M.mes.2.oligo, xlim = xl, ylim = yl.o)
                abline  (v=0, h=0)
        plot    (x$fc_bothrepeats, x$M.mes.3.oligo, xlim = xl, ylim = yl.o)
                abline  (v=0, h=0)
        dev.off ()

# is this specific for certain genes or just a general effect because both act on the X?
# correlate Notch-dependent foldchanges with randomized mes-4 fold changes

        a <- x$M.mes.4.amplicon
        a1 <- sample(a, length(a))
        a2 <- sample(a, length(a))
```

```
a3 <- sample(a, length(a))

yl.a <- c(-0.5, 1.5)
pdf     ("randomized correlation.pdf", height = 4, width = 14)
par     (mfrow=c(1,4), pch= "*")
plot    (x$fc_bothrepeats, x$M.mes.4.amplicon, xlim = xl, ylim = yl.a)
        abline  (v=0, h=0)
plot    (x$fc_bothrepeats, a2, xlim = xl, ylim = yl.a)
        abline  (v=0, h=0)
plot    (x$fc_bothrepeats, a3, xlim = xl, ylim = yl.a)
        abline  (v=0, h=0)
plot    (x$fc_bothrepeats, a4, xlim = xl, ylim = yl.a)
        abline  (v=0, h=0)
dev.off ()
```

script_5 (cell cycle genes).r

```
# open Workspace containing Notch data
# read in a list of cell cycle genes

cc <- read.delim("cellcycle.txt")

# extract interesting categories for the plot

CDK             <- as.factor(as.character(cc$Gene.WB.ID[cc$category == "CDK"]))
cyclin          <- as.factor(as.character(cc$Gene.WB.ID[cc$category == "cyclin"]))
inhibitor       <- as.factor(as.character(cc$Gene.WB.ID[cc$category == "inhibitor"]))
phosphatase     <- as.factor(as.character(cc$Gene.WB.ID[cc$category ==
                "phosphatase"]))

# mark in correlation plot and MA plot

cols <- c("orange", "green", "blue", "red")

pdf("cell cycle - fc correlation.pdf")
plot    (cont.ann$fc_repeat1, cont.ann$fc_repeat2,
        pch = "*", xlim = c(-2,4), ylim = c(-2,4),
        main = c("regulation of cell cycle genes by Notch"),
        xlab = "first repeat, fold change (log2)",
        ylab = "second repeat, fold change (log2)")
abline  (h = 0, v = 0))
points  (cont.ann$fc_repeat1[cont.ann$WBid %in% CDK],
        cont.ann$fc_repeat2[cont.ann$WBid %in% CDK],   pch = 19, col = cols[1])
points  (cont.ann$fc_repeat1[cont.ann$WBid %in% cyclin],
        cont.ann$fc_repeat2[cont.ann$WBid %in% cyclin],          pch = 19, col =
cols[2])
points  (cont.ann$fc_repeat1[cont.ann$WBid %in% phosphatase],
        cont.ann$fc_repeat2[cont.ann$WBid %in% phosphatase],
        pch = 19, col = cols[3])
points  (cont.ann$fc_repeat1[cont.ann$WBid %in% inhibitor],
        cont.ann$fc_repeat2[cont.ann$WBid %in% inhibitor], pch = 19, col = cols[4])
legend  ("bottomright", legend = c("CDK", "cyclin", "phosphatase", "inhibitor"),
col = cols, pch = 19, bty = "n")
dev.off()

pdf("cell cycle - MA plot.pdf")
plot    (cont.ann$avgExpr, cont.ann$fc_both,
        pch = "*", xlim = c(2,14), ylim = c(-2,4),
        main = c("regulation of cell cycle genes by Notch"),
         xlab = "average expression", ylab = "fold change (from both repeats)")
points  (cont.ann$avgExpr[cont.ann$WBid %in% CDK],
```

```
        cont.ann$fc_both[cont.ann$WBid %in% CDK],
        pch = 19, col = cols[1])
points  (cont.ann$avgExpr[cont.ann$WBid %in% cyclin],
        cont.ann$fc_both[cont.ann$WBid %in% cyclin],
        pch = 19, col = cols[2])
points  (cont.ann$avgExpr[cont.ann$WBid %in% phosphatase],
        cont.ann$fc_both[cont.ann$WBid %in% phosphatase],
        pch = 19, col = cols[3])
points  (cont.ann$avgExpr[cont.ann$WBid %in% inhibitor],
        cont.ann$fc_both[cont.ann$WBid %in% inhibitor],
        pch = 19, col = cols[4])
legend  ("topright", legend = c("CDK", "cyclin", "phosphatase", "inhibitor"),
col = cols, pch = 19, bty = "n")
dev.off()
```

Generation of a mouse monoclonal MEX-3 antibody

Clearly, the precise role of MEX–3 in the stem cell compartment remains to be determined. Since RNAi of *cki-2* does not rescue *puf-8 mex-3* double mutants (Fig. 20B), MEX–3 likely regulates other mRNAs apart from *cki-2* in stem cells. However, RNA–co–IPs and microarray analysis using available MEX–3 antibodies yielded unexpectedly few targets at low enrichement rates (observation of Björn Biedermann). The MEX–3 transgene available in the lab (see Methods) does not rescue the embryonic lethal phenotype of embryos born from *mex-3* mutant mothers, although it can replace MEX–3 in *mex-3 gld-1 gld-2; glp-1* mutants. Since the basis of this discrepant behaviour is not clear, this transgene is not a perfect tool to isolate bona fide MEX–3 targets. We thus decided to raise a monoclonal antibody against MEX–3. Considerations for peptide design were presence of the sequence in all *C.elegans* MEX–3 isoforms, location outside the KH domains, lack of conservation in mouse, low Methionine, Tryptophane or Cystein content to facilitate peptide production, low chance of posttranslational modification by prediction algorithms, and high charge as a predictor for surface exposure. Based on these criteria, three peptides (4068610, FYRRAFGNSNPFNQKE + C; 4068611, DPEDIAQFLNYRTSIGVQ + C; and 4068612, CDHNDHTLVPING) were injected by the FMI monoclonal antibody facility (Fig. 24A). All antibody clones that specifically recognized MEX–3 in immunofluorescence assays and Western blot analysis (Fig. 24B and 24C; Table 7) were directed against peptide 4068611, DPEDIAQFLNYRTSIGVQ. It will be curious to see whether *cki-2* mRNA can be co–immunoprecipitated with MEX–3 from *gld-1 gld-2; glp-1* animals using this antibody, and which additional MEX–3 targets mRNA–co–IPs and genome wide analysis of targets will yield.

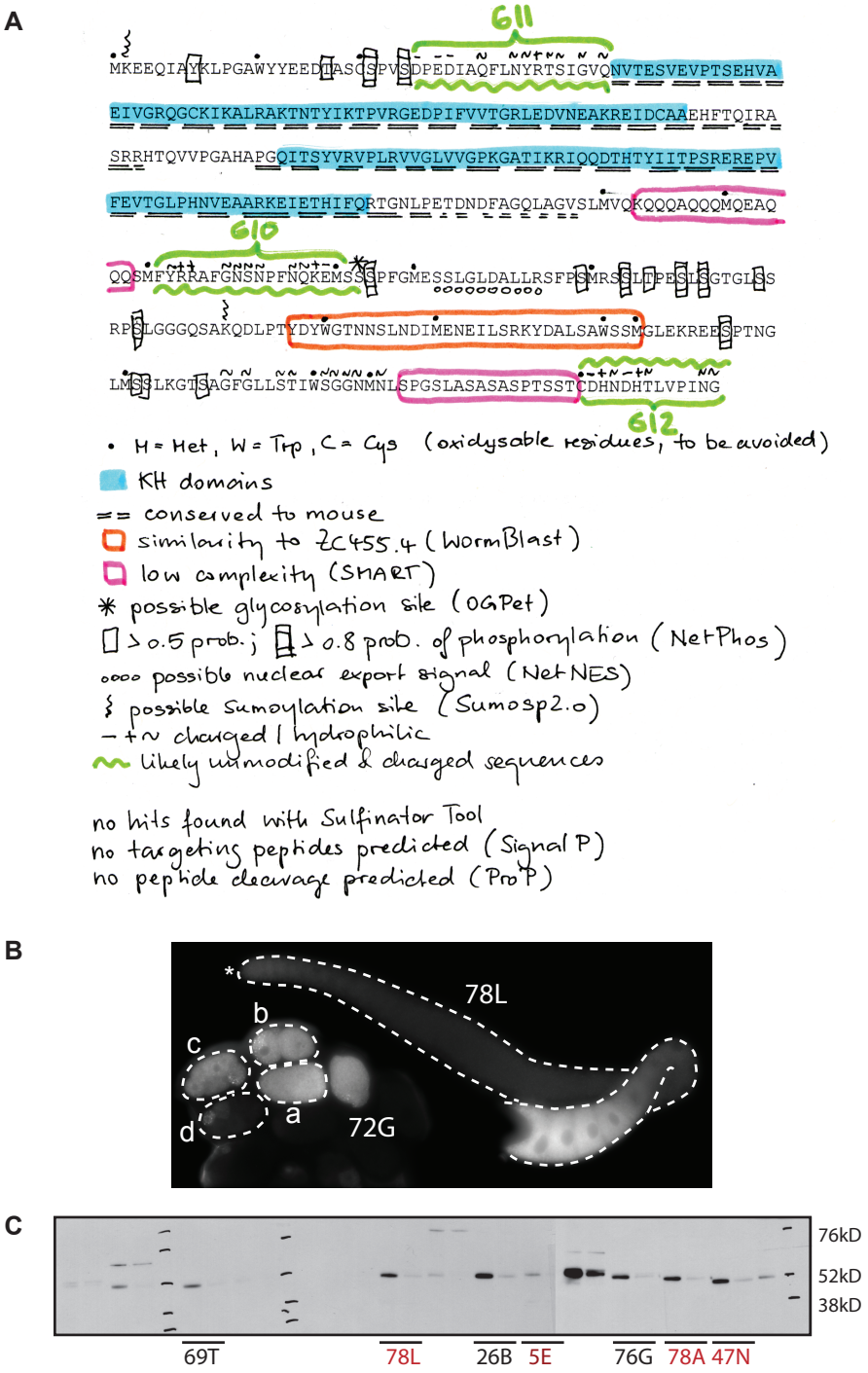


Figure 24. **Generation of a monoclonal mouse antibody against MEX-3.**
(A) MEX-3 protein sequence with domains and possible modifications indicated. Peptides 610, 611 and 612 were chosen to be unmodified, unconserved, and surface exposed. (B) Dissected gonad and embryos (outlined) stained with supernatant from clone 72G (embryos) and 78L (germline). Pictures shown are representative for all clones that performed well in immunofluorescence (see Table 7). In the germline, MEX-3 is expressed in stem cells (asterisk) and oocytes. In embryos, MEX-3 localizes to P-granules and to the AB lineage. a,b,c,d: 1, 2, 4, and ~10 cell stage embryo. (C) Western blot using indicated clones on lysate from N2 (left band) and mex-3(RNAi) treated (right band) animals. Clones 78L, 5E, 78A, and 47N detect a single band that disappears upon *mex-3*(RNAi) and perform well in immunofluorescence (see Table 7).

Table 7. *Performance of individual MEX-3 antibody clones.*

clone		pept.	immunofluorescence	Western (N2 vs *mex-3*(RNAi))
5	E	611	mex–3, no background	recognizes MEX–3, no background
76	G	611	mex–3, no background	recognizes MEX–3, no background
78	L	611	mex–3, no background	recognizes MEX–3, no background
47	N	611	mex–3, no background	recognizes MEX–3, no background
46	V	610	mex–3, no background	recognizes MEX–3 but also sth else
12	B	611	mex–3, no background	recognizes MEX–3 but also sth else
55	G	611	mex–3, no background	recognizes MEX–3 but also sth else
85	E	611	mex–3, no background	no signal
22	C	611	mex–3, no background	not tested
47	H	611	mex–3, no background	not tested
78	A	611	mex–3, somatic gonad	recognizes MEX–3, no background
26	B	611	mex–3, somatic gonad	recognizes MEX–3, no background
69	T	611	mex–3, sth. else in mid pachytene	recognizes MEX–3, no background
68	M	611	mex–3, somatic gonad, funny epitope in late pachytene nuclei	recognizes MEX–3 but also sth else
62	H	611	mex–3, nuclei, particularly around the bend	recognizes right size weakly, but not much depleted in RNAi
62	D	612	mex–3, high cytoplasmic background in germline, gut	not tested
21	F	610	mex–3, nuclear epitope that is also cytoplasmic distally, gets off nuclei when dividing, in TZ	not tested
72	G	611	mex–3, somatic gonad membranes	not tested
30	L	611	mex–3, sth else as well	not tested
70	N	612	mex–3, high cytoplasmic background (smallish points) in central gonad	not tested
73	A	610	not tested	not tested
44	O	612	nuclei in gonad, gut, punctae, somatic gonad sheeth	not tested
8	D	612	punktae in oocytes (few), distal, pachytene (many). Very likely centrosomes.	not tested
36	D	612	somatic gonad membrane, punctae in cytoplasm, nuclear epitope in embryos, p granules in embryos	not tested
88	H	612	somatic gonad, nuclei until bend, p granules	not tested
72	H	all	weakly p–granules	not tested
29	D	611	centrosomes, punctae in rachis, maybe sth in oocyte nuclei	not tested
7	P	610	decorates entire outside of the gonad (but does not look like membrane staining)	not tested
13	A	all	dots in all tissues. In the germline, they are absent from the rachis.	not tested
68	F	610	granular in germline, membranes, nuclei from bend on	not tested
17	G	612	maybe weak mex–3, sth else	not tested
94	K	611	no staining	no signal
58	C	611	no staining	not tested
82	N	611	no staining	not tested
52	W	all	no staining	not tested

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Curriculum Vitae

Curriculum Vitae
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University Education

2000 - 2006	Universtät Wien, Austria Molecular Biology Main Subjects: Genetics, Microbiology and Immunology, Interdisciplinary Focus (Science Communication, IPR, Science Theory))
2003 - 2006	Universität Wien, Austria Scandinavian Studies
2003	Universitetet Bergen, Norway Molecular Biology

Master Thesis

2005 – 2006 “Muscle migration and target selection in the *Drosophila* embryo”

Research Institute of Molecular Pathology, Vienna, Austria
Laboratory: Dr. Barry Dickson
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PhD Thesis

2006 – 2011 “Regulation of the self-renewal vs. differentiation decision in the *C.elegans* germline”

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Publications

FBF represses the Cip/Kip cell cycle inhibitor CKI-2 to promote self-renewal of germline stem cells in *C. elegans*.
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(2011, accepted by EMBO Journal)

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Schnorrer F, Kalchhauser I, Dickson BJ.
Dev Cell. 2007 May;12(5):751-66.

Awards/Funding

2003	Erasmus scholarship (Bergen, Norway)
2004	Summer school scholarship "Norwegian Language and Litterature" (Agder University College, Kristiansand, Norway)
2007-2009	Böhringer Ingelheim PhD Fellowship

Conference participations

2005	19th European Drosophila Research Conference (Eger, Hungary)
2007	USGEB (Basel)
2007	ELSO Conference (Dresden, Germany)
2007	EMBO Workshop on intracellular RNA localization and localized translation (Ciocco, Italy)
2008	European Worm Meeting (Carmona, Spain)
2008	Worm Development and Evolution (Madison, Wisconsin, USA)
2009	Joint PhD Meeting with the MRC London (Emmetten, Switzerland)
2009	Cold Spring Harbour Conference on Stem cells (CSH, USA)

Teaching Experience

2006	Lange Nacht der Forschung, IMP, Vienna
2008 and 2009	Tutoriat „Einführung in die Biologie", University Basel
2008 and 2009	Tage der Genforschung, FMI, Basel
2009	Supervision of Master Student Asja Moerkamp
2010	Open Doors, FMI, Basel

Non-scientific work experience

1999 and 2000	Bank für Arbeit und Wirtschaft AG, Vienna
2001	Österreichische Post AG, Vienna
2004	Book Review for „Falter" (Trojanische Saaten, Jeffrey M.Smith)

Non-scientific Activities

2000-2006	Scout leader at Gruppe 21, Liechtenstein, Vienna (age group 5-7 y. 2000 – 2003, 13-16 y. 2003 – 2006)
Sports:	Boxing, Swimming, Outdoor activities
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Languages and additional skills

German (mother tongue), English (fluently), Norwegian (good skills), French (moderate skills)
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