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Analysis of the core mechanisms underlying transdifferentiation in *C. elegans*

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RÉSUMÉ

INTRODUCTION

La transdifférenciation (ou reprogrammation cellulaire directe) est la conversion directe d'un type cellulaire entièrement différencié en un autre, avec ou sans division cellulaire. La transdifférenciation naturelle (Td) a été caractérisée chez *C. elegans* par notre laboratoire, qui a démontré que la cellule rectale Y de l'hermaphrodite se transdifférencie en motoneurone PDA avec une efficacité, une robustesse et une irréversibilité élevées (Jarriault et al., 2008). Par criblage de mutagenèse et d'ARNi, des facteurs de transcription (*sox-2* / *SOX*, *ceh-6* / *POU*, *sem-4* / *SALL*), des modificateurs de chromatine (*egl-27* / *MTA*, *jmjd-3.1* / *KDM6*, *set-2* / *SETD1*) et d'autres gènes développementaux (*egl-5* / *HOX*) ont été identifiés comme impliqués dans Y-to-PDA Td ; de plus, les étapes cellulaires et les réseaux moléculaires qui les contrôlent sont en train d'être déchiffrés. Par exemple, cet événement se produit sans division cellulaire et consiste en un processus par étapes qui commence par l'effacement de l'identité initiale, avant une nouvelle re-différenciation en un type de cellule différent (Kagias et al., 2012 ; Richard et al., 2011 ; Zuryn et al., 2014).

Ces mécanismes élucidés par le laboratoire sont-ils conservés ou varient-ils selon les différents événements de Td? Le but de ma thèse est d'examiner si d'autres événements de plasticité cellulaire se produisent naturellement dans le ver, et de définir quels mécanismes fondamentaux, le cas échéant, et quelles variations spécifiques à l'événement peuvent être trouvés. Un accent particulier est mis sur le rôle de la division cellulaire dans la Td.

RÉSULTATS

1) Identification d'autres événements naturels de Td chez *C. elegans*

La détermination de la lignée cellulaire embryonnaire et post-embryonnaire chez *C. elegans* (Sulston and Horvitz, 1977 ; Sulston et al., 1983) a fourni un outil puissant pour suivre sans ambiguïté le sort des cellules individuelles. Cette traçabilité nous a permis d'identifier plusieurs événements de Td putatives survenant au cours du développement larvaire de *C. elegans*, à savoir Y-to-PDA chez les vers mâles, K-to-DVB et G1-to-RMH. Le processus Y-to-PDA chez les mâles est intéressant car, contrairement à Y-to-PDA chez les hermaphrodites, il comprend une division cellulaire; K-to-DVB est un autre événement de Td dans le rectum du ver, où la cellule rectale K se divise et donne naissance à un neurone GABAergique appelé DVB ; G1-to-RMH Td est observé dans la tête du ver, où la cellule de pore excrétoire G1, une cellule épithéliale qui forme initialement un canal par un processus complexe d'auto-pliage membranaire, se divise pour former deux neurones sœurs pendant qu'elle est remplacée par la cellule G2. Ces événements se produisent entre les stades larvaires tardifs L1 et L2 et tous

partagent des caractéristiques communes: avant Td, la cellule initiale est une partie fonctionnelle d'un organe qui se connecte à l'extérieur et forme un tube.

Pour étudier ces processus, nous avons recherché dans la littérature et sélectionné des marqueurs pour identifier ces cellules chez les vers vivants ainsi que des marqueurs de leur identité cellulaire pour caractériser l'identité des cellules d'intérêt, que nous avons ensuite validés *in vivo* en utilisant des transgènes disponibles ou fabriqués dans le laboratoire (Abdus-Saboor et al., 2011 ; McIntire et al., 1997 ; Pereira et al., 2015 ; Vidal et al., 2015). Cette analyse de l'identité différenciée terminale des cellules initiale et finale, confirmée également par les différences de morphologie cellulaire, nous a permis de conclure que Y-to-PDA chez les mâles, K-to-DVB et G1-to-RMH sont événements naturels de Td.

2) Identification des facteurs de reprogrammation conservés requis pour d'autres événements de Td

Pour identifier les principaux facteurs impliqués dans différents événements de Td chez le ver, nous avons croisé des mutants ou des souches de knockdown pour les facteurs Y-to-PDA avec des souches rapporteuses pour mes cellules d'intérêt. En utilisant cette approche, nous avons constaté que certains facteurs de reprogrammation Y-to-PDA sont également nécessaires dans Y-to-PDA chez les mâles et dans K-to-DVB. Les facteurs de transcription *sox-2 / SOX*, *sem-4 / SALL* et *egl-5 / HOX* sont nécessaires pour ces événements de Td, ainsi que *ceh-6 / POU* pour K-to-DVB. D'un autre côté, le modificateur de la chromatine *egl-27 / MTA* est moins nécessaire en présence de la division cellulaire pour Y-to-PDA chez les mâles, alors qu'il n'est pas nécessaire pour la formation de DVB. Ces résultats concordent avec l'hypothèse selon laquelle l'occurrence de la division cellulaire facilite les changements de chromatine en soi, ce qui pourrait expliquer la moindre importance de certaines activités de remodelage de la chromatine (Ladewig et al., 2013). Concernant G1-to-RMH, nous avons quelques résultats préliminaires pour *egl-27 / MTA* et *sem-4 / SALL* et il semble qu'ils ne soient pas nécessaires pour la formation des neurones RMH. Quelques expériences supplémentaires seront nécessaires pour compléter ma caractérisation de ces autres événements de Td naturels. Ainsi, cependant, mes résultats montrent que, au moins dans trois contextes cellulaires différents dans la partie postérieure de *C. elegans*, des facteurs de reprogrammation conservés, qui pointent vers un mécanisme commun, sont nécessaires.

3) Détection fine des mécanismes sous-jacents au Td en présence de division cellulaire

L'implication de la division cellulaire dans Y-to-PDA chez les mâles, K-to-DVB et G1-to-RMH nous a incités à étudier son rôle lors de la Td et son interaction avec les facteurs de reprogrammation. Nous nous sommes concentrés sur K-to-DVB, un événement de Td la seule autre cellule rectale du ver hermaphrodite changeant d'identité.

Premièrement, nous avons observé que la division de K est orientée et asymétrique, donnant naissance une cellule fille postérieure K.p, dépourvue de ce fait des composants des jonctions apicales, mais présentant néanmoins plusieurs caractéristiques épithéliales. Comme la voie de signalisation Wnt est connue pour orienter le fuseau mitotique (Schlesinger et al., 1999) et pour réguler plusieurs divisions cellulaires chez *C. elegans* où les cellules filles acquièrent des destins différents (Sawa et Korswagen, 2013), nous avons analysé l'orientation de la division de K dans le mutant *lin-17 / FZD*. Nous avons constaté que l'orientation de la division de K est modifiée dans 30% des cas par rapport au type sauvage. Nous avons vérifié si cela pouvait être corrélé à un défaut de formation DVB : nous avons constaté que dans le contexte mutant *lin-17 / FZD*, DVB est absent dans plus de 90% des animaux. Ainsi, nous n'avons pas pu trouver de corrélation directe entre l'orientation modifiée de la division de K et l'absence de DVB. Pour tester directement si l'orientation de la division de K pourrait avoir un impact sur la Td, nous avons utilisé un mutant *goa-1 / GNAO1* (sous-unité de protéine G α o1), impliqué dans l'orientation du fuseau (Gotta et Ahringer, 2001) mais ne faisant pas partie de la voie de signalisation Wnt. Même si l'orientation de la division de K a été modifiée chez certains vers dans ce contexte mutant, la formation de DVB n'est pas affectée du tout. Ainsi, l'orientation modifiée de la division de K dans le mutant *lin-17 / FZD* pourrait alors être un effet secondaire, non responsable de l'échec de la formation de DVB.

Ayant exclu un rôle direct de l'orientation de la division de K et pour comprendre ensuite quelle voie Wnt est nécessaire dans ce contexte (Gómez-Orte et al., 2013 ; Sawa et Korswagen, 2013), nous avons étudié l'implication potentielle d'autres gènes de la voie Wnt dans K-to-DVB. Nous avons constaté que, comme dans les cellules T du ver (Herman et Horvitz, 1994), le couple *lin-44 / WNT* - *lin-17 / FZD* est important pour la formation de DVB, avec des impacts fonctionnels différents. L'absence de *lin-44 / WNT* se traduit par certains vers avec des identités inversées entre les cellules filles antérieure (K.a) et postérieure (K.p), mais peu d'absence de DVB. Ces résultats suggèrent que le *lin-44* seul a un rôle permissif dans la formation d'un neurone DVB et un rôle instructif dans la détermination de quelle cellule fille de K se transformera par la suite en DVB. Ils suggèrent également une possible redondance avec d'autres gènes Wnt dans ce contexte. En aval de la voie, nous avons constaté que *sys-1* / β-caténine est nécessaire pour la formation de DVB et *wrm-1* / β-caténine est également impliquée mais la pénétrance du défaut est faible. Enfin *pop-1*, le seul *TCF* chez *C. elegans*, est impliqué. Au contraire, d'autres voies Wnt, comme la voie PCP, *lin-18 / RYK* et la voie Wnt non canonique ne sont pas nécessaires pour K-to-DVB. Ainsi, nos résultats démontrent que les composants de la voie d'asymétrie canonique Wnt/β-caténine spécifiques à *C. elegans* sont impliqués dans K-to-DVB. Cette conclusion est également cohérente avec les résultats

précédents, car les voies canoniques Wnt ne sont pas impliquées dans l'orientation du fuseau, mais conduisent plutôt à une régulation transcriptionnelle (Gómez-Orte et al., 2013).

4) Caractérisation de la relation entre les facteurs de reprogrammation et la voie de signalisation Wnt

L'implication des mêmes facteurs de plasticité cellulaire dans différents événements de Td, impliquant également ou non Wnt, nous a incités à caractériser la relation entre eux pendant K-to-DVB. Nous avons trouvé deux classes de gènes: *sox-2/ SOX*, *ceh-6 / POU* et *egl-5 / HOX*, qui sont nécessaires à la fois pour la division de K et après ; *sem-4 / SALL* et la voie Wnt qui ne sont pas nécessaires pour que la division de K se produise, mais après celle-ci et/ou pour la réguler. Ainsi, différents facteurs de reprogrammation et la signalisation Wnt jouent des rôles différents lors de la Td K-to-DVB.

Puisque l'absence *sem-4 / SALL* et *lin-17 / FZD* n'empêche pas la division de K mais conduit à absence de DVB, nous avons examiné plus en détail leurs phénotypes, pour identifier leurs possibles différences fonctionnelles. En marquant l'expression de différents gènes rapporteurs rectaux et épithéliaux et neuronaux chez les mutants *sem-4 / SALL* et *lin-17 / FZD*, nous avons constaté que K.p reste épithéliale rectale dans les deux cas, et ce sans co-activation de gènes pan-neuronaux ou GABAergiques. Comme les phénotypes des deux mutants sont indiscernables, nous avons émis l'hypothèse que la voie de signalisation Wnt pourrait agir dans K.p par la régulation de *sem-4 / SALL* : cependant, l'analyse de l'épistase et l'examen d'une éventuelle régulation transcriptionnelle croisée ont démontré que ce n'était pas le cas. Considérant que la forte perte de fonction *lin-17 / FZD* et les souches nulles *sem-4 / SALL* présentent des phénotypes similaires, avec une pénétrance similaire (DVB absent dans plus de 90%), et que *sem-4 / SALL* n'est pas régulé par la voie Wnt (et vice-versa), nous avons finalement émis l'hypothèse que ces deux gènes pourraient agir à travers deux voies parallèles et non redondantes. Pour effectuer l'analyse des doubles mutants, ne pouvant utiliser les allèles forts/nuls, nous avons choisi de combiner la mutation *wrm-1* / β-caténine Ts avec un allèle mutant hypomorphique *sem-4 / SALL*, qui présente une pénétrance inférieure au mutant nul. Ceci a révélé une augmentation synergique du pourcentage de vers dépourvus du neurone DVB par rapport aux mutants simples (de 8% et 2,5% dans les mutants simples *sem-4 /SALL* et *wrm-1* / β-caténine respectivement à 46% dans le double mutant). Dans l'ensemble, ces résultats suggèrent que la signalisation Wnt et le facteur de reprogrammation *sem-4 / SALL* agissent dans des voies parallèles non redondantes lors de la Td K-to-DVB.

Nous nous sommes ensuite concentrés sur la relation entre la voie de signalisation Wnt et *sox-2 / SOX*, comme cela a été fait pour *sem-4 / SALL*. Nous avons d'abord construit un double mutant avec l'allèle *wrm-1* / β-caténine Ts et avec la *sox-2* KD médiée dans la souche *sox-*

2::gfp KI, en utilisant la stratégie de nanocorps anti-GFP (Wang et al., 2017). Fait intéressant, comme pour *sem-4 / SALL*, nous avons trouvé un effet synergique en l'absence simultanée de *wrm-1* / β-caténine et de régulation négative de *sox-2 / SOX*, suggérant à nouveau des rôles parallèles convergeant probablement sur des cibles communes afin de reprogrammer K en DVB. Certaines observations nous ont permis de faire l'hypothèse que l'une des cibles communes pourrait être le gène *lim-6 / LMX*, un sélecteur terminal important pour l'expression de l'identité spécifique DVB (Hobert et al., 1999). La régulation de l'expression du gène *lim-6 / LMX* a attiré notre attention pour deux raisons: tout d'abord, nous avons constaté que l'expression *lim-6 / LMX* commence dans K.p peu de temps après la division de K et qu'elle est augmenté en parallèle de la diminution de *sox-2 / SOX* ; deuxièmement, le 4ème intron de *lim-6*, suffisant pour l'expression dans DVB (Hobert et al., 1999), contient des sites de liaison putatifs pour TCF, et certains d'entre eux chevauchent des sites de liaison pour SOX2. Ces observations nous ont incités à émettre l'hypothèse d'un rôle répressif de SOX-2 sur l'expression de *lim-6 / LMX* et sa compétition avec TCF, agissant lui comme activateur, pour la liaison aux régions régulatrices de *lim-6*. En effet, nous avons constaté que *lin-17 / FZD* et *pop-1 / TCF* sont tous deux requis pour l'expression de *gfp* pilotée par cet intron et qu'en mutant les sites de liaison de TCF contenus dans cette séquence, l'expression de *gfp* est perdue ou considérablement diminuée. Dans une approche complémentaire, nous testons si l'expression de *lim-6 / LMX* en DVB est empêchée par la surexpression de *sox-2 / SOX*. Enfin, pour vérifier l'hypothèse du mécanisme moléculaire, nous essayons de produire des protéines SOX-2 et POP-1 *in vitro* pour tester leur liaison aux sondes *lim-6* (intron 4) et leur compétition, via un EMSA. La démonstration d'un tel mécanisme fournirait pour la première fois un exemple de la façon dont des facteurs de reprogrammation conservés et une voie de signalisation peuvent synergiser lors de la reprogrammation naturelle d'une cellule *in vivo* pour réguler étroitement et efficacement la temporalité de la re-différenciation.

CONCLUSIONS

Nos travaux démontrent l'existence d'un mécanisme intégrant à la fois des facteurs de reprogrammation conservés et d'une voie de signalisation de manière synergique - en parallèle et de manière non redondante - lors d'événements de reprogrammation naturels. Il donne également un exemple de la façon dont la régulation de la division cellulaire peut avoir un impact sur la transdifférenciation, en définissant quelle cellule fille adoptera une nouvelle identité , impliquant de nouvelles activités facilitant le processus et rendant également le processus plus rapide et moins dépendant de certaines activités de remodelage de la chromatine. La compréhension complète de la façon dont les différents acteurs contribuent aux événements de reprogrammation des cellules naturelles pourrait éclairer la façon de

rendre les processus de reprogrammation *in vitro* plus efficaces et plus complets, en vue par exemple d'applications thérapeutiques.

RÉSUMÉ EN ANGLAIS

INTRODUCTION

Transdifferentiation (or direct cell reprogramming) is the direct conversion of one fully differentiated cell type into another, with or without cell division. Natural transdifferentiation (Td) has been characterised in *C. elegans* by our lab, which has demonstrated that the hermaphrodite's Y rectal cell transdifferentiates into the PDA motor neuron with high efficiency, robustness and irreversibly (Jarriault et al., 2008). Through mutagenesis and RNAi screens, reprogramming transcription factors (i.e. *sox-2*/*SOX*, *ceh-6*/*POU*, *sem-4*/*SALL*), chromatin modifiers (i.e. *egl-27/MTA, jmjd-3.1/KDM6, set-2/SETD1*) and other developmental genes (i.e. *egl-5/HOX*) have been identified as involved in Y-to-PDA Td; in addition, cellular steps and molecular networks controlling them are being deciphered. For instance, this event occurs without cell division and consists in a step-wise process that starts with the erasure of the initial identity, before re-differentiation into a different cell type (Kagias et al., 2012; Richard et al., 2011; Zuryn et al., 2014)

Are these mechanisms unravelled by the lab universal or do they vary among different Td events? The goal of my PhD is to examine if other plasticity events occur naturally in the worm, and to define what core mechanisms, if any, and what event-specific variations can be found. A special focus is put on the role of cell division in Td.

RESULTS

1) Identification of other natural Td events in *C. elegans*

The determination of the embryonic and post-embryonic cell lineage in *C. elegans* (Sulston and Horvitz, 1977; Sulston et al., 1983) has provided a powerful tool to unambiguously follow the fate of individual cells. This traceability has allowed us to identify several putative Td events occurring during *C. elegans* larval development, namely Y-to-PDA in male worms, K-to-DVB and G1-to-RMH. Y-to-PDA in males is an interesting process because, differently from Y-to-PDA in hermaphrodites, it is preceded by a cell division; K-to-DVB is another Td event in the rectum of the worm, where the rectal cell K divides and gives rise to a GABAergic neuron called DVB from its posterior daughter K.p; G1-to-RMH Td is observed in the head of the worm, where the excretory pore cell G1, an epithelial cell which initially forms a channel through a specialised membrane auto-folding process, divides to form two sister neurons while it is replaced by G2 cell. Both these last two events occur between late L1 and L2 larval stages and all of them share common characteristics: before Td the initial cell is a functional part of an organ that connects to the exterior and forms a tube.

To study these processes, we have screened for and found reporters in the literature to identify those cells in living worms and at the same time we have selected cell identity marker genes to characterise the identity of the cells of interest, which we have further validated *in vivo* using available or lab-made transgenes (Abdus-Saboor et al., 2011; McIntire et al., 1997; Pereira et al., 2015; Vidal et al., 2015). The different terminal differentiated identity of the initial and final cell in all the cases, confirmed also by the differences in cellular morphology, allowed us to conclude that Y-to-PDA in males, K-to-DVB and G1-to-RMH are *bona fide* Td events.

2) Identification of conserved reprogramming factors required for other Td events

To identify core factors involved in different Td events in the worm, we have crossed mutants or knockdown strains for the Y-to-PDA factors with reporter strains for my cells of interest. Using this approach, we have found that some Y-to-PDA reprogramming factors are also required for Y-to-PDA in males and in K-to-DVB. *sox-2/SOX*, *sem-4/SALL* and *egl-5/HOX* transcription factors are necessary for these Td events in the rectum of *C. elegans*. In K-to-DVB, we have observed that also *ceh-6/POU* is important. On the other hand, *egl-27/MTA* chromatin modifier is less required in presence of cell division in Y-to-PDA in males, while it is not necessary for DVB formation. These results agree with the hypothesis that occurrence of cell division facilitates chromatin changes *per se*, which could explain the bypassing of the requirement of some chromatin remodelling activities (Ladewig et al., 2013). Concerning G1-to-RMH we have some preliminary results for *egl-27/MTA* and *sem-4/SALL* and it seems that they are not necessary for RMH neurons formation. A few more experiments will be necessary to complete my characterisation of these additional natural Td events. However, my results show that, at least in three different cellular contexts in the rectum of *C. elegans*, conserved reprogramming factors, which may represent a core mechanism, are required.

3) Fine probing of the mechanisms underlying Td in presence of cell division

The involvement of cell division in Y-to-PDA in males, K-to-DVB and G1-to-RMH has prompted us to investigate its role in Td and its interplay with the reprogramming factors. We have been focusing on K-to-DVB, the Td event taking place in the rectum of the hermaphrodite worm together with Y-to-PDA. First, we have observed that K division is oriented and asymmetric, giving rise to the posterior daughter K.p lacking apical junction components, but still displaying several epithelial features. As Wnt signalling pathway is known to orient the mitotic spindle (Schlesinger et al., 1999) and to regulate several cell divisions in *C. elegans* where the daughter cells acquire different fates (Sawa and Korswagen, 2013), we have analysed the orientation of K division in *lin-17/FZD* mutant. We have found that the orientation of K division is altered in 30% of the cases compared to the wild type. We have checked if this could be correlated to a defect in DVB differentiation from K.p and we have found that in *lin-17/FZD* mutant background more than 90% of the animals lack DVB. Thus, we could not find a direct

correlation between the altered orientation of K division and the absence of DVB. To directly test if the orientation of K division could have an impact on Td, we have used a *goa-1/GNAO1* (G protein subunit α o1) mutant, involved in spindle orientation (Gotta and Ahringer, 2001) but not part of the Wnt signalling pathway. Even though the orientation of K division has been altered in some worms in this mutant background, DVB formation has not been affected at all. Thus, the altered orientation of K division in *lin-17/FZD* mutant might then be a secondary effect, not causative of the failure in DVB formation.

Having excluded a direct role of the orientation of K division and to understand then which variant of the *C. elegans* Wnt pathway is necessary in this context (Gómez-Orte et al., 2013; Rose and Gönczy, 2013), we have characterised which other genes of the Wnt pathway are involved in K-to-DVB. This information is also important to dissect how the Wnt signalling pathway and the reprogramming factors are involved together in DVB formation from a rectal cell. We have found that, like in the T cells (Herman and Horvitz, 1994), the couple *lin-44/WNT*-*lin-17/FZD* is important for DVB formation, with different functional impacts. The absence of *lin-44/WNT* results in some worms with inverted identities between the anterior K.a and the posterior K.p daughters, but little DVB absence. These results suggest that *lin-44* alone has a permissive role in the formation of a DVB neuron and an instructive role in the determination of which K daughter will subsequently adopt a DVB fate. They also suggest a possible redundancy with other Wnt genes in this context. Downstream in the pathway, we have found that *sys-1*/β-catenin is required for DVB formation and *wrm-1*/β-catenin is required but the penetrance of the defect is low. Finally *pop-1*, the only *TCF* in *C. elegans*, is involved. On the contrary, other Wnt-related pathways, like the PCP pathway, *lin-18/RYK* and the non-canonical Wnt pathway are not required for K-to-DVB. Thus, our results demonstrate that components of the *C. elegans*-specific canonical Wnt/β-catenin asymmetry pathway are involved in K-to-DVB Td. This conclusion is also consistent with the previous results, as the canonical Wnt pathways are not involved in spindle orientation, but rather lead to a transcriptional output (Gómez-Orte et al., 2013).

4) Characterization of the relationship between reprogramming factors and Wnt signalling pathway

The requirement of “core” reprogramming transcription factors in different Td events, where Wnt is or is not involved, has prompted us to characterise the relationship between them and the Wnt signalling pathway in K-to-DVB. We have examined the phenotype of the mutants where DVB is absent and found two classes of genes: *sox-2/SOX*, *ceh-6/POU* and *egl-5/HOX*, which are required both for K division and after it; *sem-4/SALL* and Wnt signalling pathway which are not required for K division to occur, but after it. Thus, different reprogramming factors and Wnt signalling play different roles in K-to-DVB Td.

Since absence of *lin-17/FZD* and *sem-4/SALL* does not preclude K division but leads to No DVB, we have examined in more detail their phenotypes, to identify possible different roles if any. By scoring the expression of different reporter genes for the rectal-epithelial and for the neuronal identity in *sem-4/SALL* and *lin-17/FZD* mutants, we have found that K.p remains rectal epithelial in both the cases, also failing to activate pan-neuronal and GABAergic genes. As the phenotypes of the two mutants are indistinguishable, we have hypothesised that Wnt signalling pathway might act in K.p through the regulation of *sem-4/SALL*: however, epistasis analysis and examination of possible cross transcriptional regulation have demonstrated that this is not the case. Considering that *lin-17/FZD* strong loss of function and *sem-4/SALL* null strains display similar phenotypes, down to the penetrance of the No DVB phenotype (over 90% in both backgrounds), and that *sem-4/SALL* is not regulated by the Wnt pathway, we have finally hypothesised that those two genes could drive K-to-DVB Td through two parallel and non-redundant pathways. To perform the double mutant analysis, we could not use the strong/null alleles, but we have chosen to combine *wrm-1*/β-catenin Ts mutation with a hypomorphic *sem-4/SALL* mutant allele, that shows a lower penetrance than the null. The scoring of the double mutant strain has revealed a synergistic increase in the percentage of worms lacking the DVB neuron compared to the single mutants (from 8% and 2.5% in *sem-4* and *wrm-1* single mutants respectively to 46% in the double mutant). Overall, these results suggest that Wnt signalling and the reprogramming factor *sem-4/SALL* act in non-redundant parallel pathways to drive K-to-DVB Td.

Next, we have focused on the relationship between Wnt signalling pathway and *sox-2/SOX*, as done for *sem-4/SALL*. We have first used the same approach and built a double mutant with the *wrm-1* Ts allele and with *sox-2* KD mediated in *sox-2::gfp* KI strain, using the anti-GFP nanobody strategy (Wang et al., 2017). Interestingly, like for *sem-4/SALL*, we have found a synergistic effect in simultaneous absence of *wrm-1* and downregulation of *sox-2/SOX*, suggesting again parallel roles probably converging on common targets in order to reprogram K into DVB. Some observations has allowed us to hypothesise that one of the common targets could be *lim-6/LMX* gene, a terminal selector in DVB important for the expression of terminal differentiation genes (Hobert et al., 1999). Regulation of *lim-6/LMX* gene expression has attracted our attention for two reasons: first of all, we found that *lim-6*/*LMX* expression starts in K.p a few hours after K division and it is paralleled by a decrease in *sox-2*/SOX expression; secondly, *lim-6* 4th intron, sufficient to drive reporter expression in DVB (Hobert et al., 1999), contains putative TCF binding sites, some of them overlapping with SOX2 binding sites. These observations have prompted us to precisely hypothesise a repressive role of SOX-2 on *lim-6/LMX* expression and a competition with POP-1, acting as an activator, for the binding to *lim-6* regulatory regions. Indeed, we have found that both *lin-17*/*FZD* and *pop-1/TCF* are required

for *gfp* expression driven by this intron and that, by mutating the TCF binding sites contained in this sequence, *gfp* expression is lost or dramatically decreased. These results demonstrate that POP-1 binding to *lim-6 intron 4* is required for DVB-specific *lim-6* expression in K.p. In a complementary approach, we are testing whether *lim-6/LMX* expression in DVB is prevented by overexpressing *sox-2/SOX*. Finally, to verify the hypothesised molecular mechanism, we are trying to produce SOX-2 and POP-1 proteins *in vitro* to test their binding to *lim-6 intron 4* probes and competition through an EMSA. The demonstration of such a mechanism would provide for the first time an example of how conserved reprogramming factors and a signalling cue may integrate in a cell in a natural reprogramming context *in vivo* to tightly and efficiently regulate the timing of re-differentiation.

CONCLUSIONS

Our work demonstrates the existence of both conserved reprogramming factors and of event-specific signalling cues that can synergistically contribute - in parallel and in a non-redundant manner - to natural reprogramming events. It also gives an example of how regulated cell division can impact on transdifferentiation, by defining which daughter cell will adopt a new identity, involving new activities facilitating the process, and likely also making the process faster and less dependent on some chromatin remodelling activities. The complete understanding of how different actors contribute to natural cell reprogramming events could shed light on how to make *in vitro* reprogramming processes more efficient and complete.

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LIST OF ABBREVIATIONS

Most abbreviations used are generally adopted in scientific literature in their respective fields. Gene and protein names are written according to the nomenclature guidelines for each species. For *C. elegans*, gene names are written in lower-case and italics, while protein names in upper-case. For mouse, gene names are written in italics with the first letter in upper-case, whereas protein names are in upper-case. For human genes, upper-case letters and italics are used and protein names are written like for *C. elegans* and mouse. The respective nomenclature rules were followed for *Drosophila*, *Danio rerio*, amphibian and avian genes and proteins.

5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ACD	Asymmetric Cell Division
ACh	Acetylcholine
AMso	AMphid socket
aPCK	atypical Protein Kinase C
AzaC	5-azacytidine
BAM	*Ascl1, Brn2*, *Myt1l*
BCHE	ButyrylCHolinEsterase
BEC	Biliary Epithelial Cell
BMP	Bone Morphogenetic Protein
CASD	Cell-Activation and Signalling-Directed (transdifferentiation)
CDK	Cyclin-Dependent Kinase
CGC	Caenorhabditis Genetics Center
CRMP1	Collapsin Response Mediator Protein 1
CS	Corpuscles of Stannius
CSC	Cancer Stem Cell
CSF	CerebroSpinal Fluid
DE	Distal early (tubule renal cell)
DIC	Differential interference contrast
Dlg	Discs-large
Dsh	Dishevelled (in *Drosophila*)
DVL	Dishevelled (in mammals)
EGF	Epidermal Growth Factor
EM	Electron Microscopy

ESC	Embryonic stem cell
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FZ	Frizzled (in *Drosophila)*
FZD	Frizzled (in mammals)
GAD	Glutamic Acid Decarboxylase
gDNA	genomic DNA
GMC	Ganglion Mother Cell
GPCR	G Protein Coupled Receptor
GRN	Gene Regulatory Network
GSC	Germline Stem Cell
h	hours
hiPSC	human induced Pluripotent Stem Cell
HMG	High-Mobility Group
hpa	hours post amputation
HSC	Hematopoietic Stem Cell
ICM	Inner Cell Mass
Id	Inhibitor of Differentiation
IGF	Insulin-like Growth Factor
Insc	Inscuteable
IWM	International Worm Meeting
KI	Knock in
Lgl	Lethal (2) giant larvae
LIF	Leukaemia Inhibitory Factor
lincRNA	large intergenic non-coding RNA
lncRNA	long non-coding RNA
MCM	Mystery Cell of the Male
MCT	Multipotency-to-Commitment Transition
MEF	Mouse Embryonic Fibroblast
MET	Mesenchymal-to-Epithelial Transition
miRNA	microRNA
Mud	Mushroom body defect
MW	Molecular Weight
NGM	Nematode Growth Media
NSC	Neural Stem Cell
NuMA	Nuclear Mitotic Apparatus

iPS(C)	induced Pluripotent Stem (Cell)
PCP	Planar Cell Polarity
PEC	Pigment Epithelial Cell
PH	Post Hatching
PHD	PHasmid D (neuron)
PHso	PHasmid socket
Pins	Partner of Inscuteable
PSC	Pluripotent Stem Cell
OSKM	*Oct4/Sox2/Klf4/c-Myc*
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
RIMBP2	RIMS binding protein 2
RPE	Retinal Pigment Epithelium
RTK	Receptor Tyrosine Kinase
SC	Stem Cell
SCNT	Somatic Cell Nuclear Transfer
SD	Standard Deviation
SOP	Sensory Organ Precursor (cell)
SVZ	SubVentricular Zone
TAD	Topologically Associated Domain
Td	Transdifferentiation
TGFβ	Transforming Growth Factor-β
VGAT	Vesicular GABA Transporter
VPC	Vulval Precursor Cell
VZ	Ventricular Zone

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I. INTRODUCTION

1 Cellular reprogramming

1.1 The concepts of cell identity and cell plasticity

The specialised identity of a cell is translated into a specific morphology, cell function and behaviour, and is determined by a set of intrinsic molecular properties, such as a cell state-specific gene regulatory network (GRN) (Enver et al., 2009; Rivera and Ren, 2013; Rothman and Jarriault, 2019) which can be regulated by extrinsic cues. Transcription factors are considered the master regulators of cell identity, thus the over 200 cell types in the human body are the result of the differential expression of combination of transcription factors building up different GRNs (Enver et al., 2009; Rivera and Ren, 2013). Transcription factors activate the expression of differentiation genes which are directly responsible of the phenotypical and functional features of a cell type (Smith et al., 2016). The characterisation of the GRN of embryonic stem cells (ESCs) has significantly contributed to the understanding of the role of transcription factors, but also of chromatin regulators and non-coding RNAs in the regulation of the network (Li and Belmonte, 2017). More recently, the knowledge of the cell lineage has been proposed as another element to define the identity of a cell, giving important information on its lineage relationship with other cell types (Morris, 2019). The complete cell lineage is today known only for two animals, in which it is also invariant: the nematode worms *Caenorhabditis elegans* and *Panagrellus redivivus* (Sternberg and Horvitz, 1981, 1982; Sulston and Horvitz, 1977; Sulston et al., 1983).

The several cell types in an animal body are obtained through embryonic and post-embryonic development starting from the fertilised egg, the zygote. The capacity of the zygote and of the first blastomeres (depending on the species) to form all embryonic and extraembryonic lineages is termed **totipotency** in vertebrate development (Rothman and Jarriault, 2019). Totipotency is considered the maximum degree of cellular potential (i.e. the range of cell types that a cell can give rise to), but other levels of potential are described and are usually associated with stem cells (Figure 1). Indeed, **stem cells** are classically defined by two properties: the self-renewal capacity to maintain the stem cell population and the cellular potential to give rise to daughter cells with different, more differentiated identities which can be considered the “effector” cells of a tissue (Rothman and Jarriault, 2019; Wagers and Weissman, 2004). This definition applies to adult somatic stem cells, but less to pluripotent stem cells (PSCs) and to any transient cell type during development with a certain degree of cellular potential. Even though self-renewal is not a property of PSCs *in vivo*, as they form the

transient Inner Cell Mass (ICM) of the blastocyst, they have the potential to give rise to all the somatic embryonic lineages and the germline which defines their **pluripotency** (Hackett and Surani, 2014; Rothman and Jarriault, 2019). On the contrary, adult somatic stem cells are usually **multipotent** or even **unipotent** when they can differentiate into either several different cell types (like hematopoietic stem cells, HSCs) or only one cell type (like muscle stem cells) (Ng and Alexander, 2017; Rothman and Jarriault, 2019; Sambasivan and Tajbakhsh, 2015).

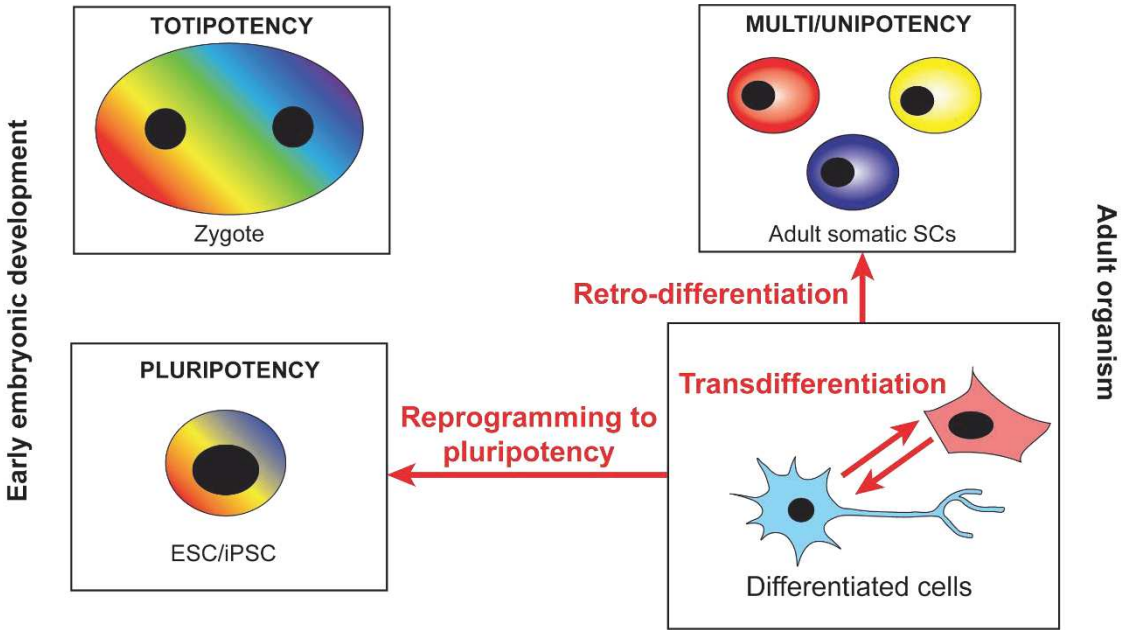

Figure 1. Schematic representation of the concepts of reprogramming to pluripotency, retro-differentiation, transdifferentiation and of cellular potential with all its possible levels and the representative cell types for each level. The rainbow-coloured zygote represents its totipotency, while the blue, red and yellow colours of the ESC represent its pluripotency to give rise to the three germ layers, ectoderm, mesoderm and endoderm.

Overall, the ability of a cell to give rise to cells with different identities is referred to as **cellular plasticity**. Even though cellular plasticity is traditionally considered a property of stem cells, it is not restricted to them. From the second half of the XX century, key observations and experiments demonstrated that cellular plasticity can naturally occur or artificially be induced in committed and even in terminally differentiated cells. Thus, “terminal” differentiation cannot be used anymore with the meaning of “irreversible”, but it rather means “stable” (Holmberg and Perlmann, 2012; Sánchez Alvarado and Yamanaka, 2014). Cellular plasticity events observed in committed but not differentiated cells are called **transdetermination**, which consists in a change in commitment and was first described in *Drosophila melanogaster* imaginal disc cells (Blau, 1992; Hadorn, 1968; Schubiger, 1971); when cellular plasticity is observed in differentiated cells, it can lead either to **retro-differentiation** or to **transdifferentiation**. The former consists in the loss of the differentiated state with reversal to a progenitor or even stem cell identity (culminating in **reprogramming to pluripotency**); dedifferentiation is often used

as a synonym of retro-differentiation, however dedifferentiation specifically reflects the loss of differentiated features and does not imply automatically the gain of cellular potential. Moreover, as we will see later, retro-differentiation can also occur in progenitor cells which in some regeneration contexts can give rise to stem cells (Merrell and Stanger, 2016). Transdifferentiation occurs when a stably differentiated cell loses its identity to gain another terminal differentiated cell identity (for the original definition of transdifferentiation see Selman and Kafatos, 1974 and Okada, 1986; Rothman and Jarriault, 2019) (Figure 1). In some variants of the definition of transdifferentiation the initial cell type is a somatic cell, thus cell fate conversions starting from germ cells would not be considered as transdifferentiation (Takahashi and Yamanaka, 2016).

The change of identity of a more differentiated into either a less differentiated cell type or another differentiated cell type is generally called **cellular reprogramming** (Holmberg and Perlmann, 2012). In the field, the most common terms used are reprogramming to pluripotency (Yamanaka and Blau, 2010) to indicate the reversion of a differentiated cell type to a PSC state, and transdifferentiation, as defined above, to indicate the direct conversion between two differentiated cell types without pluripotent intermediate. Some scientists use the term lineage reprogramming either as a synonym of transdifferentiation (Jessen et al., 2015; Morris, 2016), or to indicate both transdifferentiation and transdetermination (Graf and Enver, 2009), or even including both transdifferentiation and dedifferentiation/reprogramming to pluripotency (Xu et al., 2015; Zhou and Melton, 2008). For this Thesis, the terms reprogramming to pluripotency and transdifferentiation will be used with the meanings first described above to avoid any ambiguity. Finally, as also pointed out by G. Eguchi and R. Kodama (Eguchi and Kodama, 1993), I will not use the term transformation to indicate non-malignant cell fate changes, as transformation of animal cells denotes a change to a cancer cell phenotype. It is now accepted that cell reprogramming occurs during cancer development (Xiong et al., 2019), however reprogramming (or transdifferentiation) and transformation in their strict meaning cannot be used as synonyms.

1.2 The maintenance of cell identity

The advent of cellular reprogramming has increased the interest in the mechanisms defining and maintaining cell identity (Gascón et al., 2017). In fact, even if it occurs, reprogramming of differentiated cells is a rare event in nature (Gurdon, 2013; Merrell and Stanger, 2016). It can be induced in many different cell types by altering the expression of transcription factors, chromatin regulators, non-coding RNAs or with chemicals (described below; Pennarossa et al., 2016; Slack, 2007; Xu et al., 2015). However, using these approaches, reprogramming efficiency may vary, and the process may be incomplete. This suggests that there are

mechanisms that maintain the identity of differentiated, mature cells and have to be overcome to allow reprogramming: indeed, more differentiated cells are more difficult to be reprogrammed (Pasque et al., 2011). Moreover, different cell types are differentially prone to be reprogrammed by the same transcription factor(s), demonstrating the importance of the initial cellular context (Gascón et al., 2017; Riddle et al., 2016). The stability of a differentiated cell identity is fundamental to ensure tissue function in physiological conditions (Merrell and Stanger, 2016): in support to this, cell identity is perturbed in pathological conditions like degenerative diseases, metaplasia and cancer (Roy and Hebrok, 2015; Slack, 2007).

What are the mechanisms maintaining cell identity? Answering to this question could provide useful information both for understanding the onset of certain diseases and for improving reprogramming strategies for therapeutic treatments (Gascón et al., 2017).

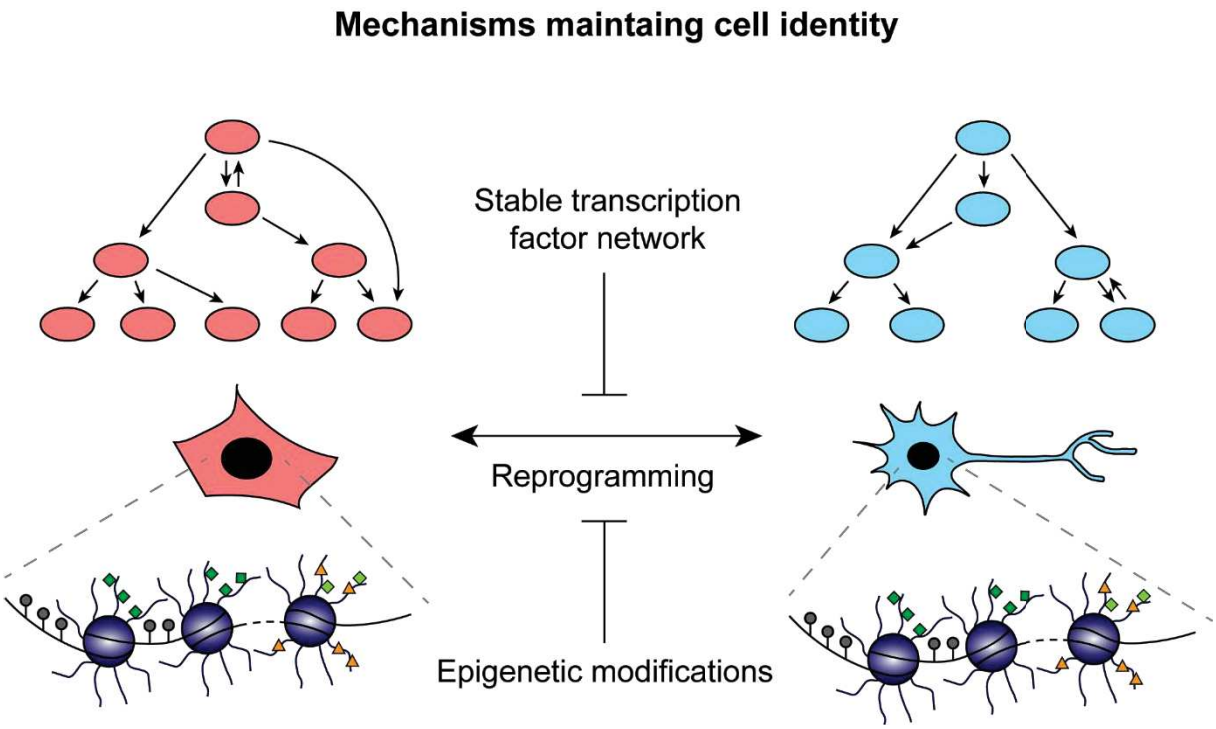

Figure 2. Schematic summary of the two main mechanisms maintaining cell identity and preventing cellular reprogramming: the presence of cell-specific stable transcription factor network and epigenetic modifications.

The evidence that even differentiated cells retain cell plasticity and silenced genes can be reactivated support the idea that the differentiated identity of a cell is actively maintained, as proposed for the first time by H. Blau and D. Baltimore (Blau, 1992; Blau and Baltimore, 1991). They suggested the presence of positive and negative regulators in different cell types that actively and continuously maintain the cell type-specific transcriptional state. Subsequent studies demonstrated that these regulators may be some of the transcription factors that are

also required to initially drive terminal differentiation. This was confirmed in different animal model organisms, from *C. elegans* to mammals (Holmberg and Perlmann, 2012).

In *C. elegans*, work from several labs (reviewed by Hobert, 2008) demonstrated that a class of autoregulated transcription factors, called terminal selectors, are required both to initiate and to maintain the expression of terminal differentiation genes (also called effector genes) in different neuronal subtypes (Hobert and Kratsios, 2019). For instance, TTX-3 and CEH-10 terminal selectors drive the expression of terminal differentiation genes in AIY neurons and maintain AIY identity by also activating their own expression (Bertrand and Hobert, 2009). Interestingly, in *C. elegans* the loss of only one or two terminal selectors can lead to the loss of terminal differentiation of specific neurons and to the ectopic expression of other effector genes: the terminal selector UNC-3 was shown to be required both for the activation of terminal differentiation genes in ASI neurons and to repress other differentiation programmes (Kim et al., 2005). On the contrary it seems that in “higher” organisms, such as vertebrates but also *Drosophila*, a more complex regulation is usually required with combinations of several transcription factors building up a stable transcription factor network and ensuring the maintenance of differentiated cell identity (Figure 2). This stability is based on redundant interactions among transcription factors that are reinforced during differentiation (Holmberg and Perlmann, 2012). A few exceptions are observed and, for instance, the depletion of *Pax5* alone in mature B cells leads to their dedifferentiation, allowing them to be reprogrammed to T cells *in vivo* (Cobaleda et al., 2007) and the loss of *Foxl2* in the ovarian follicle cells results in their transdifferentiation into Sertoli-like and Leydig-like cells (Uhlenhaut et al., 2009).

Together with transcription factors, another category of proteins is responsible for maintaining the differentiated cell identity: DNA and chromatin modifiers (Figure 2) (Flavahan et al., 2017; Holmberg and Perlmann, 2012; Merrell and Stanger, 2016; Paksa and Rajagopal, 2017). These factors are responsible for establishing and propagating the epigenetic information which consists in “heritable changes in gene function that occur without alteration to the DNA sequence” (Probst et al., 2009). Active mechanisms have been described for the maintenance of histone modifications at specific sites especially after cell division (Probst et al., 2009). These mechanisms rely on the double nature of histone modifier complexes often being both readers of pre-existing modifications and writers of new marks, thus also enabling the crosstalk between different histone modifications and building up a robust network. The existence of feedback mechanisms between histone modifications appear conserved in metazoans, having been described in *C. elegans* as in mammals (Zhang et al., 2015). H. Sawa and colleagues demonstrated the importance of histone mark readers and writers in both establishing and maintaining differentiated cell identities of different cell types *in vivo* in *C. elegans* (Shibata et al., 2010). On the other hand, DNA methylation at cytosines is present only in vertebrates

where it also contributes to establishment and maintenance of cell identity, with both active and passive mechanisms described for its removal (Bogdanović and Lister, 2017).

Altogether these studies demonstrate that the identity of differentiated cells is robustly maintained through the continuous presence of several transcription factors and chromatin regulators which interact genetically and physically to build up a stable transcription factor network and a “rigid” chromatin configuration (Vierbuchen and Wernig, 2012). However, under certain conditions, the perturbation of the transcriptional network and of epigenetic marks on chromatin can lead to loss of cell identity and acquisition of another fate.

1.3 Evidence of cell plasticity observed in nature

The interest in cell plasticity and in its potential applications in disease modelling, drug discovery and regenerative medicine has significantly grown after the generation of induced pluripotent stem (iPS) cells in 2006 (Cohen and Melton, 2011; Graf, 2011 for the increase in citations after 2006; Takahashi and Yamanaka, 2006, 2016). Nevertheless, the first idea of cell plasticity in non-stem cells date back to the XIX century thanks to the observation of natural phenomena, namely the lens regeneration in newts after lentectomy, by C. Bonnet, V. Colucci and Gustav Wolff (Merrell and Stanger, 2016). Today, accumulating data demonstrate that cell plasticity manifests itself in normal development, regeneration and in pathological conditions. This evidence led to a re-interpretation of Conrad H. Waddington’s epigenetic landscape (*The strategy of the genes*, 1957) which was depicting the differentiation path of cells from the pluripotent state as a marble on the top of a slope rolling down into different groves representing the possible irreversible differentiation states. The irreversibility of the differentiation states cannot be accepted anymore, and more recent representations show that the marble can go back even at the top of the slope (Ladewig et al., 2013).

1.3.1 Cell plasticity during development

1.3.1.1 *Cell plasticity during development in invertebrates: C. elegans*

The stereotyped cell lineage of *C. elegans* has allowed the discovery and study of cell identity switches taking place during *C. elegans* post-embryonic larval development both in hermaphrodites and in males (Sulston and Horvitz, 1977; Sulston et al., 1980). The occurrence of transdifferentiation was demonstrated in the *C. elegans* hermaphrodite where the Y rectal epithelial cell gives rise to a motor neuron called PDA (Jarriault et al., 2008). This process proceeds in discrete steps with the Y cell first dedifferentiating without acquiring pluri/multipotency and then irreversibly re-differentiating (Richard et al., 2011).

Another transdifferentiation event occurring in the male worm was described more recently and it consists in the sex-specific conversion of a pair of glial cells, called PHso1 (phasmid socket), into sensory neurons, named PHD for phasmid D neurons (Molina-García et al., 2018. This work is a preprint, but it clearly demonstrates the change in PHso1 identity in males and not in hermaphrodites). In both Y-to-PDA Td and PHso1-to-PHD Td cell division does not occur.

1.3.1.2 *Cell plasticity during development in invertebrates: Drosophila*

Cell plasticity in the form of transdetermination has been described during the development of another invertebrate model organism, *Drosophila melanogaster*. One event takes place during embryogenesis: the body segments or compartments in which the developing embryo is organised are usually made up of different cells that cannot cross segment boundaries, being already committed to specific fates. However, live imaging experiments revealed that a group of epidermal cells, called “mixer cells”, can reprogram and cross compartment boundaries (Gettings et al., 2010).

Another plasticity event occurs at the onset of metamorphosis in *Drosophila*: some progenitor cells in the midgut migrate onto the renal tubules and switch their identity to become renal progenitor cells. This event is fundamental for the development of the adult renal tubules and the viability of the animal, and it requires the activation of the homeodomain transcription factor *Cut* (Xu et al., 2018).

1.3.1.3 *Cell plasticity during development in vertebrates: Zebrafish*

Cell plasticity during development has been recently described in *Danio rerio*. Like in *Drosophila*, live imaging experiments allowing to trace single cells *in vivo* demonstrated that some differentiated distal early (DE) tubule renal cells form the Corpuscles of Stannius (CS) gland cells through transdifferentiation. These cells are generated in the renal tubule and then are extruded and proliferate (Naylor et al., 2018).

Transdifferentiation is suggested to be also the source of different skin pigment cells in Zebrafish, where black pigment cells called melanophores give rise to a population of white pigment cells called melano-leucophores, switching the pigment synthesis pathway (Lewis et al., 2019). Whether this switch could be really defined as transdifferentiation might require a careful consideration of what cell identity is, and when a change in a cell state (in this case in some synthesis pathways) could be considered a change in cell identity. In other words, it means to establish whether a switch in a few genes is sufficient to talk about an identity change.

1.3.1.4 *Cell plasticity during development in vertebrates: mammals*

An example of cell plasticity during embryonic development has also been discovered in mammals: the formation of coronary arteries from venous endothelial cells. In the mouse, clonal analysis allowed to demonstrate that differentiated cells of the sinus venosus are the major contributor of coronary vessel endothelial cells with an intermediate step of proliferation and migration. The analysis of cell identity-specific markers showed that sinus venosus cells dedifferentiate before starting proliferation and re-differentiate into coronary vessels after having invaded the myocardium. The authors suggest that this process might occur for the development of vessels in other organs such as the retina and the kidney, as plasticity of venous cells is supported by their capacity to also give rise to lymphatic vessels (Red-Horse et al., 2010).

1.3.2 Cell plasticity during regeneration

Regeneration is probably the context where cell plasticity has been mostly described in physiological conditions. Regeneration through dedifferentiation or transdifferentiation of differentiated cells has been observed across phyla, even in adult organisms (Merrell and Stanger, 2016). The development of lineage tracing tools and clonal analysis has been key for the demonstration of these phenomena especially in vertebrates (Graf, 2011). The study of cell plasticity in regeneration has highlighted the importance of instructive, non-cell autonomous signalling in cell fate changes and suggests the existence at least in some cases of new differentiation paths not deployed during development (Rajagopal and Stanger, 2016).

1.3.2.1 *Cell plasticity during regeneration in invertebrates*

The best characterised example of cell plasticity during regeneration in invertebrates comes from the *Drosophila* testis (reviewed by Merrell and Stanger, 2016). In this organ, germline stem cells (GSCs) self-renew, to maintain their pool, and give rise to transit-amplifying cells called gonialblasts which ultimately differentiate into spermatogonia and the spermatocyte lineage. Interestingly, upon ablation of the GSC population, gonialblasts and spermatogonia, but not spermatocytes, are sufficiently plastic to retro-differentiate and form new GSCs (Brawley and Matunis, 2004). A similar behaviour was described in the ovary, where cells called cystocytes derived from the ovary GSCs can retro-differentiate to repopulate a depleted GSC niche (Kai and Spradling, 2004). Thus, in *Drosophila* gonads, progenitor cells retain cellular plasticity to retro-differentiate and reconstitute the stem cell pool upon its loss.

1.3.2.2 *Cell plasticity during regeneration in Zebrafish*

Cell plasticity in Zebrafish has been described in several tissues after injury, including the heart and the brain but also in the notochord during development. Depending on the context, either dedifferentiation followed by re-differentiation into the same initial cell type or transdifferentiation contributes to regeneration (Alvarado and Tsonis, 2006; Garcia et al., 2017; Jopling et al., 2011).

Heart regeneration in Zebrafish takes place through dedifferentiation and subsequent proliferation of dedifferentiated cells. Cardiomyocytes lose cell-to-cell contacts and expression of terminal differentiation genes and start to express cell cycle progression genes before re-differentiating. This process allows full restoration of the organ when up to 20% of the ventricle is amputated (Jopling et al., 2010). Zebrafish can also regenerate the fins through cell dedifferentiation which leads to blastema formation (Alvarado and Tsonis, 2006). The blastema is defined as a “mesenchymal growth zone that undergoes proliferation, differentiation and morphogenesis” by J. Brockes, one of the leader scientists in amphibian regeneration research (Brockes and Kumar, 2003). This transient tissue is a common feature of regeneration in several contexts in fish, amphibians and mammals. In Zebrafish, dedifferentiated blastema cells are lineage-restricted, i.e. they maintain the memory of their initial origin: no transdifferentiation occurs during fin regeneration (Tu and Johnson, 2011).

Transdifferentiation ensures regeneration in at least three different tissues in Zebrafish. During development, it is observed in the notochord. This transient embryonic structure is made up of giant vacuolated cells which have membrane invaginations termed caveolae important for the notochord integrity. Genetically driven loss of caveolae leads to the collapse of vacuolated cells, thus affecting the notochord structure. However, under these conditions, the sheath cells, epithelial cells surrounding the vacuolated cells, transdifferentiate to give rise to new vacuolated cells and restore development (Garcia et al., 2017). Another example of transdifferentiation in Zebrafish is the plasticity of radial glia-like cells observed in the retina, where upon injury these cells called Müller cells dedifferentiate into multipotent progenitors which will contribute to retina regeneration (Bernardos et al., 2007). Finally, biliary epithelial cells (BECs), also called cholangiocytes, transdifferentiate into hepatocytes to ensure liver regeneration. These epithelial cells dedifferentiate by losing their tubular morphology, proliferate and re-differentiate into hepatocytes. Interestingly, lack of biliary cells impairs hepatocyte regeneration (He et al., 2014).

1.3.2.3 *Cell plasticity during regeneration in amphibians*

Dedifferentiation and transdifferentiation are common during regeneration in amphibians, especially salamanders. In the newt eye, retina regeneration is achieved through dedifferentiation and proliferation of retinal pigment epithelium (RPE) cells, while lens regeneration requires proliferation and transdifferentiation of pigment epithelial cells (PECs) of the dorsal iris (Alvarado and Tsonis, 2006). Interestingly, the ability of PECs to transdifferentiate into lens cells under certain conditions is conserved in vertebrates (including chicken and human), even though it occurs *in vivo* only in the newts (Tsonis et al., 2004).

Dedifferentiation of differentiated cells contributes to blastema formation in both newts and axolotls during limb and tail regeneration (Figure 3). Lineage tracing studies demonstrated that in axolotls, depending on the initial cell types forming the blastema, memory of the initial cell identity can either be maintained during re-differentiation or be lost, leading to transdifferentiation (Kragl et al., 2009). For instance, dedifferentiated Schwann cells contribute only to nerve regeneration, while dermis cells can also give rise to connective tissues including cartilage. A drastic lineage switch from ectoderm-derived to mesoderm-derived cells occurs during axolotls tail regeneration, where spinal cord cells can generate muscle and cartilage (Echeverri and Tanaka, 2002). Thus, the capacity of differentiated cells to contribute to cell types different from the initial one seems context and species-specific (Joven et al., 2019). Moreover, concerning the regeneration of muscle cells it was shown that while dedifferentiation of pre-existing muscle takes place in the newt *Notophthalmus viridescens*, in the axolotl resident stem cells are recruited (Sandoval-Guzmán et al., 2014).

Regeneration of limb and tail through cell plasticity (blastema formation) is also observed in anurans amphibians like frogs. However, differently from urodeles which can regenerate through adulthood and after repetitive insults (Eguchi et al., 2011), this capacity is restricted to young tadpoles, before metamorphosis occurs. The change in regenerative capacities during development in frogs makes these animals a powerful model to understand the mechanisms underlying this switch (Gurley and Sánchez Alvarado, 2008).

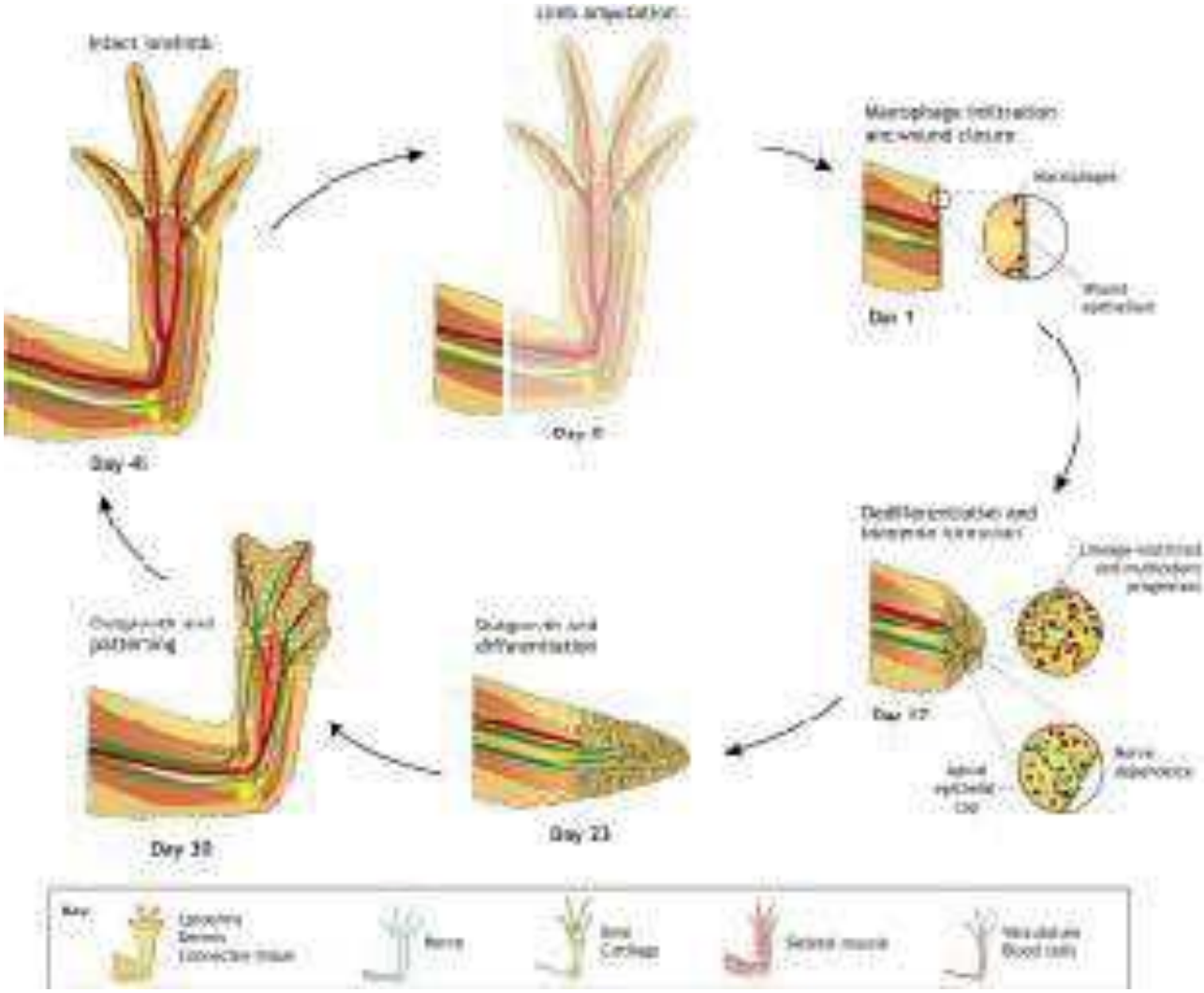

Figure 3. Steps of salamander limb regeneration. After amputation and recruitment of macrophages, regeneration occurs thanks to dedifferentiation of cells from different tissues which contribute to the blastema. Dedifferentiated cells proliferate and afterwards re-differentiate to reconstitute the limb. Depending on the cell types and on the salamander species, cells may re-differentiate keeping a memory of their initial identity or in other cases may transdifferentiate (Joven et al., 2019).

1.3.2.4 *Cell plasticity during regeneration in mammals*

Several examples of retro/dedifferentiation contributing to regeneration have been described in mammals. For instance, in the hair follicle, differentiated epithelial cells can retro-differentiate and occupy the stem cell niche; in the airway, differentiated secretory cells retro-differentiate into stem cells after stem cell pool ablation; partial dedifferentiation is also observed in nerves and kidney, where differentiated Schwann cells and tubule cells respectively acquire immature/stem cell features and proliferate. Finally, in the intestinal crypt progenitor cells, but not differentiated ones, can retro-differentiate into LGR5+ stem cells and

repopulate the stem cell niche after loss of stem cells (Clements et al., 2017; reviewed by Rajagopal and Stanger, 2016) .

Similar to limb regeneration in amphibians but to a lesser extent, digit-tip regeneration through blastema formation was observed in mice and children at the first phalange (Alvarado and Tsonis, 2006). Moreover, some examples of transdifferentiation were characterised in the ear, in the pancreas, in the liver and in the enteric nervous system (Jessen et al., 2015; Laranjeira et al., 2011). In the ear, sensory hair cells are regenerated through transdifferentiation of supporting cells either requiring (Bramhall et al., 2014) or not requiring (Lin et al., 2011) cell division. Transdifferentiation of adult pancreatic α-cells into β-cells was described in mice upon severe loss of β-cells (Thorel et al., 2010), while in the liver hepatocytes can transdifferentiate to produce BECs after damage (Yanger et al., 2013). This regeneration process appears to proceed in the opposite direction to the regeneration of the liver in Zebrafish where BECs transdifferentiate into hepatocytes (see above). Finally, enteric glia was shown to form neurons after injury in the enteric nervous system in mice (Laranjeira et al., 2011).

1.3.3 Cell plasticity in disease

1.3.3.1 *Cell plasticity in cancer*

Cell plasticity is recognised as a key feature of cancer cells, occurring in cancer initiation, development and even recurrence after treatments. Both progenitor cells and differentiated cells in somatic tissues are considered the source of cancer stem cells (CSCs), tumour cells that like normal stem cells are able of self-renewal and to differentiate, thus allowing tumour initiation and growth. Differently from normal stem cells, CSCs abnormally differentiate giving rise to cells that sustain tumour growth like pericytes, which are not generated by the normal somatic counterpart. Given that transcription factors and chromatin regulators are the guardians of cell identity, it comes as no surprise that their dysregulation is observed in cancer and is responsible for cancer cell plasticity. Moreover, it has been shown that loss of tumour suppressor genes can lead to reprogramming to CSCs or even to transdifferentiation. These phenomena are described in many different cancer types, from acute myeloid leukaemia, to glioblastoma, breast carcinomas, non-small cell lung cancer, prostate cancer, colorectal cancer, and osteosarcoma. Reprogramming in tumour cells can be caused by different tumour treatments, leading to recurrent and more aggressive cancers. Understanding the mechanisms underlying cell plasticity in those cases is of fundamental importance for successful cancer eradication (Xiong et al., 2019).

1.3.3.2 *Cell plasticity in degenerative diseases*

Cell plasticity in the form of dedifferentiation of terminally differentiated cells has been described as one of the causes of type-2 diabetes mellitus. In mouse models of diabetes, it was shown that loss of β-cell function is not mainly due to β-cells death, but rather to their acquisition of a progenitor-like state with expression of stemness genes including *Oct4* and *Nanog* (Talchai et al., 2012). Furthermore, other diseases in which cell plasticity has been suggested to be one of the causes include cholestatic liver injury and neural injuries (Merrell and Stanger, 2016). For instance neural cell reprogramming was described in retinal degeneration (Marc et al., 2007).

Cell plasticity can also appear as a consequence of degeneration and chronic inflammation when it is driven by cell fusion. These conditions increase the number of cell fusion events observed *in vivo*, for instance in the brain, in the liver or in the skeletal muscle. Cell fusion often involves bone marrow-derived cells either endogenous or transplanted, which leads to the generation of tetraploid cells. Interestingly, the fusion to bone marrow-derived cells reprograms the fusion partner cell improving cell and thus tissue function in some contexts (e.g. in the liver) (Pesaresi et al., 2018).

1.4 Experimental evidence of cell plasticity

Complementary to the natural evidence of cellular plasticity is the experimental evidence obtained in laboratories since the 1950s of the last century. The pioneer in this work is J. Gurdon, who performed somatic cell nuclear transfer (SCNT) experiments with differentiated cells, followed by A. Miller and F. H. Ruddle in the 1970s with the cell fusion experiments and by R. L. Davis, H. Weintraub and A. B. Lassar in the 1980s with transcription factor-mediated reprogramming, culminated in 2006 with K. Takahashi and S. Yamanaka who succeeded in the generation of iPS cells from differentiated fibroblasts (reviewed by Graf, 2011). Moreover, another demonstration of cell plasticity in differentiated cells dates back to the 1990s by Volker Schmid's lab when they showed that when medusa muscle cells are placed in culture, thus losing interactions with the extracellular matrix, they start to proliferate and transdifferentiate into smooth muscle and nerve cell (Reber-Müller et al., 1994). In those years when lineage tracing techniques were still not developed, this experimental evidence was the strongest catalyser for the revolution in the concepts of cell identity and plasticity and for the interest in cellular reprogramming.

1.4.1 Somatic Cell Nuclear Transfer

In 1958 J. Gurdon managed to obtain *Xenopus laevis* tadpoles after transplantation of tadpoles' intestinal cell nuclei into enucleated eggs (Gurdon et al., 1958). Those tadpoles developed through metamorphosis to adulthood and were fertile females and males (Gurdon and Uehlinger, 1966). This experiment demonstrated cell plasticity of differentiated cells importantly implying that during differentiation genes are neither lost nor irreversibly inactivated, as it was initially postulated by A. Weismann in the XIX century (Weismann. *The Germ-Plasm: A Theory of Heredity*, 1893; Laskey and Gurdon, 1970; Takahashi and Yamanaka, 2016). Moreover, thanks to nuclear transfer experiments by J. Gurdon and before by Briggs and King (who did the experiments on another frog species, but not with differentiated cell nuclei) two key concepts emerged: the higher resistance to reprogramming of nuclei from older animals (Briggs and King, 1953), and the perdurance of an epigenetic memory of the initial cell identity in the reprogrammed nucleus (Ng and Gurdon, 2005). The low efficiency (1.5%) in the generation of tadpoles from SCNT into eggs was also due to the fast DNA replication and cell division driven by the egg, to which differentiated somatic nuclei are not used. Thus, nuclear transfer experiments in not dividing growing oocytes, instead of eggs, allowed to characterise some factors involved in reprogramming resistance such as histone variants, histone modifications and DNA methylation (Gurdon, 2013). For the discovery that differentiated cells can be reprogrammed, John Gurdon was awarded the Nobel Prize in Physiology or Medicine in 2012 with Shinya Yamanaka (nobelprize.org). In the 1990s, SCNT also made possible animal cloning of mammals such as mouse and the famous Dolly the sheep (Wilmut et al., 1997; reviewed by Graf, 2011) (Figure 4).

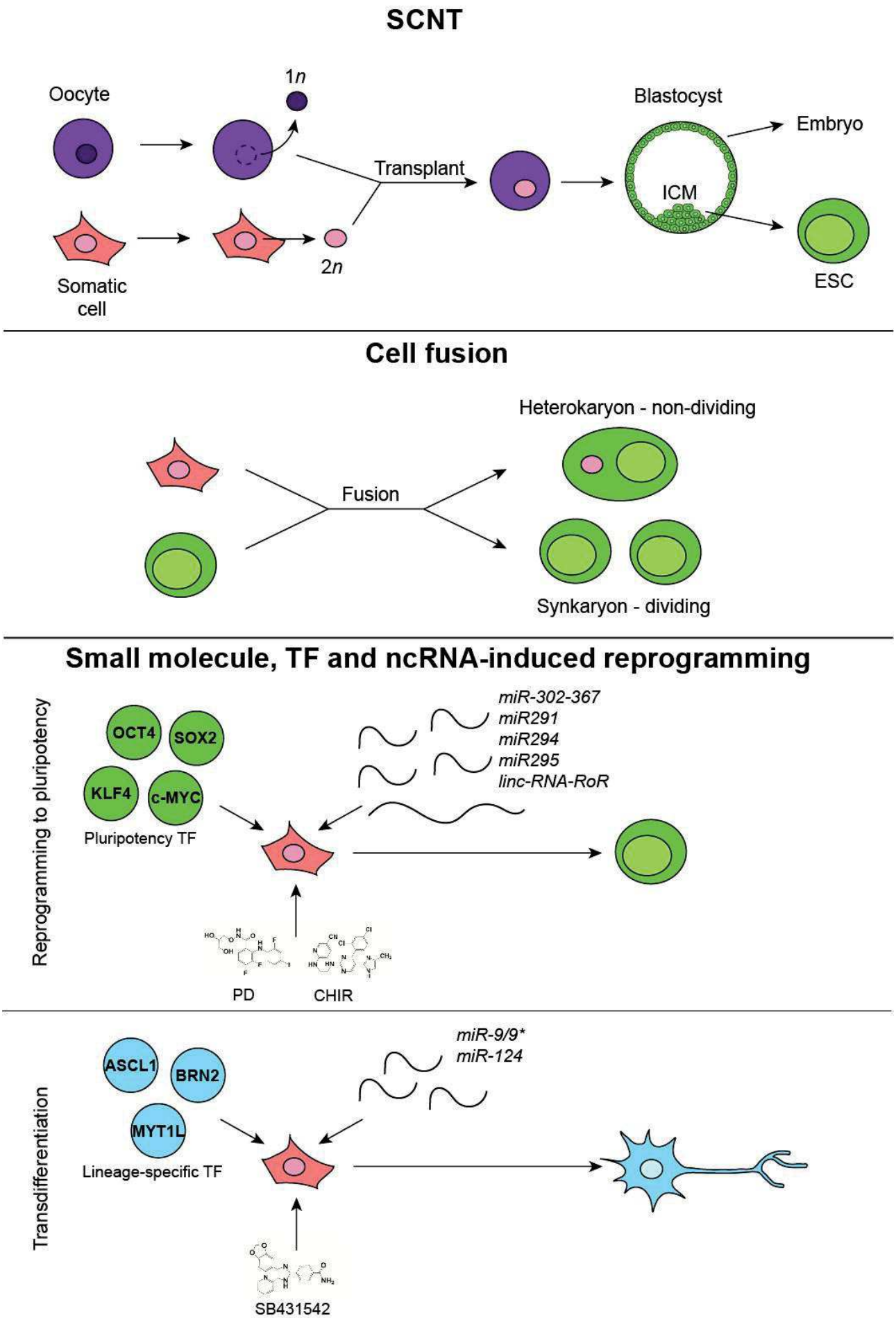

Figure 4. Summary of the experimental evidence of cell plasticity. Transcription factors, small molecules and ncRNAs can be used alone or in combination to increase the efficiency.

1.4.2 Cell fusion

The second experimental demonstration of cell plasticity are the results of cell fusion experiments (Figure 4). The first such experiment was performed by A. Miller and F. H. Ruddle by fusing embryonal carcinoma cells to thymus cells to obtain *in vivo* teratocarcinomas. The carcinoma identity was overwriting the thymus cell identity (Miller and Ruddle, 1976). Few years later, H. Blau performed other cell fusion experiments between human and mouse cells obtaining non-dividing heterokaryons. She observed that mouse muscle cells could reactivate the expression of muscle genes in the nuclei of human cells such as amniocytes, keratinocytes and hepatocytes (reviewed by Graf, 2011), suggesting that muscle cells contained factors required for the maintenance of their identity also capable of establishing it. Finally, in 2001 heterokaryons were made by fusing T cells with ESCs demonstrating the dominance of the pluripotent cell nucleus over the other (Tada et al., 2001). More generally, the dominant fate after cell fusion is dictated by the cell which contributes with more regulatory factors (Vierbuchen and Wernig, 2012).

As described previously in the context of cell plasticity in disease, cell fusion occurs and leads to cell plasticity also *in vivo* (Pesaresi et al., 2018). These findings paved the way for applications of cell fusion-mediated cell reprogramming in regenerative medicine. For instance, M. P. Cosma's lab demonstrated that the fusion of retinal neurons with hematopoietic stem and progenitor cell reprograms them to a proliferative precursor stage that can differentiate into new functional neurons, thus partially repairing retinal damage (Sanges et al., 2013).

1.4.3 Chemical (small molecule)-induced reprogramming

The capacity of some chemicals (i.e. small molecules) to induce cell plasticity and reprogramming is demonstrated and importantly paved the way for the cloning of the first transcription factor capable of reprogramming, *MyoD* (Davis et al., 1987). 5-azacytidine (AzaC), a chemotherapeutic drug which inhibits DNA methylation, was shown to drive cellular reprogramming when added to fibroblast cultures leading to transdifferentiation into muscle cells, adipocytes and chondrocytes (Taylor and Jones, 1979). After the achievement of reprogramming to pluripotency, other chemicals have been used alone or in combination with transcription factor overexpression to circumvent the requirement for some transcription factors and/or to increase reprogramming efficiency and speed (Figure 4). Small molecules can drive reprogramming by modulating transcription factors, epigenetic regulators (DNA and histone methyltransferases, histone demethylases and histone deacetylases), signalling pathways and metabolism, this last especially for reprogramming to pluripotency where a switch from oxidative to glycolytic metabolism is required (Li et al., 2013).

Small molecules hold a great promise for cell therapy by preventing the risk associated with genetic manipulations required for sustained transcription factor overexpression.

1.4.4 Transcription factor-induced reprogramming

Transcription factors are known key regulators of cellular identity during development (Cohen and Melton, 2011) and in 1987 Weintraub's lab demonstrated that they can even force the conversion of a differentiated cell type into another (Davis et al., 1987; Graf and Enver, 2009). The transcription factor *MyoD* was able alone to convert fibroblasts into muscle cells. Few years later, T. Graf's lab demonstrated the capacity of another transcription factor, GATA-1, to reprogram avian cells (Kulessa et al., 1995). In 2006 K. Takahashi and S. Yamanaka succeeded in converting fibroblasts into pluripotent stem cell by overexpression of a combination of four transcription factors, the famous OSKM (*Oct4/Sox2/Klf4/c-Myc*) cocktail (Takahashi and Yamanaka, 2006). The research in transcription factor-induced reprogramming field has dramatically expanded after that year and many protocols have been developed to improve the low efficiency of reprogramming to pluripotency and also to obtain several cell types from different starting cells through transdifferentiation.

1.4.4.1 *Transcription factor-induced reprogramming to pluripotency*

The pioneer work of Takahashi and Yamanaka led to the identification of four factors able to convert differentiated mouse fibroblasts back to pluripotency (Figure 4), followed the year after by the reversion to pluripotency of different human somatic cells (reviewed by Yamanaka and Blau, 2010). The knowledge of the pluripotency network acquired through the studies on ESCs (Ng and Surani, 2011) allowed to identify other candidate transcription factors and to test them for induction of pluripotency. For instance, generation of hiPSCs was also achieved by using other pluripotency factors such as *NANOG* and *LIN28* which could replace *KLF4* and *MYC*. Moreover, it was shown that *Sox2* can be replaced with *Sox1* and *Sox3*, *Klf4* with *Klf2* or *Klf5*, and *c-Myc* with the other two members of the MYC family in mouse cells (Takahashi and Yamanaka, 2016). On the contrary it is more difficult to replace *Oct4* with other transcription factors, but it is possible to bypass its requirement with a small molecule targeting G9a histone methyltransferase (Shi et al., 2008). Co-expression of other factors such as *Utf1*, *Sall4* or *Lin28* with OSKM was demonstrated to enhance reprogramming efficiency. Finally, modulation of the expression of epigenetic modifiers can enhance (or impede) reprogramming to pluripotency by modifying chromatin accessibility (reviewed by Takahashi and Yamanaka, 2016; for the role of *Sall4* see Tsubooka et al., 2009), in support to the role of epigenetics in cell plasticity and cell identity maintenance. Even though most protocols to reprogram somatic cells to pluripotency were developed in cell cultures, reprogramming to pluripotency was also

performed *in vivo* in transgenic animals with doxycycline inducible OSKM factors. The overexpression of these factors led to the reprogramming to pluripotency of cells in several tissues and as it happens for iPSC transplantation resulted in teratoma formation (Pesaresi et al., 2018).

iPSCs are undoubtedly an outstanding resource for biomedical research, including disease modelling, drug discovery, toxicology tests and regenerative medicine (Morris, 2016). The possibility of *ex vivo* expansion (a huge number of cells is required for transplantation) and of autologous transplantation using patient-derived iPSCs makes them a powerful tool for cell therapy (Pesaresi et al., 2018).

1.4.4.2 *Transcription factor-induced transdifferentiation*

Transdifferentiation has attracted the interest of researchers for its *in vivo* applicability, preventing the issues of genetic instability due to long-term *in vitro* culture and of transplantation, together with avoiding the risk of teratoma formation associated to iPSC intermediates (Xu et al., 2015). Moreover, it has the advantage for disease modelling of not resetting the age of the initial cell like iPSC reprogramming does (Mertens et al., 2015).

Even though it was initially thought that transdifferentiation could be possible only between related cell types sharing lineage history (and thus epigenetic features), in the last ten years several labs showed that inter-germ layer transdifferentiation can be artificially induced by overexpression of combinations of transcription factors (Vierbuchen and Wernig, 2012). Thus, while the first transdifferentiation protocols published aimed to interconvert related cell types such as B cell into macrophages (Xie et al., 2004), pancreatic cells into hepatocytes (Shen et al., 2000) and pancreatic exocrine cells into β-cells (Zhou et al., 2008), in 2010 M. Wernig's lab succeeded in converting fibroblasts into neurons by overexpression of three neuronal transcription factors (Vierbuchen et al., 2010) (Figure 4). Today through different lineage-restricted transcription factors, we can reprogram mouse or human fibroblasts into macrophages, adipocytes, cardiomyocytes, chondrocytes, hepatocytes, different types of neurons (glutamatergic, dopaminergic, motor, GABAergic), astrocytes, oligodendrocyte progenitors, endothelial cells, hematopoietic progenitors and pancreatic cells; astrocytes, hepatocytes, microglia and T cells can be reprogrammed into neurons; different endocrine and exocrine pancreatic cells can be interconverted (reviewed by Xu et al., 2015; Matsuda et al., 2019; Tanabe et al., 2018; Zhu et al., 2016). The favourite starting cell type for many *in vitro* reprogramming protocols are fibroblasts, as this cell type is readily available and thus of interest for regenerative medicine where autologous transplantation would solve the issue of immune rejection (Cohen and Melton, 2011). However, the lineage relationship of the starting cell type with the desired final cell type is another element to consider, because, even though

inter-germ layer conversions are possible, reprogramming of lineage-related cells might be easier (Gascón et al., 2017).

Transcription factor-induced transdifferentiation has been performed i*n vivo* in animal models, like *C. elegans* and mice. In the latter, a special effort has been made to obtain cells of therapeutic relevance *in vivo* from other cells resident in the organ of interest. For instance, pancreatic exocrine cells were converted into β-cells (Zhou et al., 2008, the first protocol of a series); cardiac fibroblasts were converted into cardiomyocytes (Qian et al., 2012; Song et al., 2012); astrocytes were converted into neuroblasts which in presence of signalling molecules and deacetylase inhibitor could differentiate into functional neurons (Niu et al., 2013); astrocytes and NG2 cells were converted into glutamatergic and GABAergic neurons (Guo et al., 2014).

In *C. elegans* several transdifferentiation events have been induced *in vivo* in differentiated cells by overexpression of transcription factors known to play a role in differentiation. J.H. Rothman and colleagues converted pharynx cells and somatic gonad into gut-like cells by overexpressing *elt-7* transcription factor (or *elt-2*, with lower efficiency). Together with demonstrating that terminally differentiated cells retain plasticity, these studies show that the success of reprogramming by a specific transcription factors depends on the initial cell identity: the overexpression of *elt-7* in the whole body driven by a heat-shock promoter led to *in vivo* transdifferentiation of the pharynx and the somatic gonad but not of other cells (Riddle et al., 2013, 2016). Moreover other *in vivo* reprogramming experiments in *C. elegans* demonstrated that the success of reprogramming and cell plasticity also depend on the developmental stage: while early blastomeres are prone to be reprogrammed, more difficult is to change the identity of differentiated (and post-mitotic) cells in the larva or adult worm. It was shown that a quite sharp change in plasticity, the so called multipotency-to-commitment transition (MCT), occurs at mid-embryogenesis in *C. elegans* and it appears to be a common transition in different animals' embryos (Rothman and Jarriault, 2019). According to this, expression of OSKM pluripotency transcription factors in mammalian differentiated cells for less than 6 days, under the appropriate culture conditions, increases the efficiency of transdifferentiation compared to direct expression of lineage specific-transcription factors without inducing pluripotency (Efe et al., 2011; Kim et al., 2011). It suggests that OSKM could increase cell plasticity in differentiated cells. This strategy is called "cell-activation and signalling-directed transdifferentiation" (CASD) and it seems an interesting alternative to persistent transcription factor overexpression to drive transdifferentiation (Firas and Polo, 2017). However, it is controversial whether the cells pass through a transient pluripotent state (Bar-Nur et al., 2015; Maza et al., 2015), which would preclude the occurrence of transdifferentiation according to its definition, or not (Efe et al., 2011; Kim et al., 2011). It has been suggested that these contrasting results could be due to

the different systems (e.g. culture conditions during the process) used in these studies (Ebrahimi, 2016; Firas and Polo, 2017). Thus, whether CASD reprogramming is actually transdifferentiation or pluripotent reprogramming followed by directed differentiation is not clear yet.

1.4.5 Non-coding RNA-induced reprogramming

Non-coding RNAs are non-translated RNAs involved in the regulation of gene expression through several possible mechanisms. They are components of the regulatory network underlying cell identity and the understanding of their function in different cell types has led to test their potential in cellular reprogramming. Both small non-coding RNAs, especially microRNAs (miRNAs), and long non-coding RNAs (lncRNAs) have been implicated as it was shown that their level changes during the reprogramming process (Li and Belmonte, 2017; Sherstyuk et al., 2018). Thus, the overexpression (or downregulation) of miRNAs or lncRNAs can induce reprogramming or increase reprogramming efficiency (Figure 4). Non-coding RNAs have been used both to drive reprogramming to pluripotency and transdifferentiation (Takahashi and Yamanaka, 2016; Xu et al., 2015). For reprogramming to pluripotency, different ESCs miRNA clusters (miR-302-367 or miR291, miR294, miR295) were used alone or in combination with OSKM factors to enhance reprogramming efficiency, and overexpression of the lincRNA-RoR increased reprogramming efficiency by inhibiting p53 and by acting as a sponge to sequester miRNAs against core pluripotency transcription factors (Li and Belmonte, 2017; Sherstyuk et al., 2018; Takahashi and Yamanaka, 2016).

The same principle was applied for transdifferentiation: neuronal-specific miRNAs can induce neuronal identity in fibroblasts, while cardiac-specific miRNAs induce cardiomyocytes. The overexpression of miRNAs alone is sufficient to drive transdifferentiation, however the process is not as efficient as transcription factor-induced reprogramming. The mechanisms through which regulation of miRNA expression induces reprogramming are not always known, but it is possible that they act by downregulating genes for alternative cell fates or by regulating the expression of chromatin modifiers. Indeed, some evidence for both mechanisms exists (Pfaff et al., 2017; Xu et al., 2015).

2 Mechanisms underlying cellular reprogramming

Through the description of different cellular reprogramming events, it emerges that the principal cell intrinsic factors which can maintain cell identity and drive reprogramming are transcription factors and chromatin regulators. More precisely, while transcription factors are both required to maintain cell identity and to actively drive reprogramming, chromatin regulators are mainly involved in cell identity maintenance. A mechanistic description of how reprogramming is achieved through the regulation of those factors requires more investigation and could provide insights on how to improve reprogramming efficiency, completeness and eventually speed. In the last 20 years advances in understanding mechanisms of natural and induced cellular reprogramming have been made; however, there are several questions which today are only partially answered. For instance, it is not always clear what the sequential cellular and molecular steps during reprogramming are, the role of cell division and the contribution of signalling pathways, especially during reprogramming *in vivo*. Mechanisms at play during natural cell plasticity events might not be the same as mechanisms during *in vitro* induced reprogramming (Merrell and Stanger, 2016). Moreover, they might significantly differ even from one reprogramming event to the other, natural or induced.

In the next pages I will focus on the mechanisms involved in several different cell plasticity contexts and I will discuss what is known about the role of cell division during reprogramming.

2.1 Mechanisms underlying natural reprogramming events

The cellular and molecular characterisation of reprogramming events taking place during invertebrate and vertebrate development and regeneration has allowed to deepen the understanding of mechanisms underlying natural cell reprogramming. Importantly, both cell-autonomous and non-cell-autonomous factors (environmental cues) have been identified which control cell plasticity.

2.1.1 Cell-autonomous factors involved in cell plasticity

The cell-autonomous factors regulating cell plasticity *in vivo* include:

- Tumour suppressors like the retinoblastoma *Rb, p53* and *Arf* (Pomerantz and Blau, 2013). Those genes are important regulators of cell cycle, but are also known to directly regulate acquisition and maintenance of the differentiated cell identity (Molchadsky et al., 2010; Pesaresi et al., 2018).
- Transcription factors, both lineage-specific and pluripotency associated-transcription factors (Merrell and Stanger, 2016; Poss, 2010).

- Chromatin modifiers and histone variants (Merrell and Stanger, 2016).

2.1.1.1 *Tumour suppressors controlling cell plasticity: Rb, p53, Arf*

RB is a tumour suppressor whose regulation is implicated in regeneration. It generally acts as a transcriptional repressor and controls the cell cycle entry by preventing the G1/S transition, through inhibition of the E2F transcription factors. It is inactivated through phosphorylation by G1 cyclin-dependent kinases (CDKs). RB may have a role during regeneration dependent on or independent of cell cycle regulation: on one hand inactivation of RB is required for limb regeneration in newts by regulating cell cycle re-entry (Tanaka et al., 1997), on the other hand it controls differentiation by regulating the expression of tissue-specific transcription factors as shown in mouse muscle cells (Pajcini et al., 2010; Pomerantz and Blau, 2013). Finally, it was demonstrated that *Rb* inhibits reprogramming to pluripotency independently of cell cycle regulation (Kareta et al., 2015). Interestingly, data from our lab show that loss of the *C. elegans Rb* orthologue *lin-35* rescues Y-to-PDA transdifferentiation defects in a Y-to-PDA mutant background, independently of cell cycle (S. Becker and S. Jarriault, unpublished). This evidence suggests that the role of *Rb* in maintaining the differentiated identity independently of cell cycle regulation might be highly conserved.

The other primary tumour suppressor which regulates cell cycle progression and not only is p53, the “prototypical tumour suppressor” (Pomerantz and Blau, 2013). p53 is a transcriptional regulator usually expressed at low levels, whose activity is regulated mostly through post-translational modifications in response to different cellular stresses such as those inducing DNA damage. Differently from *Rb*, *p53* seems an evolutionary novelty appeared in concomitance with adult somatic stem cells in vertebrates, while the ancestral genes in invertebrates like Hydra and nematodes closer resemble the other family members *p63* and *p73*. Interestingly, in highly regenerative urodeles only *p53* family member was identified. *p53* pathway can interact with *Rb* pathway at the level of E2F transcription factors and their genes are often mutated in human cancer. Activation of p53 can trigger various responses depending on the context, including activation of the DNA repair machinery, cell cycle arrest, induction of autophagy, apoptosis or senescence. The tumour suppressor ARF is upstream to p53 and can lead to its stabilisation through inhibition of MDM2 (Pomerantz and Blau, 2013; Vousden, 2000). Inhibition of p53 activity occurs during the initial phases of regeneration in amphibians when dedifferentiation and proliferation take place (Yun et al., 2013) and it relies on sustained ERK signalling (Yun et al., 2014). However, p53 activity has to be re-established during the re-differentiation phase (Yun et al., 2013). This regulation of p53 in amphibians, which is not observed in mammals, could be due to the presence of some variations in the amino acid composition (which in humans are associated with cancers) and/or to the absence of the

tumour suppressor *Arf* (reviewed by Tanaka, 2016). Thus, the dynamic regulation of a vertebrate conserved tumour suppressor in amphibians allows cell plasticity in this Class which in turn enables regeneration. On the contrary, p53/ARF pathway appears as a barrier to reprogramming and regeneration in mammals: J. Pomerantz and H. Blau's labs demonstrated that transient, concomitant downregulation of *Rb* and *Arf* in mammalian muscle cells leads to dedifferentiation and proliferation, while the sole downregulation of *Rb* is not enough (Pajcini et al., 2010). In agreement with that, the reverse experiment that introduced human *ARF* in Zebrafish inhibited fin regeneration (Hesse et al., 2015).

In conclusion, tumour suppressor pathways play a key role in cell plasticity during regeneration and some variations in their components and their regulation between vertebrate Classes can, at least in part, explain the different regenerative capacities between animals.

2.1.1.2 *Transcription factors involved in natural cell plasticity*

Different lineage-specific transcription factors have been shown to be required for natural cell plasticity events (summarised in Table 1). I will be considering transcription factors required at the early phases of reprogramming as they are more likely to be involved in cell plasticity of the differentiated state. This function is more difficult to be defined for those examples where a dedifferentiated intermediate is not present or identified yet.

In *Drosophila*, the change in commitment of mixer cells (transdetermination), from anterior to posterior, during dorsal closure requires the activation of the posterior compartment-specific gene *Engrailed*, when the anterior one *Patched* is still expressed (Gettings et al., 2010). A similar dynamics is observed during the midgut-to-renal transdifferentiation at metamorphosis where the renal progenitor transcription factor *Cut* starts to be expressed in *Delta+* midgut progenitors which then is irreversibly shut down (Xu et al., 2018).

Regulation of transcription factor activity was also shown in examples of cellular reprogramming in vertebrates. During Zebrafish development, transdifferentiation of DE tubule renal cells into CS gland cells requires the cytoplasmic sequestration of the renal transcription factor Hnf1b and the downregulation of the downstream transcription factor gene *irx3b* (Naylor et al., 2018). Expression of lineage-specific transcription factors was shown to be required for transdifferentiation during lens regeneration in newts (Figure 6). *Six3* (*sine oculis homeobox homologue 3*) and *Pax6* (*paired box 6*) are both transcription factors regulating eye and lens development (Alvarado and Tsonis, 2006). The level of expression of the transcription factor *Six3* rather than its specific localisation in the dorsal iris PECs accounts for the dorsal iris-specific transdifferentiation capacity. *Pax6* is also required but together with upregulation of *Six3* (Grogg et al., 2005). Thus, these examples illustrate the involvement of transcription

factors in cell plasticity, even though how they contribute to reprogramming is not completely clear. If *Engrailed*, *Cut*, *Six3* and *Pax6* have positive roles during reprogramming, *irx3b* must be downregulated. A more detailed characterisation of those events is necessary to understand whether these transcription factors are required either to broadly change the transcriptome or to regulate only a few genes, and whether they could both activate the new programme and silence the initial one.

The same conclusion on the importance of transcription factors comes from studies in mammals, for instance during pancreas regeneration where regulation of key lineage-specific transcription factors leads to interconversion of endocrine cells (Chera et al., 2014; Thorel et al., 2010). Interestingly the genes involved in these processes are transcription factors that instruct differentiation during development and which remain express in terminally differentiated cells to maintain their identity (Puri et al., 2015). *Pdx1* and *Nkx6.1* are upregulated during α-cell to β-cell conversion (Thorel et al., 2010) and the endocrine transcription factor *Ngn3* is upregulated during δ-cell to β-cell transdifferentiation, while *FoxO1* is downregulated (Chera et al., 2014). The requirement of *FoxO1* downregulation in enforcing β-cell fate during stress (Talchai et al., 2012) suggests that not only the presence, but also the stoichiometry of different transcription factors is required to define the identity of lineage-related cells and in different conditions. In support to this notion, an extreme example is given by the regulation of *Oct4* expression in mESCs: changing the levels of OCT4 and thus its amount relative to its binding partners leads to differentiation to primitive endoderm and mesoderm (if upregulated) and to trophectoderm (if downregulated) (Niwa et al., 2000). Thus, the sharing of some transcription factors among cells with common lineage histories can explain why natural cell fate conversions are more common between developmentally related lineages than across germ layers, both in development and regeneration.

Some families of transcription factors are involved in regeneration across different vertebrate Classes: one example is *Msx1* (*muscle segment homeobox 1*). In the context of limb regeneration, the urodele orthologue *Msx1* is required for myotubes dedifferentiation (Kumar et al., 2004). Interestingly, orthologues of *Msx1* in Zebrafish, namely *msxB* and *msxC*, in *Xenopus* and in mouse are upregulated during fins, limb, tail, heart and digit-tip regeneration suggesting conserved roles of this factor family in cell plasticity during regeneration (Alvarado and Tsonis, 2006; Endo et al., 2000; Raya et al., 2003). Importantly, the regeneration-specific expression of *msxB* and *msxC* during heart regeneration, completely absent during heart development, allowed the authors to define them as molecular markers of regeneration (Raya et al., 2003). Another factor involved in dedifferentiation during (retina) regeneration in Zebrafish is *ascl1* (Ramachandran et al., 2010), one of the factors used for *in vitro* reprogramming of fibroblasts into neurons (Vierbuchen et al., 2010).

Another group of factors which could have conserved role through evolution in cell plasticity are pluripotency-associated factors such as *Oct4*, *Sox2*, *Klf4*, *c-Myc* and *Sall4*. Their expression in different combinations was shown in the blastema in newts, frogs and Zebrafish (Christen et al., 2010; Maki et al., 2009; Neff et al., 2005). Bulk RNA sequencing experiments of cells at different times during lens and limb regeneration in the newt showed that *Sox2* and *Klf4* are upregulated during the initial phases and *c-Myc* after, probably according to its function in cell proliferation. The authors of this study suggest that the expression of these factors regulates cell plasticity, similarly to what the mammalian counterparts do in reprogramming to pluripotency, but the absence of *Oct* prevents the gain of pluripotency (Maki et al., 2009). In *Xenopus*, upregulation of *sall4* expression was detected in the blastema of regeneration-competent limb before metamorphosis (Neff et al., 2005). The other pluripotency factors were not upregulated during *Xenopus* limb or fish fin regeneration, but their expression was detected in those tissues even if at levels not comparable to PSCs (Christen et al., 2010). In this study, they suggest that this low level of pluripotency-associated transcription factors in the blastema might confer multipotency instead of pluripotency during regeneration. Interestingly, differently from *Xenopus* and newt limbs, in fish fins *oct4* is also expressed and together with *sox2* it is required for regeneration. A role for *Oct4* is suggested as well in hepatocyte-to-BEC transdifferentiation in rodents by an *in vitro* study using organoids (Doffou et al., 2018). Finally, during retina regeneration in Zebrafish, upregulation of the pluripotency factors *oct4*, *sox2*, *nanog*, *klf4* and c-*myc* is detected, preceded by *ascl1* and *lin-28* upregulation (Ramachandran et al., 2010).

Invertebrate orthologues of some mammalian pluripotency factors are also required for *in vivo* cell plasticity. Our lab demonstrated that Y-to-PDA natural transdifferentiation occurring during *C. elegans* larval development requires *sox-2/SOX*, *ceh-6/POU* (the family of *Oct4*) and *sem-4/SALL* (Figure 5). These factors are necessary for the initiation of transdifferentiation, i.e. Y dedifferentiation; in absence of those genes the Y cell retains its rectal epithelial morphology, gene expression and function continuing to form the rectum of the worm with the other rectal cells (Kagias et al., 2012). However, these transcription factors are not associated with pluripotency in this context, as Y remains unipotent (Richard et al., 2011), and moreover their expression pattern is not compatible with a pluripotency function. *C. elegans* lacks the POUV family to which mammalian *OCT4* belongs, and *ceh-6* is a POUIII factor, closer to mammalian *Brn1*, *Brn2, Brn4* and *Oct6* (Bürglin and Ruvkun, 2001). These later reprogramming factors were shown to have reprogramming activities, but more towards transdifferentiation than reprogramming to pluripotency (Malik et al., 2018). For instance, both *Oct4* and *Brn2* are POU family genes, but the second one can contribute to transdifferentiation of fibroblast into neurons (Vierbuchen et al., 2010) and not to pluripotency (Malik et al., 2018). The same observations

can also be made for the SOX family (Julian et al., 2017). Thus, it is likely that these families of transcription factors retain a remarkable reprogramming capacity through evolution, but to a different extent and with a different output depending on the specific factor and on the organism. Interestingly, sequence analyses suggest that the POUV family might have evolved from the POUIII family, making the Y-to-PDA reprogramming factor *ceh-6* the closest *C. elegans* orthologue of the pluripotency factor *OCT4* (Bürglin and Ruvkun, 2001).

In conclusion, both lineage-specific and pluripotency-associated transcription factors play key roles during natural reprogramming.

2.1.1.3 *The role of chromatin modifiers*

As chromatin modifiers are important for the maintenance of cell identity, they must be regulated to confer cell plasticity during natural reprogramming events. Indeed, this was shown in many instances and confirmed by *in vitro* reprogramming (see 2.2; Paksa and Rajagopal, 2017) (Table 1).

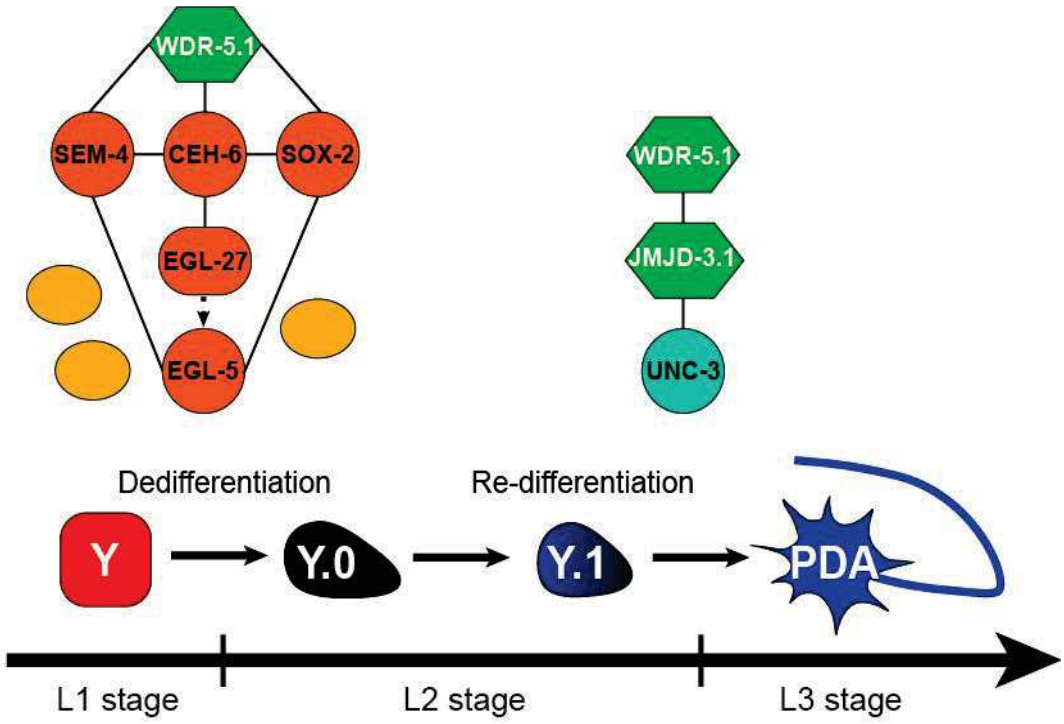

Figure 5. Y-to-PDA transdifferentiation steps and nuclear factors involved. Y rectal epithelial cell dedifferentiates and re-differentiates into the PDA motor neuron in a stepwise manner from the L1 to the L3 larval stages. Different factors are involved at different steps. In orange, core Y-to-PDA reprogramming factors (all transcription factors except EGL-27, high Y-to-PDA defect in their absence); in yellow other reprogramming factors required for the initiation of Y-to-PDA; in green, chromatin modifiers; in light blue, PDA terminal selector. Lines between factors indicate physical interactions as seen through coIP experiments. Arrows indicate a genetic relationship.

In invertebrates, histone modifiers have been shown to be required for natural reprogramming during development. In *C. elegans* Y-to-PDA transdifferentiation requires the sequential activity of two factors, the H3K27me2/3 demethylase JMJD-3.1 and the H3K4 methyl transferase complex Set1 (Trithorax in *Drosophila*). The former is necessary for the re-differentiation step, while the latter is required during both dedifferentiation and re-differentiation. Interestingly WDR-5.1 of the Set1 complex associates with both initiation factors such as SOX-2, CEH-6 and SEM-4 and re-differentiation factors such as the pro-neural terminal selector UNC-3 (Hobert, 2016; Zuryn et al., 2014). Moreover, initiation of transdifferentiation requires the MTA (metastasis-associated protein) orthologue EGL-27, a component of chromatin modifier complexes (Kumar and Wang, 2016), which also interacts with initiation transcription factors SOX-2, CEH-6 and SEM-4 as seen in HeLa cells (Kagias et al., 2012) (Figure 5). The reprogramming defects associated with chromatin remodelling mutants are lower than the defects in transcription factors mutants, except for *egl-27/MTA*, supporting their role in ensuring robustness of the process and the role of transcription factors in driving it by selecting the target sequences (Zuryn et al., 2014). The high PDA defect observed in *egl-27/MTA* mutants might be due to its role upstream of the HOX gene *egl-5*, which is also known to be required for the initiation of Y-to-PDA transdifferentiation (Kagias et al., 2012). However, the function of EGL-27 as a component of chromatin modifier complexes is not demonstrated, thus we cannot rule out that other functions of this protein might be responsible of the absence of PDA observed in *egl-27/MTA* mutant worms. Indeed, MTA proteins are known to have extra-nuclear functions in vertebrates (Kumar and Wang, 2016).

In *Drosophila*, the expression of *Polycomb* (*Pc*) is downregulated to allow the activation of *Engrailed* for the reprogramming of mixer cells during dorsal closure. Pc is responsible of the methylation of H3K27, associated to transcriptional silencing. Its downregulation in mixer cells is controlled by JNK signalling pathway (Roumengous et al., 2017). Thus, differently from Y-to-PDA in this event a chromatin modifier is upstream to the activation of a reprogramming transcription factor.

A similar mechanism to the one occurring in Y-to-PDA transdifferentiation has been demonstrated in the blastema during fish fin regeneration. Like in Y-to-PDA, the existence of bivalent H3K4me3 and H3K27me3 was demonstrated at genes induced during regeneration, and a Zebrafish orthologue of the demethylase JMJD3 is required to demethylate H3K27me3 resolving the bivalent domains. Importantly, this is necessary to activate the expression of genes involved in regeneration and thus for regeneration to occur (Stewart et al., 2009). In fin regeneration the Polycomb subunit EZH2 is also required, expressed early in the blastema, but it is not clear whether it is needed for dedifferentiation or not (Dupret et al., 2017). Instead, components of the NuRD complex are required for blastema proliferation and re-differentiation

(Pfefferli et al., 2014). Moreover, DNA demethylases expression is detected during blastema formation and the levels of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) decrease accordingly in dedifferentiated blastema cells, 36 hours post amputation (hpa) (Hirose et al., 2013). Later on, 72 hpa, *de novo* DNA methyltransferases and especially *dnmt3aa* are upregulated and the level of DNA methylation is restored (Takayama et al., 2014). Thus, an active, dynamic regulation of DNA methylation occurs during blastema-mediated regeneration of the Zebrafish fin.

Concerning regeneration in salamanders, transcriptomic analyses identified many different chromatin modifier activities which are upregulated in blastema during lens regeneration in newts: histone acetyltransferases, histone deacetylases, histone demethylases and DNA methyltransferases (Maki et al., 2010a). In agreement with these expression profiles, a dynamic change in histone marks during dedifferentiation was observed both in the dorsal and ventral iris: H3K4me3 and H4K5Ac increase, while H3K9Ac decreases. The level of the repressive mark H3K27me3 remains constant in the dorsal iris while it increases in the regeneration incompetent ventral iris (Maki et al., 2010b). Functional tests would further confirm histone modifiers' role during dedifferentiation leading to blastema formation. Finally, the linker histone variant B4 typical of oocytes was shown to be required, but probably not at the dedifferentiation step as its knockdown affects the expression of transcription factors associated with the acquisition of the lens identity (*MafB* and *Pax6*) (Maki et al., 2010c).

Overall, these examples support the importance of chromatin modifying activities in the regulation of cell plasticity (Paksa and Rajagopal, 2017) and highlight their conservation across phyla.

2.1.2 Non-cell-autonomous factors involved in cell plasticity: the role of signalling pathways

Extracellular cues are often required in parallel to or for the regulation of cell intrinsic factors involved in cellular reprogramming, including transcription factors and chromatin regulators. These cues activate conserved signalling pathways which also regulate embryonic development.

Different signalling pathways may contribute to dedifferentiation, proliferation or re-differentiation and patterning in the same regenerative event such as the newt lens regeneration (Figure 6) and the fish retina regeneration. In this last context, upstream EGF pathway is required for the Wnt signalling activation through stimulation of β-catenin nuclear localisation and for downregulation of the Notch signalling pathway at the initial phase of reprogramming. Feedback loops between EGF and WNT as well as EGF and Notch ensure

the right timely coordination between cell dedifferentiation, proliferation and re-differentiation, this last requiring Notch (Wan et al., 2012). Notch could also be downstream to another signalling pathway, Hippo, in mammalian hepatocytes: two studies demonstrated that Notch activation is required for hepatocyte-to-BEC transdifferentiation (Yanger et al., 2013) and that the transcriptional regulator YAP, a target of Hippo signalling pathway, is nuclear in BECs and upstream to Notch (Yimlamai et al., 2014).

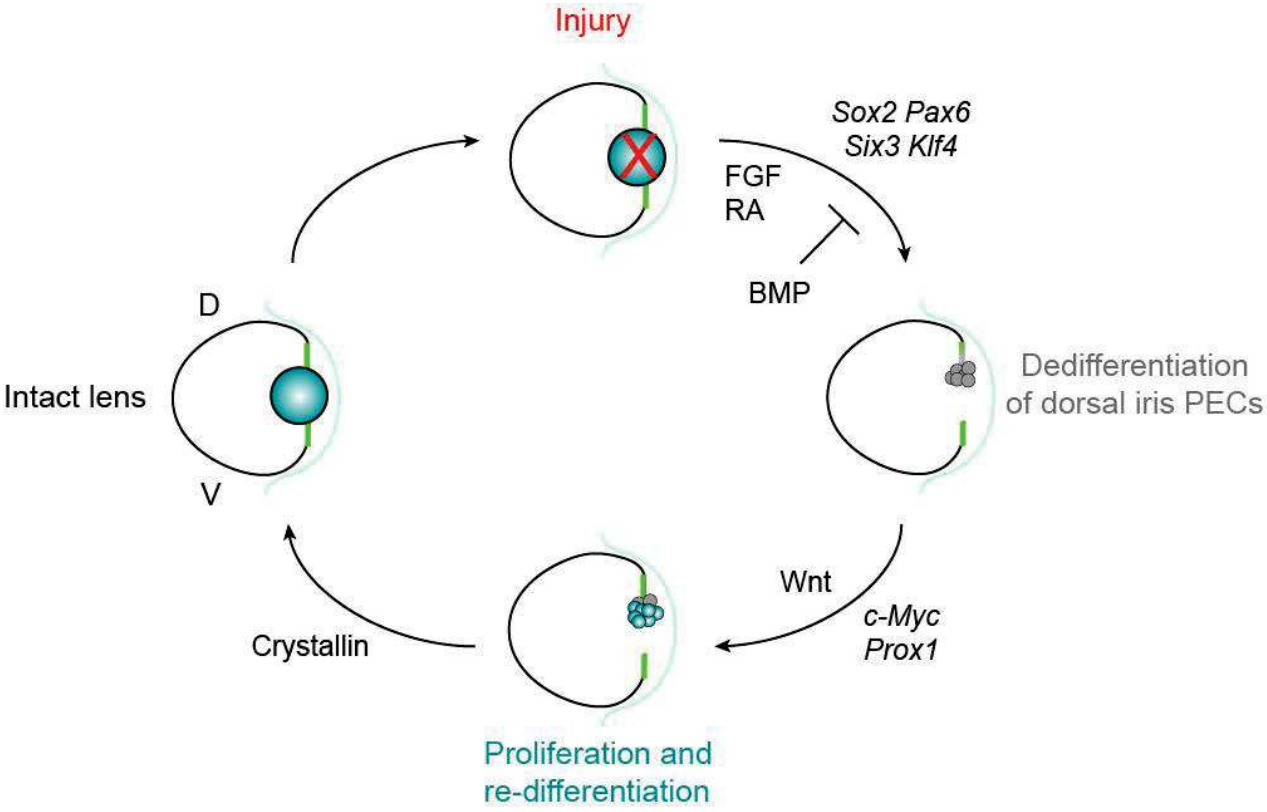

Figure 6. Transcription factors and signalling pathways required for transdifferentiation during lens regeneration in the newt. *Sox2*, *Pax6*, *Six3* and *Klf4* are expressed in the dorsal iris PECs during dedifferentiation, when FGF and RA signalling are activated. Later, during proliferation and re-differentiation, *c-Myc* and *Prox1* are expressed and the Wnt signalling is required (Barrero & Belmonte, 2011 and see text).

2.1.2.1 *The Wnt signalling pathway*

The highly conserved Wnt signalling pathway is required in *Drosophila* for the activation of the gene *Cut* in midgut progenitors leading to their conversion into renal progenitors (for the description of the Wnt signalling pathway components and variants see 3.3.1). Wnt/Wingless (Wg in *Drosophila*) functions as a spatial cue to select which midgut cells will transdifferentiate into renal cells when receiving another signal, the ecdysone hormone, at the onset of metamorphosis. The molecular mechanisms for this gene activation were investigated: the downstream intracellular factors of both pathways (TCF and Br-Z2 respectively) bind together

to *Cut* promoter and an enhancer localised into the second intron and thus activate its transcription (Xu et al., 2018).

In vertebrates, the Wnt signalling pathway mediates Müller cells dedifferentiation during retina regeneration in Zebrafish (Ramachandran et al., 2011). In other contexts, like the fish fin and the newt lens regeneration, the Wnt signalling pathway is required after the initial dedifferentiation phase for proliferation or re-differentiation of the blastema cells (Hayashi et al., 2006; Wehner and Weidinger, 2015). Interestingly, in newts the canonical Wnt signalling pathway is required for proper differentiation of lens during development too, suggesting similar mechanisms between development and regeneration in the re-differentiation phase (Henry et al., 2013). A comparable effect of Wnt was also described in mammals for the regeneration of sensory hair cells from supporting cells: the Wnt signalling pathway is required for transdifferentiation and for the expression of *Atoh1,* a bHLH transcription factor required for hair cell differentiation (Bramhall et al., 2014). What appears common among these last processes where the cellular dynamics are different (with or without a dedifferentiated intermediate and cell proliferation) is that the Wnt signalling pathway activates the expression of transcription factors associated with the final identity.

2.1.2.2 *The Notch signalling pathway*

Another highly conserved pathway is the Notch signalling pathway. Notch has been shown to be involved in several cell plasticity events in Zebrafish, both during regeneration and development. For instance, it is required for fin and heart regeneration where it precedes the expression of *msxB* and *msxC* transcription factors (however, a direct regulation was not shown; Raya et al., 2003). Moreover, the activation of the Notch signalling pathway is required for transdifferentiation of DE tubule renal cells into CS gland cells during normal development. Interestingly, this activation is observed a few hours before the appearance of CS cell markers and modulation of the Notch signalling pathway at different timepoints before transdifferentiation leads to opposite effects in the numbers of CS cells generated (Naylor et al., 2018). A time-dependent effect of Notch on transdifferentiation outcomes has also been observed in Y-to-PDA Td in *C. elegans* (T. Daniele PhD Thesis and Daniele et al., *in prep.*).

An apparently contradictory role of Notch signalling in liver regeneration is suggested by two different models, Zebrafish (He et al., 2014) and mouse (Yanger et al., 2013). As anticipated in the previous chapter, in the former organism BEC-to-hepatocyte transdifferentiation occurs, while in the latter hepatocyte-to-BEC is observed. In both cases activation of the Notch signalling pathway is required. The authors of the last study, in Zebrafish, suggest that the apparent contradiction is solved by the fact that a dedifferentiated intermediate, expressing progenitor markers, is formed. Thus, Notch should be required for dedifferentiation and

emergence of a bipotent intermediate which in one case gives rise to hepatocytes and in the other to BECs (He et al., 2014). This hypothesis is also in agreement with the demonstration that YAP activation in hepatocytes, upstream to Notch, activates a liver progenitor programme (Yimlamai et al., 2014).

Finally, in hair cell regeneration in mouse, the Notch signalling pathway seems to have a negative role against transdifferentiation: indeed, inhibition of the pathway increases transdifferentiation of supporting cells without requiring any cell division (Lin et al., 2011).

2.1.2.3 *Growth factor/RTK signalling*

Different families of growth factor ligands (e.g. Epidermal Growth Factor EGF, Fibroblast Growth Factor FGF, Insulin-like Growth Factor IGF) act by binding to receptor tyrosine kinase (RTK) and can lead to activation of intracellular signalling cascades, such as the MAPK, PLCγ and PI3K. They are generally known to promote cell proliferation, survival and to regulate cell differentiation (*Molecular Biology of the Cell*, B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter). Indeed, they are involved in those regeneration contexts where blastema formation is required, for instance Zebrafish fin and retina regeneration, newt lens and limb regeneration and axolotl limb regeneration (Henry et al., 2013; Knapp and Tanaka, 2012; Simon and Tanaka, 2013; Wehner and Weidinger, 2015).

While in Zebrafish fin FGF is required for blastema proliferation, in the retina EGF is required for dedifferentiation of Müller glial cells (Knapp and Tanaka, 2012; Wehner and Weidinger, 2015); in the newt expression of different FGF ligands and FGFR receptors in the dedifferentiating dorsal iris suggests that in this context FGF signalling is also required for dedifferentiation (Henry et al., 2013). Moreover, an *in vitro* study showed that unidentified factors present in the serum allow sustained activation of ERK in newt myotubes which leads to p53-dependent cell cycle re-entry and dedifferentiation. On the contrary, mammalian cells cannot sustain the prolonged activation of ERK signalling and this could be an explanation of their incompetency to regenerate (Yun et al., 2014).

Finally, in *Drosophila* mixer cells reprogramming, the JNK kinase downstream to the MAPK pathway is required for the downregulation of *Polycomb*, which in turn allows the activation of *Engrailed* expression necessary for the acquisition of the new identity, as anticipated above (Roumengous et al., 2017).

2.1.2.4 *BMP signalling*

The TGFβ/BMP (Transforming Growth Factor-β/Bone Morphogenetic Protein) signalling pathway is another major pathway which is involved in cell renewal and differentiation (Henry

et al., 2013). As for growth factor signalling, BMP signalling has also been identified in blastema-mediated regeneration. BMP was shown to be required for Zebrafish fin regeneration, *Xenopus* tail regeneration, mouse digit-tip regeneration and its downregulation for lens regeneration in newts.

In Zebrafish it is required for blastema proliferation (Wehner and Weidinger, 2015). In frog tadpoles during tail regeneration, BMP signalling directly regulates the expression of *Msx1* transcriptional repressor required for dedifferentiation; moreover, BMP signalling is also upstream to Notch signalling in this context. Activation of the BMP pathway is sufficient for regeneration of all the frog tail tissue, while Notch allows the regeneration of notochord and spinal cord (Beck et al., 2003). A link between BMP signalling and *Msx1* expression is also observed in mice digit-tip regeneration, but in this case *Msx1* seems upstream and not downstream to BMP4 production (Han et al., 2003).

In the newt iris, BMP signalling has not a positive but rather a negative role on the capacity of the iris cell to transdifferentiate. It was shown that exposing the dorsal iris to BMP ligands affects regeneration and, accordingly, inhibiting the pathway in the ventral iris allows transdifferentiation of ventral PECs. The activation of BMP signalling in the ventral and not in the dorsal iris agrees with a ventralising role of this signalling pathway observed during development. Moreover, the authors suggest that downregulation of the pathway in the dorsal iris is required for upregulation of *Six3* and *Pax6* transcription factors which drive dorsal iris-to-lens transdifferentiation (Grogg et al., 2005).

2.1.2.5 *Retinoic acid signalling*

Retinoic acid (RA), a derivate of vitamin A which targets nuclear receptors, is required together with FGF signalling for dedifferentiation of dorsal iris cells during lens regeneration in newts. The expression of its receptors RARα and RARγ is undetected in uninjured lens, while they are expressed in the dedifferentiated regenerating tissue (Henry et al., 2013).

RA was shown to be also required for proper regeneration of Zebrafish fin; however, in this context is not required for cell dedifferentiation but for later patterning of the fin (White et al., 1994).

Table 1. Summary of factors involved in different natural cell plasticity contexts.

Context	Dedifferentiated intermediate and cell division?	Transcription factors involved	Epigenetic factors involved	Signalling pathways required	References
Y-to-PDA Td, *C. elegans* development	Dedifferentiated intermediate, no cell division	*ceh-6/POU, sox-2/SOX, sem-4/SALL, egl-5/HOX, unc-3*	*jmjd-3.1* Set1 complex (*set-2 wdr-5.1*)	Notch	Jarriault et al., 2008; Kagias et al., 2012; Richard et al., 2011; Zuryn et al., 2014; Daniele et al., unpublished
Remodelling of segment boundaries, *Drosophila* development	No dedifferentiated intermediate, no cell division	*Engrailed*	*Polycomb*	JNK	Gettings et al., 2010; Roumengous et al., 2017
Midgut-to-renal Td, *Drosophila* development	No dedifferentiated intermediate, no cell division	*Cut*		Wnt and ecdysone hormone	Xu et al., 2018
Retina regeneration, Zebrafish	Dedifferentiated intermediate, cell division (blastema formation)	*ascl1, lin-28 (oct4, sox-2, nanog, klf4, c-myc*)	*apobec2* ? (DNA demethylation)	EGF/MAPK, Wnt, Notch	Ramachandran et al., 2010, 2011; Wan et al., 2012; Zhu et al., 2018
Fin regeneration, Zebrafish	Dedifferentiated intermediate, cell division (blastema formation)	*msxB, msxC*	*kdm6b.1 (JMJD3)*, *gadd45*, *dnmt3aa*	Notch, FGF, BMP, RA (for patterning)	Alvarado and Tsonis, 2006; Stewart et al., 2009; Hirose et al., 2013; Raya et al., 2003; Zhu et al., 2018; Wehner and Weidinger, 2015; White et al., 1994
Liver regeneration, Zebrafish	Dedifferentiated intermediate, cell division	*sox9b*		Notch	He et al., 2014

Heart regeneration, Zebrafish	Dedifferentiation, cell division (no transdifferentiation)	*msxB*, *msxC*		Notch	Alvarado and Tsonis, 2006; Raya et al., 2003
Tail regeneration, *Xenopus*	Dedifferentiated intermediate, cell division (blastema formation)	*Msx1*		BMP, Notch	Alvarado and Tsonis, 2006
Limb regeneration, *Xenopus*	Dedifferentiated intermediate, cell division (blastema formation)	*sall4*			Neff et al., 2005
Lens regeneration from iris, newt	Dedifferentiated intermediate, cell division (blastema formation)	*Klf4, Sox2, Myc, Pax6, Six3*	*p300*, *HDAC2/5*, *Dnmt1, Jmj* (need functional test)	FGF, RA, thrombin for dediff., Wnt for re-diff., BMP (inhibition)	Hayashi et al., 2006; Henry et al., 2013; Maki et al., 2010; Natalia Vergara et al., 2018
Limb regeneration, newt	Dedifferentiated intermediate, cell division (blastema formation)	*Msx1*, *Klf4, Sox2, Myc*		FGFs, thrombin, glial growth factor, transferrin (both proposed for re-diff.)	Alvarado and Tsonis, 2006; Kumar et al., 2004
Digit-tip regeneration, mouse and human	Dedifferentiated intermediate, cell division (blastema formation)	*Msx1*		BMP	Alvarado and Tsonis, 2006; Han et al., 2003
Regeneration of β-cells from α-cells, mouse	No, or partial dedifferentiation, no cell division	*Pdx1*, *Nkx6. 1*			Jessen et al., 2015; Merrell and Stanger, 2016; Thorel et al., 2010
Regeneration of β-cells from δ-cells, mouse	Dedifferentiation intermediate, cell division	*Ngn3* up, *FoxO1* down		BMP, PI3K (suggested by transcriptional profiles)	Chera et al., 2014; Jessen et al., 2015
Regeneration of sensory hair cells from	No cell division	*Atoh1*		Notch (inhibition), Wnt	Jessen et al., 2015; Lin et al., 2011; Bramhall et al., 2014

supporting cells in vestibular and cochlear epithelium of the ear, mouse					
Biliary epithelial cells from hepatocytes, mouse	No dedifferentiated intermediate, no cell division	*Hes1*, *Sox9* downstream to Notch		Notch	Yanger et al., 2013

All these examples demonstrate the importance of signalling cues in natural reprogramming events and show that they may be required for either dedifferentiation, cell proliferation or re-differentiation, depending on the cell-specific context.

2.2 Mechanisms underlying induced-reprogramming events

Studying the mechanisms underlying *in vitro* cellular reprogramming, even though artificially induced, could provide useful information to understand cell identity acquisition, maintenance and cell plasticity (Buganim et al., 2013). *In vitro* studies allow to perform several cellular and molecular biology assays and in recent years have revealed a lot of interesting molecular events taking place in the nuclei of mammalian cells during reprogramming. A common theme in *in vitro* reprogramming is the capacity of reprogramming transcription factors to both silence the initial transcriptional programme and activate the final one. Different molecular mechanisms have been proposed by the study of iPSC reprogramming and transdifferentiation. However, in all the induced reprogramming events, like in the natural ones, the role of transcription factors as drivers of the process is demonstrated together with the integration of the transcription factor network with context-specific signalling pathways.

2.2.1 Mechanisms of reprogramming to pluripotency

Most efforts to understand the mechanisms underlying reprogramming to pluripotency have focused on transcription factor-induced reprogramming instead of cell fusion or SCNT-mediated reprogramming, taking advantage of the knowledge of the factors used and of the possibility to modulate their expression with this method (Buganim et al., 2013).

The formation of iPSC colonies from fibroblasts after transduction with OSKM factors takes 1.5 weeks. Different intermediate steps have been observed at the cellular and molecular level, following the initial inductive signal: the first event is the downregulation of the fibroblast marker Thy already 1 or 2 days after transgenes induction; by day 3, mesenchymal-to-epithelial transition (MET) occurs as shown by upregulation of *E-cadherin*; after 3 days pluripotency-associated markers such as SSEA1 and alkaline phosphate are detected; however, only after 8 to 10 days the endogenous *Oct4* and *Sox2* genes are expressed, with different timings. Importantly, cells that activate SSEA1 are more prone to complete reprogramming while cells that fail to downregulate fibroblast Thy marker become refractory. Gene expression profiles of cells at those different steps revealed two waves of gene transcription, the first occurring by day 3 and the second after day 9, with an intermediate window with few transcriptional changes. The downregulation of somatic genes occurs early, while the upregulation of most pluripotency genes is achieved later. During those two transcriptional waves, cells behave in a more homogenous way compared to the intermediate phase. According to these results, the authors of this work suggest the existence of an early deterministic phase, followed by a more stochastic intermediate phase and a final deterministic phase (Polo et al., 2012). Another study looking at single cell gene expression (but of only 48 genes) suggested instead that the first phase of reprogramming is stochastic (Buganim et al., 2012). These opposite results are probably due to the different approaches followed by the two studies. More investigations are required to establish how reprogramming to pluripotency proceeds, but it is highly probable that this depends on the conditions (including starting cell type, method of transcription factor delivery and factors used). Another study exploiting cellular barcoding demonstrated that reprogramming to iPSCs happens in a deterministic manner at least in lineage-related cells, suggesting that the initial cell population is heterogenous respect to its tendency to be reprogrammed and that this ability is inherited through cell division (Yunusova et al., 2017). Interestingly, the removal of some epigenetic barriers allows to achieve reprogramming in a deterministic way, homogenous, faster and more efficient (they claim near 100% efficiency, Rais et al., 2013).

2.2.1.1 *The role of OSKM factors*

Oct4, *Sox2* and *Klf4* are three fundamental factors required for reprogramming to pluripotency, while exogenous *c-Myc* is dispensable (Zviran et al., 2019), but it enhances efficiency and speed of the conversion (Wernig et al., 2008). This finding agrees with a different role of c-MYC in mESCs, where it mainly binds to different target sequences compared to OCT4 and SOX2, leading to the definition of an OCT4-centric module and a MYC-centric module (Ng and Surani, 2011).

OCT4, SOX2 and KLF4 are pioneer transcription factors because of their capacity of binding to nucleosomal DNA (Soufi et al., 2015), a key feature shared by many factors driving cellular reprogramming (Morris, 2016). A recent study described at the molecular level how dynamically OSK transcription factors lead to iPSC reprogramming (Chronis et al., 2017). They analysed the changes in transcription, OSK transcription factors' binding, somatic transcription factors' binding, histone modifications and chromatin conformation (through RNA-seq, ChIP-seq and ATAC-seq technologies) during mouse embryonic fibroblasts (MEFs) reprogramming to pluripotency. Their results revealed the importance of cooperative binding of OSK for efficient target selection; their initial binding to open chromatin at MEF enhancers; the consequent loss of somatic transcription factors at their own binding site, their re-localisation to other sites and the downregulation of their target genes; the binding of somatic transcription factors with OSK to early pluripotency genes too, perhaps contributing to their early activation; the requirement for the gradual activation of more (core) pluripotency transcription factors to build a stable pluripotency transcriptional network only at later phases. The changes in transcription factors' binding are accompanied by changes in chromatin state, both in histone modifications and nucleosome compositions especially at enhancer regions, while promoters' states remain mostly unchanged. The downregulation of fibroblast genes could be explained by the binding of OSK to their regulatory regions with the consequent loss of binding of somatic transcription factors and changes in chromatin conformation. At the same time, a gradual change in histone marks at pluripotency genes (initially in a closed chromatin conformation and without active histone marks) prepares them for later activation since the initial phases of reprogramming. Interestingly, the pluripotency genes that are activated earlier are enriched in OSK binding sites compared to those activated only later on (Chronis et al., 2017).

Concerning c-MYC, it is required in the initial phases of reprogramming (Buganim et al., 2013). Differently from OSK, it preferentially binds to promoters than enhancer regions (Papp and Plath, 2013) and it requires cooperative binding with other factors to bind to nucleosomal DNA (it has not pioneer factor activity on its own, Soufi et al., 2015). c-MYC was shown to prevent differentiation, activate the expression of miRNAs regulating cell proliferation and to facilitate chromatin opening thanks to the recruitment of histone modifiers and chromatin remodelling complexes (Orkin and Hochedlinger, 2011). Moreover, c-MYC is known for its capacity to recruit the CDK pTEFb which releases RNA pol II from pausing, suggesting a general role of this factor in activating transcription by increasing the rate of transcriptional elongation (Buganim et al., 2013; Papp and Plath, 2013; Young, 2011). Finally, a recent study showed that *Myc* is required for activating biosynthetic pathways necessary to establish pluripotency and that, even though its overexpression may be dispensable, the presence of the endogenous gene is required for reprogramming to pluripotency (Zviran et al., 2019).

2.2.1.2 *The role of chromatin modifiers*

ESC chromatin state is uniquely open and dynamic compared to differentiated cells (Orkin and Hochedlinger, 2011). Thus, during somatic cell reprogramming to iPSCs different chromatin modifications must occur and are the consequence of OSK-mediated recruitment of chromatin modifiers on DNA. These modifications are essential for creating the PSC dynamic chromatin state. The changes in chromatin observed during reprogramming include a genome-wide resetting of histone tail modifications and a massive DNA demethylation and chromatin reorganization, which also result in X chromosome re-activation, a marker of mESC state (Nashun et al., 2015; Papp and Plath, 2013). A more recent study analysed the overall dynamics of chromatin topology during reprogramming and found that it is highly dynamic, with several switches between A (active) and B (inactive) compartments. They showed that, even though topologically associated domains (TADs) are normally stable among different cell types, few TAD borders are altered. Importantly they also showed that many chromatin changes precede changes in gene expression, reinforcing the results of other studies which demonstrated that chromatin architecture is not simply a consequence of gene expression (Stadhouders et al., 2018).

The first chromatin change detected after OSKM overexpression is in histone modifications at regulatory regions even before full activation of gene expression. Deposition of H3K4me2 histone mark is observed early at the promoters of several pluripotency genes, including *Sall4*, while H3K27me3 is gradually lost. It is proven that the enzymes responsible for these modifications physically interacts with OSK: for instance, WDR5 interacts with OCT4 and UTX H3K27me3 demethylase associates with OCT4, SOX2 and KLF4 (reviewed by Buganim et al., 2013). This interaction is reminiscent of the interaction described between *C. elegans* WDR-5.1 with orthologues of OCT4 and SOX2 (Zuryn et al., 2014; see above).

Later during the reprogramming process, other modifications are necessary. Among the factors involved, BAF (SWI/SNF) chromatin remodelling complex is required for demethylation of endogenous pluripotency genes such as *Oct4*, *Nanog* and *Rex1*, thus enhancing reprogramming efficiency. Moreover, other histone modifiers participate in resetting the somatic cell genome to a pluripotent state: for instance, both H3K9 methyltransferases EHMT1 and SETDB1 and Polycomb complexes PRC1 and PRC2 are necessary for iPSC generation to silence somatic genes. On the contrary the H3K9 methyltransferase SUV39H represents a barrier to reprogramming probably through a general alteration of transcriptional regulation (Buganim et al., 2013).

Histone variants and histone chaperons have been shown to have an impact on reprogramming too. Study of SCNT-mediated reprogramming contributed to the understanding

of the role of histone variants in reprogramming to iPSCs. Histone variants associated with active transcription, such as H3.3, favour reprogramming; on the contrary, histone variants associated with transcriptional repression, such as macroH2A, constitute a barrier to reprogramming (Nashun et al., 2015). Since both transcriptional activation and repression of different target genes are required for reprogramming, it would be interesting to know how the dynamic regulation of the deposition of these variants impacts on changes in gene expression associated with reprogramming.

Finally, changes in DNA methylation must occur to establish the pluripotent stem cell state. Several studies showed that low methylation levels are a feature of the ESC state both *in vivo* and *in vitro*. Accordingly, the erasure of the somatic methylome is important for successful reprogramming while *de novo* methylation is not strictly necessary. The establishment of ESC-like DNA methylome is more efficient by reprogramming through SCNT, while many aberrations were found in OSKM-induced iPSCs. The difficult erasure of the somatic cell methylome could be one of the main barriers to complete iPSC reprogramming and methylome differences are one of the features distinguishing iPSCs from ESCs (Kim et al., 2010; Nashun et al., 2015).

2.2.1.3 *The role of signalling pathways*

Regulation of signalling pathways important for the maintenance of ESC identity *in vitro* is also required for reprogramming to pluripotency as they collaborate with OSKM to establish the pluripotency network (Hackett and Surani, 2014; Ng and Surani, 2011) (Figure 7). iPSC reprogramming can be obtained by overexpression OSKM, but at the same time it requires to plate the cells in ESC culture conditions, i.e. on feeder cells and in ESC medium (with LIF, and more recently with PD0325901 and CHIR99021, the so called 2i medium) (Stadhouders et al., 2018; Takahashi and Yamanaka, 2006). The culture conditions vary between human and mouse iPSCs reflecting the different species-specific features of these pluripotent cells (Davidson et al., 2015; Yu et al., 2007).

Derivation and maintenance of mouse iPSCs rely on activation of STAT3 transcription factor, downstream to the Leukaemia Inhibitory Factor (LIF) signalling. LIF binds to its cell-surface receptor gp130 and activates JAK kinases which in turn lead to STAT3 phosphorylation. Among the target genes of this pathway there are key pluripotency genes such as *Klf4*, *Klf2*, *Gbx2, Tfcp2l1, Esrrb* and perhaps also *c-Myc.* Indeed, STAT3 was shown to promote complete reprogramming of pre-iPSCs (Ye et al., 2014). Together with LIF, BMP4 must also be present to maintain the pluripotent state in mouse cells. BMP4 is provided by foetal calf serum (FCS) addition to the medium and acts through SMAD signalling pathway to activate *Inhibitor of Differentiation* (*Id*) genes. Interestingly, while LIF is required for the repression of non-neuronal

differentiation, BMP4 inhibits neuroectoderm differentiation (Hackett and Surani, 2014). Thus, both STAT3 and SMAD are considered part of the pluripotency core transcription factor regulatory network (Orkin and Hochedlinger, 2011).

In alternative to serum addition, the 2i medium can be used both for establishing iPSCs and for maintaining them. This medium was defined by A. Smith's lab and relies on the use of two small molecules which inhibit pro-differentiation FGF-ERK pathway (PD03 for short) and stimulate the canonical Wnt signalling pathway (CHIRON for short) by inhibiting GSK3 kinase (Ying et al., 2008). Thus, the former prevents the autocrine FGF4 signalling from driving exit from pluripotency, while the latter stabilises β-catenin which after translocation to the nucleus inhibits the transcriptional repressor activity of TCF3 at pluripotency genes (Hackett and Surani, 2014). According to a positive role of the Wnt signalling pathway on pluripotency, it was demonstrated that stimulation of the Wnt signalling pathway by addition of Wnt3a to the medium enhances reprogramming efficiency in absence of *c-Myc* overexpression (Marson et al., 2008). Interestingly, the Wnt signalling pathway does not improve reprogramming through upregulation of reprogramming factors, but the two pathways remain distinct while cooperating to achieve the conversion (Lluis and Cosma, 2009).

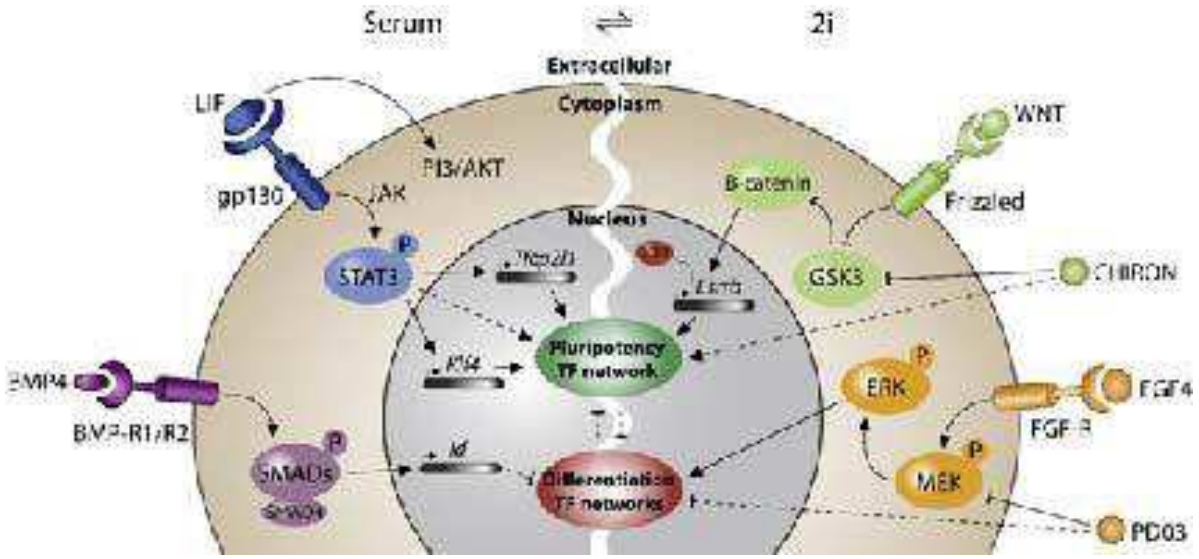

Figure 7. The pluripotency TF network is integrated by the modulation of signalling pathways *in vitro*. The core pluripotency TF integrate with JAK/STAT signalling downstream to LIF and with SMAD signalling downstream to BMP4 in LIF+serum culture conditions; they integrate with activation of Wnt signalling pathway (thanks to the small molecule CHIR99021) and inhibition of ERK signalling (thanks to the small molecule PD0325901) in 2i culture conditions (Hackett and Surani, 2014).

These studies underline the importance of external cues in the acquisition and maintenance of the pluripotent stem cell identity (Hackett and Surani, 2014). Moreover, they represent an accurate description of how signalling cues integrate with and reinforce the GRN to establish

and maintain pluripotency. Activation of signalling pathways is not dispensable, and most importantly their effect is so broad in the responding cell that the simple expression of single downstream factors is usually not enough to entirely recapitulate their activity (as described for STAT3; Hackett and Surani, 2014).

2.2.2 Mechanisms of induced transdifferentiation

Transcription factor-induced transdifferentiation has been used as a powerful system to study mechanisms underlying induced transdifferentiation, offering the same advantages of transcription factor-induced reprogramming to pluripotency (Vierbuchen and Wernig, 2012). For instance, the inter-germ layer reprogramming of mouse fibroblasts into neurons through overexpression of *Ascl1*, *Brn2* and *Myt1l* (BAM) was extensively characterised revealing similarities and differences when compared to reprogramming to pluripotency (Treutlein et al., 2016; Wapinski et al., 2013, 2017). Analysis of the transcriptome at different steps during fibroblasts-to-neuron transdifferentiation highlighted a continuum of intermediate states at the transcriptional level, in which it is possible to delineate two gene regulatory events: an initiation stage where most fibroblast genes and features are lost and some neuronal genes start to be expressed; a maturation stage where the last fibroblast genes are turned off and genes required for neuronal maturation are activated. Moreover, expression of some genes associated with neural precursors is transiently activated, even though a complete gene expression profile associated with these progenitor cells is not detected (Treutlein et al., 2016). Thus, as suggested by S. Morris, an intermediate state which likely activates some developmental programmes is observed (Morris, 2016). However, at least in this case, the transient intermediate is not a canonical neural precursor (Treutlein et al., 2016). It is likely that analyses at the single cell-level of reprogramming at different steps will allow to discover the existence of progenitor-like intermediates in different contexts, especially when reprogramming is performed using pioneer factors (see 2.2.2.1) and to achieve lineally distant conversions. Interestingly, differently from this example of induced reprogramming, transcriptional analyses of blastema cells at different timings during axolotl limb regeneration showed that intermediates recapitulating limb development are formed after an initial cell state which appears blastema-specific (Gerber et al., 2018).

2.2.2.1 *The role of transcription factors and their impact on chromatin structure*

Several studies focused on fibroblast-to-neuron transdifferentiation aimed to understand how lineage-specific transcription factors can drive cell fate conversion at the molecular level. The key role of pioneer factors in initiating reprogramming described for iPSCs is also confirmed by studying transdifferentiation (Morris, 2016). For instance, ASCL1 was defined not only as a

pioneer factor, but more precisely an “on-target” pioneer factor (Wapinski et al., 2013) because it can bind to nucleosomal DNA mostly targeting its natural binding sites, with only a few off-targets (differently from OSK which show off-target binding, Soufi et al., 2012). However, the pioneer factor activity of ASCL1 is context-dependent (i.e. it depends on the starting cell type): the overexpression of *Ascl1* in cell types other than fibroblasts demonstrated that it fails to bind to its DNA target sites in other contexts. Indeed, ASCL1 can bind to nucleosomal DNA in presence of a permissive chromatin signature based on combinations of specific histone marks (Wapinski et al., 2013). According to its pioneer capacity, ASCL1 is required for the first transcriptional changes and its level of expression matters for the success of reprogramming. Activation of neuronal and muscle genes is observed in fibroblasts when *Ascl1* is overexpressed, and *Brn2* and *Myt1l* are required to direct transdifferentiation towards a neuronal fate by silencing myogenic genes and activating neuronal terminal differentiation genes (Treutlein et al., 2016).

Differently from iPSC reprogramming with OSKM factors, a hierarchical rather than a cooperative transcription factor binding mechanism is at play during BAM-mediated fibroblast-to-neuron transdifferentiation (Wapinski et al., 2013). As suggested by transcriptional profiles (Treutlein et al., 2016), in a hierarchical way ASCL1 is the first factor binding to chromatin and mediating chromatin remodelling which allows subsequent binding of BRN2 and MYT1L. While chromatin remodelling already starts 12 hours post induction, accessibility of BRN2 binding sites is achieved by day 5 (Wapinski et al., 2013, 2017). Interestingly, in agreement with what observed during reprogramming to pluripotency (Stadhouders et al., 2018), the chromatin switch induced by ASCL1 binding precedes activation of gene expression (Wapinski et al., 2017).

More demonstrations of these mechanisms have come from another work characterising transdifferentiation of human fibroblasts into muscle cells induced by *MyoD* overexpression. This study confirmed the role of transdifferentiation-driving factors in reorganising chromatin and the occurrence of large-scale chromatin remodelling before transcriptional changes occur (except for changes in locus-specific enhancer-promoter interaction which appear to require transcription). Moreover, the authors suggest that the capacity of MYOD to affect chromatin organisation might explain its ability to repress fibroblast genes, as the transcriptional activator nature of this factor cannot otherwise explain it (Dall’Agnese et al., 2019).

Since pioneer factor MYOD belongs to the bHLH family like ASCL1, M. Wernig and colleagues directly compared their activity during reprogramming to further investigate conserved functions of these factors during transdifferentiation (Lee et al., 2020). Indeed, they found both similarities and differences. What is striking is that they bind to similar sites, many of them

overlapping, with ASCL1 also binding to myogenic genes (seen by transcriptome analysis during reprogramming too, Treutlein et al., 2016) and MYOD to neuronal genes. The difference is found in their quantitative, and not qualitative binding on different sites which leads to different impacts on the chromatin state and thus to preferential activation of certain lineage-specific genes. While ASCL1 preferential binding to neuronal genes is driven by its interaction with enriched E-box DNA sequences, MYOD is driven to its sites through interaction with cofactors, including PBX. However, in the case of ASCL1 additional factors, BRN2 and MYT1L, are required while MYOD alone allows reprogramming. Interestingly, the authors found that overexpression of *MyoD* together with myogenic repressor *Myt1l* leads to conversion of some fibroblast into functional neurons (Lee et al., 2020).

Overall, these studies suggest that pioneer factors might have a broader role in enhancing cell plasticity than just promoting a dedicated expression programme, by driving a more general chromatin remodelling (Morris, 2016). This is supported by their broad chromatin binding, their role in supressing the initial expression programme (MYOD) and by the capacity of ASCL1 to give rise to muscles in absence of BRN2 and MYT1L and of MYOD to form neurons in presence of MYT1L. In her Hypothesis Article (Morris, 2016), S. Morris suggests that pioneer factors can induce reprogramming thanks to their ability to engage with developmental "upper-level" GRNs, that are GRNs which are established early during development and lineage specification. These factors are expressed early during development and are upstream to many other lineage-specific transcription factors, thus being also considered as master regulators of cell fate. On the contrary, at the bottom of the "transcription factor hierarchy", S. Morris puts the terminal selector genes, transcription factors directly regulating the expression of terminal differentiation genes (Hobert, 2008a). Terminal selectors alone should not be able to bind to closed chromatin to drive reprogramming: in an *in vivo* example of induced reprogramming in *C. elegans*, cell fate conversion mediated by CHE-1 (ASE sensory neurons terminal selector) was possible in cell types other than sensory neurons only in absence of *lin-53*/RbAp46/48 (Tursun et al., 2011), a histone chaperone found in several chromatin remodelling complexes (Vierbuchen and Wernig, 2011). However, the only cell type responding to *che-1* overexpression in a *lin-53* mutant background are germ cells, which are not somatic and have different properties, such as the potential to give rise to an entire embryo after fertilisation (Rothman and Jarriault, 2019). Thus, this cell fate conversion would not fit even into the definition of transdifferentiation given by Takahashi and Yamanaka which implies the interconversion between somatic cells (see above, Takahashi and Yamanaka, 2016). More recently, another study reinforced the hypothesis that terminal selectors are not able to bind to silent chromatin: O. Hobert and colleagues demonstrated that the overexpression of a terminal selector can change the neuronal subtype of a cell into another subtype only if the terminal

selector of the target cell is absent (Patel and Hobert, 2017). To note, the change in cell fate that can be driven by the terminal selector is not only intra-germ layer, but also among different neuronal subtypes. This result agrees with a higher capacity of reprogramming (between distant lineages) of pioneer factors compared to terminal selectors. However, more studies are required to reinforce this conclusion, especially because several terminal selectors have been identified in neurons in *C. elegans* while in other cell types and organisms they are not identified yet or sufficiently studied. It might be also possible that a sharp distinction between pioneer factors and terminal selectors is not always appropriate, and their reprogramming capacity could mainly depend on the specific transcription factor or cellular context (and their combination).

2.2.2.2 *The role of signalling pathways and cell-activation and signalling-directed transdifferentiation*

Signalling pathways activated by external cues provided by the cell culture media also play a key role in induced transdifferentiation. Like for iPSC reprogramming, overexpression of reprogramming transcription factors in the target cells *in vitro* is accompanied by addition of the medium specific for the desired cell type, at different time points depending on the protocol. Thus, N3 medium containing FGF2 is added for neural induction (Vierbuchen et al., 2010), hepato-medium is used for obtaining hepatocytes (Sekiya and Suzuki, 2011) and different media can be used sequentially to induce human β-cells (Zhu et al., 2016). For the transdifferentiation events induced *in vivo* (Niu et al., 2013; Qian et al., 2012; Zhou et al., 2008), it is probable that signals from the environment might contribute (or impede) reprogramming. Indeed, transplantation of hepatocyte-like cells obtained *in vitro* allows their full differentiation and integration into the tissue (reviewed by Morris, 2016; Sekiya and Suzuki, 2011).

An extreme demonstration of the importance of signalling pathway in driving cell fate acquisition during reprogramming is given by cell-activation and signalling-directed transdifferentiation (CASD, Figure 8). As pointed out previously (1.4.4.2), some groups demonstrated that differentiated cells can transdifferentiate through a transient overexpression of OSKM factors (to start the first wave of transcription; Wapinski et al., 2013) and directing differentiation to the desired cell type by exposing the cells to appropriate culture conditions, without passing through a pluripotent state (Efe et al., 2011; Kim et al., 2011) or with a transient pluripotent state (Bar-Nur et al., 2015; Maza et al., 2015). While these contradictory results, in terms of the cellular steps involved, might be explained by the different system used (Firas and Polo, 2017; see above), they confirm the importance of developmental signalling pathways in cell fate acquisition also in the context of transdifferentiation.

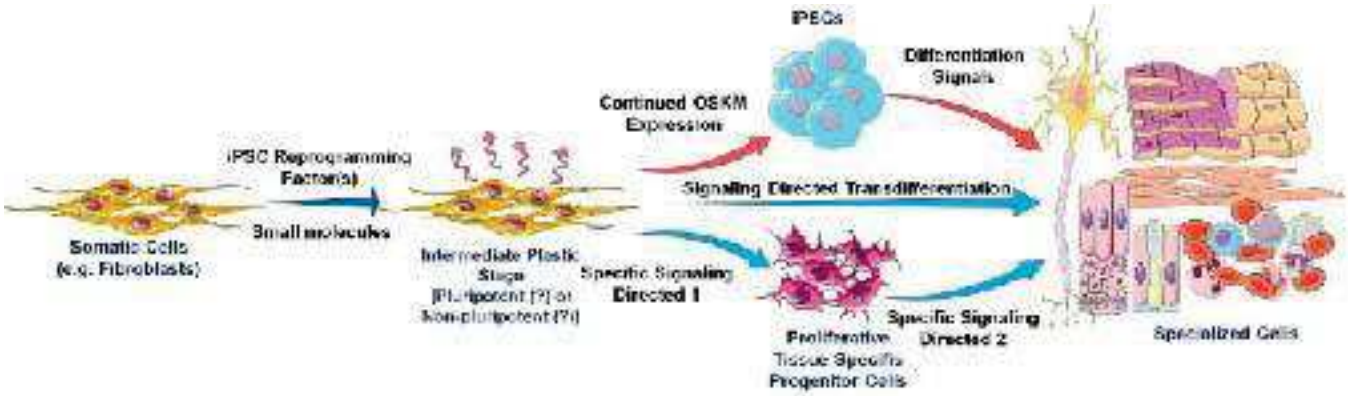

Figure 8. Cell-activation and signalling-directed transdifferentiation. Schematic of the steps required for CASD compared to reprogramming to pluripotency (Ebrahimi, 2016).

2.3 The role of cell division during cellular reprogramming

Whether DNA replication and cell division are absolutely required for reprogramming has been a long-lasting question. During induced reprogramming, depending on the protocol and on the desired cell type, a different number of cell divisions can be observed: for instance, transdifferentiation of pancreatic exocrine cells into hepatocytes does not require cell division (Shen et al., 2000), conversion of fibroblasts into neurons is achieved with maximum one cell division (Vierbuchen et al., 2010), while generation of iPSCs requires dozens of cell divisions (Hanna et al., 2009). The authors of this last work demonstrated that increasing cell proliferation and total number of cell divisions accelerates reprogramming in a proportional way, and downregulation of *p53* increases reprogramming rate with 70 cell divisions occurring before iPSCs are obtained. They argue that the higher division rate could increase the probability of stochastic events required for iPSC generation. Generally, a high number of cell division is typical of reprogramming to pluripotency. Moreover, during SCNT and cell fusion-directed reprogramming, considered for a long time as examples of reprogramming in absence of cell division, DNA synthesis takes place (Nashun et al., 2015). Conversely, cell division was shown not only to not improve reprogramming in neuronal lineages but even to decrease its efficiency (Gascón et al., 2017).

Concerning natural cellular reprogramming, the first discussion about the role of cell division dates back to 1990, when W. A. Beresford wrote a review remarkably entitled “Direct transdifferentiation: can cell change their phenotype without dividing?” (Beresford, 1990). He highlighted several examples where transdifferentiation might take place in absence of cell divisions, both in invertebrates and vertebrates. However, many of these cell identity changes were observed *in vitro* after cell isolation and not in their *in vivo* context, as lineage tracing tools were not available yet. More recently, accurate studies discovered different instances in which transdifferentiation occurs in absence of cell division (see Table 1). The knowledge of

the cell lineage (Sulston and Horvitz, 1977; Sulston et al., 1983), the small cell number and the transparency of *C. elegans* allowed to demonstrate that Y-to-PDA transdifferentiation occurs in absence of DNA replication and cell division (Jarriault et al., 2008; Richard et al., 2011), suggesting that cell division is not strictly required for a drastic change in cell identity. The occurrence and requirement of cell division seem more context-dependent and more studies are needed to understand the contribution of cell division to cell fate changes (Soufi and Dalton, 2016).

As discussed by P. Hajkova and colleagues, several possible mechanisms could explain the requirement of cell division in some circumstances or at least its capacity of enhancing cell reprogramming (as clearly shown for reprogramming to pluripotency). Their hypotheses are focused on the epigenetic changes that DNA replication can favour, considering the known impact of epigenetic barriers on reprogramming efficiency. DNA replication, with the replication fork disrupting chromatin structure, could create a window of opportunity for both pioneer and non-pioneer transcription factor binding and at the same time lead to stochastic loss of epigenetic memory (Nashun et al., 2015). These possible nuclear mechanisms are discussed mostly for induced reprogramming; it would be interesting to study how cell division might contribute to transdifferentiation in a natural context. This is one of the questions we wanted to answer to with this Thesis.

2.3.1 Cell division in the definition of transdifferentiation

There is a general agreement on the fact that transdifferentiation (differently from reprogramming to pluripotency) can occur either in presence or in absence of cell division, as discussed in the previous paragraphs and in several reviews on the topic (Pomerantz and Blau, 2004; Slack, 2007; Tosh and Slack, 2002). Absence of cell division is not a requirement even in the original definition of transdifferentiation, where the criteria are that “the differentiation state before and after the transdifferentiation can be reliably described and distinguished” using “morphological criteria […] accompanied by biochemical or molecular evidence”; and that there is “a direct ancestor-descendent relationship between cells before and after the switch” (Eguchi and Kodama, 1993). These criteria can be integrated with the initial cell type being mature, having acquired a “working character”, which means being functional in a tissue (Beresford, 1990). Thus, while we can discuss the requirement and contribution of cell division to reprogramming, we cannot exclude from being defined as transdifferentiation those events in which cell division takes place. If we want to be more precise, we could use “direct transdifferentiation” to indicate transdifferentiation events without cell division (Beresford, 1990) and simply “transdifferentiation” to describe all the events

involving or not cell division. Accordingly, in this Thesis I always use the term transdifferentiation independently of the presence (and number) or absence of cell divisions.

3 Asymmetric cell division: a source of cell fate diversity

As discussed above, cell division occurs (and is required) in some reprogramming contexts and the current vision is that it is not contrasting with the definition of reprogramming and transdifferentiation. The possibility to follow single cells *in vivo* in *C. elegans* has allowed researchers to identify putative transdifferentiation events during larval development where cell division takes place. In some of these cases, transdifferentiation appears to occur in an asymmetric cell division (ACD) setting, where the asymmetry consists in one differentiated daughter cell maintaining the identity of the mother cell and the other changing fate. ACD can occur with distinct dynamics, for instance when one of the daughter cells receives a signal after an initial symmetric division or following asymmetric localisation during mitosis of cell intrinsic or cell extrinsically-provided cell fate determinants which generate two daughter cells differing in their content and potential (Horvitz and Herskowitz, 1992; Venkei and Yamashita, 2018). The AMso-to-MCM putative transdifferentiation in the *C. elegans* male could be an example of the latter mechanism (Sammut et al., 2015), which hereafter I will refer to when talking about ACD (as a real asymmetry in content during the division is observed). AMso cells are the amphid socket cells which are glial cells supporting neurons in the head of the worm. They are fully differentiated, expressing glial markers, and developed in a typical ring-like shape projection surrounding the distal ends of neurons contacting the environment (Altun, Z.F. and Hall, D.H. 2010. Nervous system, neuronal support cells. In *WormAtlas*). In the male, they divide maintaining their shape and with the mitotic spindle parallel to the polarity axis (suggesting that a cell intrinsic mechanism of ACD might be involved, see 3.2). The daughter cell maintaining the projections remains an AMso cell, while the one not inheriting them is converted into a male-specific neuron which the authors named MCM for “mystery cells of the male”. The MCM loses the glial markers and expresses pan-neuronal and neuronal terminal differentiation genes (Sammut et al., 2015). AMso-to-MCM process is a transdifferentiation event in agreement with the original criteria which are still accepted (Beresford, 1990; Eguchi and Kodama, 1993; Okada, 1986). Nevertheless, the mechanisms underlying this conversion, and the role, if any, of the cell division, are not characterised, apart from the fact that sex-determining pathways are involved cell-autonomously (Sammut et al., 2015). It would be interesting to investigate whether there is an involvement of factors required for Y-to-PDA transdifferentiation, where cell division does not take place, and of mechanisms regulating ACD at the same time. These questions were raised by our lab using other putative

transdifferentiation events involving a cell division, with a focus on one taking place in the rectum of the worm (it will be introduced at 4.3 and addressed in the Results).

To assess the role of ACD we need to know how it occurs. In this part I will describe what ACD is, how it is regulated and how it contributes to neurogenesis in different contexts (from *C. elegans* to mouse), as all the known and putative transdifferentiation events we have found give rise to neurons.

3.1 Definition and classification of asymmetric cell divisions

ACD is defined as any cell division which gives rise to two daughter cells with different fates (Horvitz and Herskowitz, 1992) (however, as said above, I will consider as ACD the divisions where an asymmetry is already observed during the division). It allows to generate cell diversity with a specific orientation in space thanks to the underlying mechanisms, which can both impact on the distribution of cell fate determinants and on the orientation of the mitotic spindle (Knoblich, 2001). Since the definition by H. R. Horvitz and I. Herskowitz in 1992, two different mechanisms have been widely accepted and described: an intrinsic mechanism (cell-autonomous) and an extrinsic mechanism (mediated by extracellular signals from environment) (Knoblich, 2001, 2008; Venkei and Yamashita, 2018) (Figure 9). Cell intrinsic mechanisms of ACD are described during early development, while more flexible cell extrinsic mechanisms are prevalent in later development and in the adult, especially in the stem cell

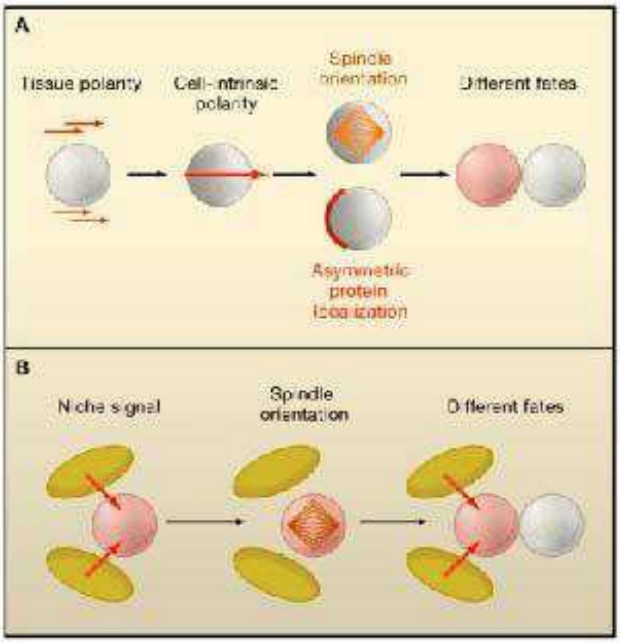

Figure 9. Classification of ACDs. A) Cell intrinsic mechanisms rely on cell intrinsic polarity. B) Cell extrinsic mechanisms rely on polarisation cues from the environment which polarise the dividing mother cell (contact with other cells or polarised signalling molecules) (Knoblich, 2008).

niche (Knoblich, 2008; Sawa, 2010). In some cases, a combination of the two mechanisms can be observed, like in *Drosophila* neurogenesis (Chen et al., 2016; Hawkins and Garriga, 1998). Indeed, the main contributions in this field were possible thanks to studies in invertebrates, *C. elegans* and *Drosophila* (Knoblich, 2010). The classification of ACD in cell intrinsic or cell extrinsic is more complicated for mammalian neurogenesis and this process will be considered in a separated chapter.

When describing cell intrinsic or cell extrinsic mechanisms of ACD, the terminology used might generate confusion, especially the concepts of cell polarity, polarity of cell division (often used in *C. elegans* literature on ACD) and orientation of cell division. Cell polarity is the asymmetric localisation of cellular components inside a cell (from Nature.com) including cytoplasmic determinants and cortex components; polarity of cell division instead refers to the relative position of two daughter cells along the body axis after ACD (as used by Herman and Horvitz, 1994 and Whangbo et al., 2000); finally the orientation of cell division must not be confused with the polarity of cell division because it concerns the spindle orientation and absolute position of the daughter cells after the division, not their respective positions considering their identity.

I will first describe the mechanisms involved in intrinsic cell polarity; secondly, I will consider cell extrinsic mechanisms of ACD; next, I will describe ACD in the context of mammalian cortical neurogenesis; finally, I will conclude with the mechanisms controlling mitotic spindle orientation in the context of ACD.

3.2 Cell intrinsic mechanisms of asymmetric cell division

Cell intrinsic mechanisms of ACD rely on the polarisation of the mother cell with asymmetric distribution of segregating determinants and on the orientation of the mitotic spindle in order to asymmetrically segregate the cell fate determinants between the two daughter cells (Knoblich, 2008). The relationship between the polarity axis and the spindle orientation determines whether the cell division will be symmetric or asymmetric (Matsuzaki and Shitamukai, 2015). The conserved proteins required for establishing cell polarity are the partitioning defective (PAR) proteins, discovered in *C. elegans* but necessary for cell polarity also in *Drosophila* and mammals. They are involved in apicobasal cell polarity and usually regulate ACD along the apicobasal axis (Morin and Bellaïche, 2011). PAR proteins directly link cell polarity and spindle orientation since they interact both with cell fate determinants and with proteins required for spindle positioning and generation of pulling force (Morin and Bellaïche, 2011).

By studying the *C. elegans* zygote, six PAR proteins have been identified which function together to establish cell polarity. PAR-1 and PAR-4 are serine/threonine kinases; PAR-5 is a 14-3-3 family protein which can recognise phosphorylated serines and threonines; PAR-3 and PAR-6 could be scaffold proteins as well, containing PDZ domains; PAR-2 is a RING finger domain protein and thus might act in the ubiquitin pathway. Finally, the atypical protein kinase C (aPKC) is required together with PAR proteins to establish cell polarity. All these proteins but PAR-2 are conserved in animals (McCaffrey and Macara, 2012). In polarised cells, some PAR proteins are asymmetrically distributed: PAR-3 and PAR-6 are enriched in the anterior cortex, while PAR-1 and PAR-2 are found posteriorly. Interestingly, they can prevent each other from localising in the wrong pole of the cell. However, the mechanisms required for establishing the polarisation are not always completely understood especially in mammalian systems. On the other hand, they are described for the *C. elegans* zygote and *Drosophila* neuroblasts (Goldstein and Macara, 2007).

3.2.1 Cell polarity in epithelial cells

PAR proteins regulate cell polarity not only in cells that will divide asymmetrically, but more in general in some epithelial sheets throughout the animal kingdom (Armenti and Nance, 2012; Goldstein and Macara, 2007). Epithelial cells are polarised cells with an apical and a basolateral domain characterised by different molecular features, especially visible in the apical localisation of adherens and tight junctions (in *C. elegans* constituted by a unique structure referred to as apical junctions). PAR-3 (and PAR-6 in *C. elegans*) is localised at the apical surface and mediates the formation and maturation of apical junctions. Studies in the worm's intestinal epithelial cells showed that PAR-3 is required for the recruitment of apical junction proteins at the apical surface while PAR-6 is needed to control their maturation. Those proteins which segregate with PAR-3 are the E-cadherin HMR-1, the α-catenin HMP-1 and the β-catenin HMP-2 (not involved in the Wnt signalling pathway, but exclusively required for cell adhesion, Korswagen et al., 2000) (Armenti and Nance, 2012). In agreement with apical junction proteins being regulated by PAR proteins, cell polarisation does not seem to depend on them (McCaffrey and Macara, 2012). However, cell adhesion proteins like cadherins play a role in regulating spindle orientation in some contexts in *Drosophila* and mammals (Chen et al., 2016). In *C. elegans*, immediately basal to apical junction proteins there are other proteins required for maturation of apical junctions: the conserved DLG-1/Discs-large and AJM-1 which colocalise. Moreover, the Scribble orthologue LET-413 localises basolaterally and is required for restriction of the apical compartment. Thus, epithelial cells in *C. elegans* are characterised from the apical to the basolateral domain by the presence of PAR-6, PKC-3, PAR-3, HMR-1/E-cadherin, HMP-1/α-catenin and HMP-2/β-catenin at the most apical domain; DLG-1 and AJM-

1 at the basal region of the apical junctions; LET-413/Scribble and LGL-1/Lgl at the basolateral domain (Armenti and Nance, 2012). While in *Drosophila* Lgl contributes to the basolateral domain by targeting Par-6, LGL-1 in *C. elegans* is not required for polarisation (Armenti and Nance, 2012; Goldstein and Macara, 2007).

3.2.2 Cell polarity in asymmetric cell division

As pointed out previously, cell polarity is key for ACD. The *C. elegans* zygote is one of the best characterised examples of this process (Figure 10). The somatic and germ cell lineages are segregated apart since the first cell division after fertilisation in *C. elegans*, starting with AB blastomere and P1 blastomere, respectively. Thus, the cytoplasmic determinants that are initially symmetrically distributed in the oocyte need to become polarised after fertilisation and to be asymmetrically partitioned between the sister cells (Campanale et al., 2017). The polarisation (in this case anteroposterior) is stimulated by the entry of the sperm into the oocyte, which provides centrosomes and a RhoGAP (CYK-4) that in turn will affect PAR proteins and cell fate determinants' distribution. At the entry site, which will become the posterior pole, the sperm centrosome recruits PAR-1 and PAR-2 to the cortex and CYK-4 converts the Rho monomeric G protein (CDC-42) in a GDP-bound inactive form inhibiting myosin. Because of this inhibition, the actomyosin meshwork in the cell cortex contracts away from the posterior together with the associated PAR-3/PAR-6/aPKC, establishing their anterior localisation. The maintenance of this polarisation is achieved through two different kinases,

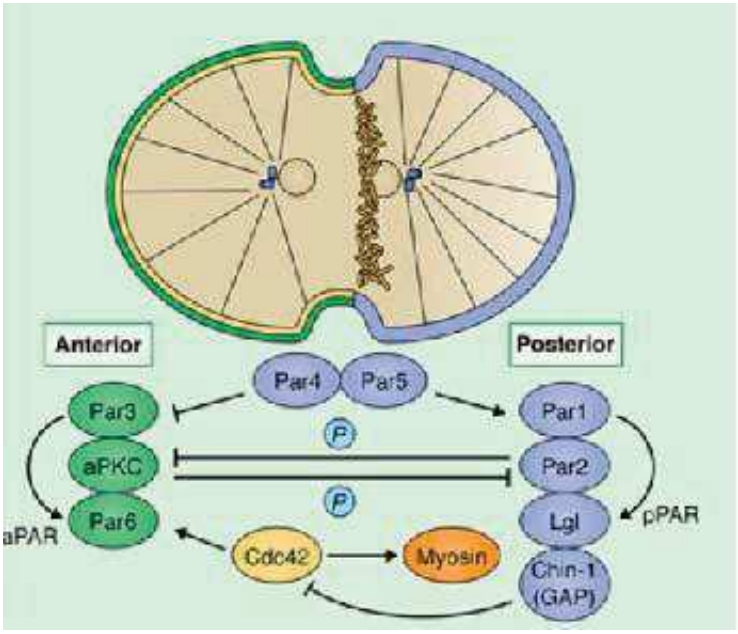

Figure 10. The ACD of *C. elegans* zygote requires PAR proteins. PAR proteins asymmetrically localise to the cell cortex and their distribution is reciprocally controlled through post-translational modifications (phosphorylation) (Campanale et al.; 2017).

aPKC at the anterior pole and PAR-1 at the posterior pole which phosphorylate target proteins to avoid their local cortical association: aPKC phosphorylates PAR-1 and PAR-2 at the anterior pole, while posteriorly PAR-2 recruits PAR-1 to phosphorylate PAR-3 excluding it from this domain (Campanale et al., 2017; Goldstein and Macara, 2007; McCaffrey and Macara, 2012). The cortical flow caused by the sperm entry and the interaction with asymmetrically distributed PAR proteins also allows asymmetrical partitioning of cell fate determinants. MEX-5, required for the anterior AB fate, is phosphorylated posteriorly by PAR-1 which probably affects its association with actin. At the same time actin movements towards the anterior allow accumulation of MEX-5 at this pole. On the contrary, PIE-1 is associated with P granules, a marker of *C. elegans* germline, which are segregated posteriorly in a PAR protein-dependent manner (Knoblich, 2010). Other cell fate determinants known for AB blastomere are MEX-3, MEX-6 and GLP-1, while for P1 blastomere SKN-1, PAL-1, MEX-1 and POS-1 have been identified (Knoblich, 2001).

In *Drosophila* neuroblasts (stem cell-like progenitors) the mechanisms at play are similar. For instance, type I neuroblasts divide asymmetrically giving rise to one neuroblast, which self-renews, and one ganglion mother cell (GMC), which terminally divides forming two neurons (Knoblich, 2010). The neuroblasts have an apicobasal polarity which requires the PAR proteins but also Lgl (Lethal (2) giant larvae), first described in this organism. These cortical factors are responsible of the asymmetric partitioning of segregating determinants such as Numb, Prospero and Brat. They localise at the basal side of the neuroblast and thus are inherited by the GMC which can leave the stem cell niche and generate two differentiating neurons (Knoblich, 2008).

In conclusion, these examples provide evidence for a conserved mechanism of PAR-mediated intrinsic ACD. However, some studies suggest that even in these cases some extrinsic cues might be required. For instance, while *Drosophila* neuroblasts have been considered an example of cell intrinsic ACD (Knoblich, 2001), it was shown that extrinsic cues are needed to polarise PAR proteins and orient the cell division axis (Siegrist and Doe, 2006 and see below). Indeed, the entry of the *C. elegans* sperm into the oocyte could also be considered a source of cues (Goldstein and Macara, 2007). This consideration would exclude the existence of purely cell intrinsic ACDs.

As cells are always in contact with other cells or the extracellular matrix in multicellular organisms, cell extrinsic mechanisms are widely adopted which both regulate cell fate and orient cell division. Several cell extrinsic ACDs occur during both development and adult tissue homeostasis in the stem cell compartments.

3.3 Cell extrinsic mechanism of asymmetric cell division

Extrinsic cues including cell-cell contacts can instruct different cell fates in sister cells and regulate spindle positioning (Knoblich, 2008; Werts and Goldstein, 2011). The signalling pathway which is able to couple cell identity and cell position in different contexts in several organisms is the conserved Wnt signalling pathway in all its variants (Loh et al., 2016), but other pathways can also be involved.

An example of ACD directed by extrinsic cues different from Wnt is the division of GSCs in the *Drosophila* ovary, already introduced previously for its remarkable plasticity in regenerating stem cells after their loss (see 1.3.2.1). In the germarium of the ovary, GSCs are in contact with two types of somatic cells called cap cells and escort stem cells. These cells form the stem cell niche of the ovary. Adherens junctions between GSCs and cap cells are required for orienting the spindle perpendicular to the contact site. At the same time the somatic cells of the ovary produce BMP ligands and probably other unidentified signals which activate a signalling cascade leading to repression of *Bam* gene in the cell that receives the signal (which remains in contact with the niche) and its activation in the cell that does not receive the signal anymore. The cell which loses the contact with the niche and activates *Bam* expression starts the differentiation to become a cystoblast, which ultimately gives rise to the oocyte with its supporting cells (Knoblich, 2008; Werts and Goldstein, 2011).

I will focus on WNT-mediated ACDs in the next paragraphs. These include ACDs regulated by the Wnt/β-catenin asymmetry pathway in *C. elegans* (a canonical, TCF-dependent pathway) and by the Planar Cell Polarity (PCP) pathway in *Drosophila* (a non-canonical, WNT dependent but TCF-independent pathway).

3.3.1 The Wnt signalling pathway and asymmetric cell division in *C. elegans*

The Wnt signalling pathway, especially a *C. elegans*-specific variant, drives several ACDs both during embryonic and post-embryonic larval development of the worm. For instance, a WNT ligand acts as an external cue to orient the ACD of the EMS blastomere during early development (4-cell stage) and of the T blast cell in the L1 larvae (Mizumoto and Sawa, 2007).

3.3.1.1 *Components and variants of the Wnt signalling pathway in C. elegans*

C. elegans has five different WNT ligands (EGL-20, LIN-44, MOM-2, CWN-1, CWN-2) and four different Frizzled receptors (LIN-17, MOM-5, MIG-1, CFZ-2), which are required in different cell types at different developmental stages, while it lacks LRP5/6 coreceptor (Cravo and van den Heuvel, 2020; Jackson and Eisenmann, 2012). The intracellular components of the

pathway include, like in other organisms, Dishevelled (DSH-1, DSH-2 and MIG-5), the β-catenin destruction complex composed by axin (AXL-1 and PRY-1), glycogen synthase kinane-3β (GSK-3), APC (APR-1) and CK1α (KIN-19), three different β-catenins (BAR-1, SYS-1 and WRM-1, a fourth one called HMP-2 is not involved in the pathway) and a single TCF homologue (POP-1). These components are not all always required, some show redundancy, and this might depend on the variant of the pathway activated or on the specific cellular context (Jackson and Eisenmann, 2012).

The *C. elegans* Wnt signalling pathways can be classified in two main groups like in other animals: the canonical Wnt pathways and the non-canonical Wnt pathways. The difference between them is the presence in the former and the absence in the latter of a transcriptional output (Figure 11) (Gómez-Orte et al., 2013; Phillips and Kimble, 2009). While in the canonical pathways the WNT ligand binds to its transmembrane FZD receptor and activates an intracellular signalling cascade which culminates in transcriptional activation (or repression), the non-canonical pathways do not require a signalling into the nucleus, but act directly on the cytoskeleton to regulate spindle positioning. Thus, the different pathways share some upstream components like WNT ligands, FZD receptor, DVL and the destruction complex, but the transcriptional activator and the β-catenins (excluding WRM-1) downstream are only part of the canonical pathways (Gómez-Orte et al., 2013; Schlesinger et al., 1999). Thanks to the upstream components, some of the pathways can be activated in parallel in some cellular contexts, coupling cell fate specification and oriented cell division (Cravo and van den Heuvel, 2020; Loh et al., 2016).

In the worm two canonical pathways have been described, which are required in different cells and with different functions. These pathways are characterised by the involvement of different β-catenins regulated through different mechanisms. The canonical Wnt/β-catenin pathway, which requires *bar-1*/β-catenin, functions as in other metazoans: the binding of WNT to FZD leads to the inhibition of the β-catenin destruction complex, stabilisation of BAR-1/β-catenin and its translocation into the nucleus where it binds to the *C. elegans* TCF homologue POP-1 resulting in transcriptional activation of Wnt-responsive genes. This pathway regulates few cell fate decisions during development and Hox genes are among its known targets: *lin-39* in vulval precursor cells (VPCs) and *egl-5* in P12 cell both required for fate specification, *mab-5* in QL neuroblast required for proper cell migration (Jackson and Eisenmann, 2012; Phillips and Kimble, 2009).

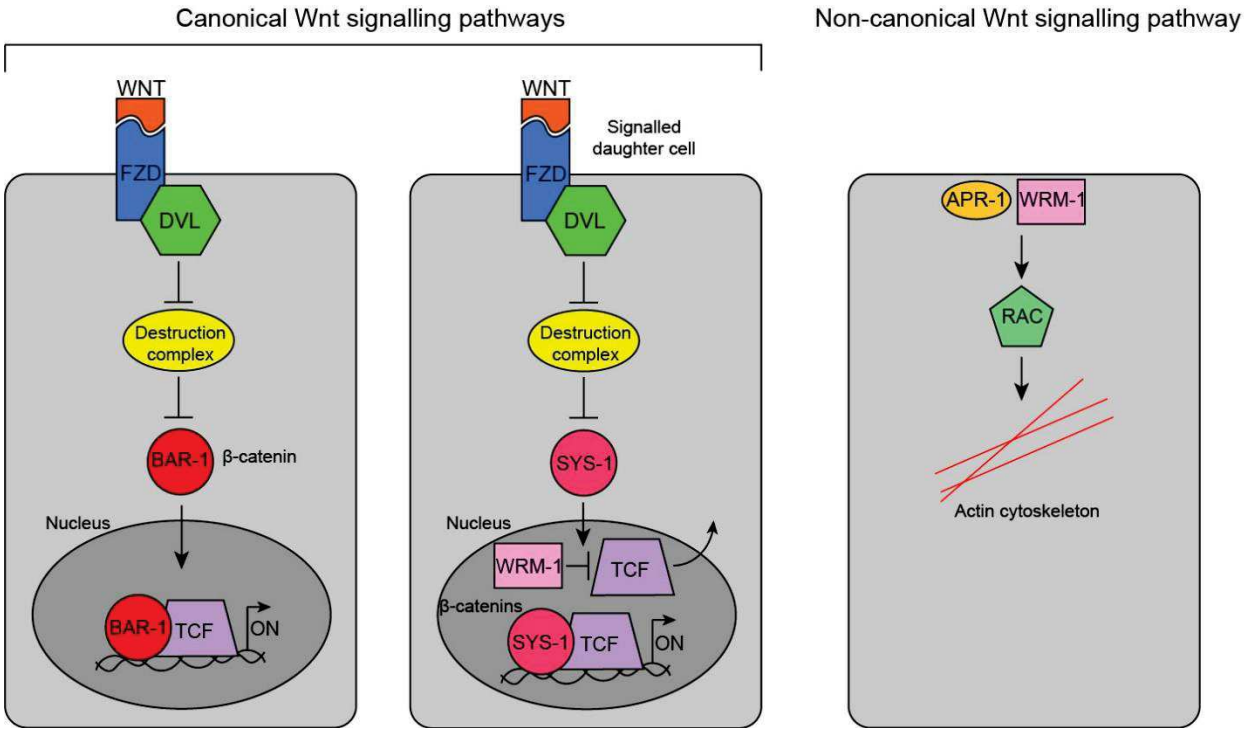

Figure 11. Variants of the Wnt signalling pathway in *C. elegans*. On the left, the canonical and conserved Wnt signalling pathway; in the middle, the canonical, *C. elegans*-specific Wnt/β-catenin asymmetry pathway; on the right, the non-canonical pathway acting in the cytoplasm on the actin cytoskeleton. The last two are involved in ACD with different functions.

The other canonical pathway is the *C. elegans*-specific variant regulating ACDs along the anteroposterior axis (Bertrand, 2016) which appears to be involved in cell fate decisions during development more often than the Wnt/β-catenin pathway does (Phillips and Kimble, 2009). Due to its role in ACD, this pathway was termed Wnt/β-catenin asymmetry pathway and two different β-catenins are involved, encoded by *sys-1* and *wrm-1* genes (Kidd et al., 2005; Rocheleau et al., 1997), with different roles (Mizumoto and Sawa, 2007; Phillips and Kimble, 2009; Robertson and Lin, 2012). In this case, the mother cell before cell division receives a polarised signal which leads to asymmetrical distribution of FZD and DVL at its membrane, at the side where the signal comes from. Daughter cells are already distinct at birth: the signalling cascade is activated only in one daughter cell (usually the posterior one), where FZD was localised. WRM-1/β-catenin is exported from the anterior cell nucleus thanks to APR-1-mediated spindle asymmetries, while it accumulates in the posterior cell nucleus where it binds to POP-1 which is consequently phosphorylated by the Nemo-like kinase LIT-1. Phosphorylated POP-1 is exported from the posterior cell nucleus, leading to an asymmetry in the nuclear POP-1 concentration between the two daughter cells. In parallel, SYS-1 (a functional homologue of human β-catenin, Kidd et al., 2005) is stabilised in the posterior cell through inhibition of the destruction complex, translocates into the nucleus and binds to the free POP-1 fraction present in the nucleus converting it from a transcriptional repressor to an

activator. The final output of these different cascades between the anterior (non-signalled) and the posterior (signalled) daughter cell is the acquisition of two different cell identities thanks to the different partners of POP-1 and thus its different nuclear activities (Bertrand, 2016; Jackson and Eisenmann, 2012; Koonyee Lam and Phillips, 2017; Phillips and Kimble, 2009; Robertson and Lin, 2012). At the same time, WNT ligands were shown to orient the spindle of the mother cell independently of transcription (Schlesinger et al., 1999) through the asymmetric localisation of FZD (Goldstein et al., 2006) in a non-canonical Wnt signalling pathway (shown in EMS cell, early embryo; see 3.3.1.2). Importantly, components of this pathway, but not of the PCP pathway, orient cell polarity in the cells where the Wnt/β-catenin asymmetry pathway is activated (Mizumoto and Sawa, 2007). The mechanisms directly linking the Wnt signalling pathway components to the spindle positioning machinery are still not well understood (Cravo and van den Heuvel, 2020).

The Wnt/β-catenin asymmetry pathway is involved in the ACD of EMS blastomere, T cells (the two best characterised, Bertrand, 2016), VPCs (though with a very characteristic mechanism, Green et al., 2008), somatic gonadal precursors Z1 and Z4 cells (independently of WNT ligands, Sawa and Korswagen, 2013), and of the other seam cells (Mizumoto and Sawa, 2007). It has been shown that WNT signal acts as a positional instructive cue in EMS, T (Goldstein et al., 2006), V5 cell division (Whangbo et al., 2000) and VPCs division (Green et al., 2008), while in the other seam cells (H0, H1, H2, V1-4 and V6) WNT ligands seem to have a permissive role (Yamamoto et al., 2011; reviewed by Sawa and Korswagen, 2013). The experimental results leading to this conclusion in seam cells might be explained by the expression of WNT antagonist *sfrp-1* (Cravo and van den Heuvel, 2020). Concerning the impact of WNT on the orientation of cell division, it is not always evident probably due to the fact that it acts redundantly with other factors (like cell-shape and extrinsic tension; di Pietro et al., 2016; Wildwater et al., 2011). Indeed spindle orientation is also affected by cell-contact polarisation mechanisms (Koonyee Lam and Phillips, 2017), as described for the oriented cell division of the AB blastomere (Sugioka and Bowerman, 2018) (see 3.5 for spindle orientation mechanisms).

In the next paragraphs I will describe three examples of ACD regulated by the Wnt/β-catenin asymmetry pathway: the first during early embryogenesis, the second during larval development and the third during terminal differentiation of a neuronal subtype during embryogenesis. The different outputs of the activation of the Wnt/β-catenin asymmetry pathway in several cellular contexts and developmental stages will highlight an important principle: the Wnt signalling effectors integrate with cell type-specific transcriptional regulators to target different genes and specify different cell types (Sawa, 2010). Thus, the same general process, the Wnt/β-catenin asymmetry pathway ACD is reused in several cellular contexts to

differently contribute to cell fate specification. This conclusion is consistent with what is observed in the context of cellular reprogramming, where reprogramming transcription factors integrate with signalling pathways activated thanks to neighbouring cells or the cell culture medium.

3.3.1.2 *The Wnt pathway in EMS asymmetric cell division*

The division of EMS blastomere is one of the best characterised and the first cell division during *C. elegans* development (at 4-cell stage) which requires the Wnt/β-catenin asymmetry pathway (Koonyee Lam and Phillips, 2017) (Figure 12). EMS division gives rise to an anterior daughter, MS, that contributes to mesoderm, and a posterior daughter, E, that contributes to endoderm (Thorpe et al., 2000). The WNT ligand is MOM-2, which is produced by the posterior neighbour P_2 cell and polarises EMS division, and the FZD receptor is MOM-5. Downstream, two DVL (DSH-2 and MIG-5), destruction complex components and SYS-1, WRM-1, LIT-1 and POP-1 are required. In the anterior daughter MS (non-signalled), POP-1 acts as a transcriptional repressor, by interacting with cofactors, and prevents the expression of endoderm genes such as *end-1* and *end-3*; conversely, in the posterior daughter E, POP-1 binds to SYS-1 and

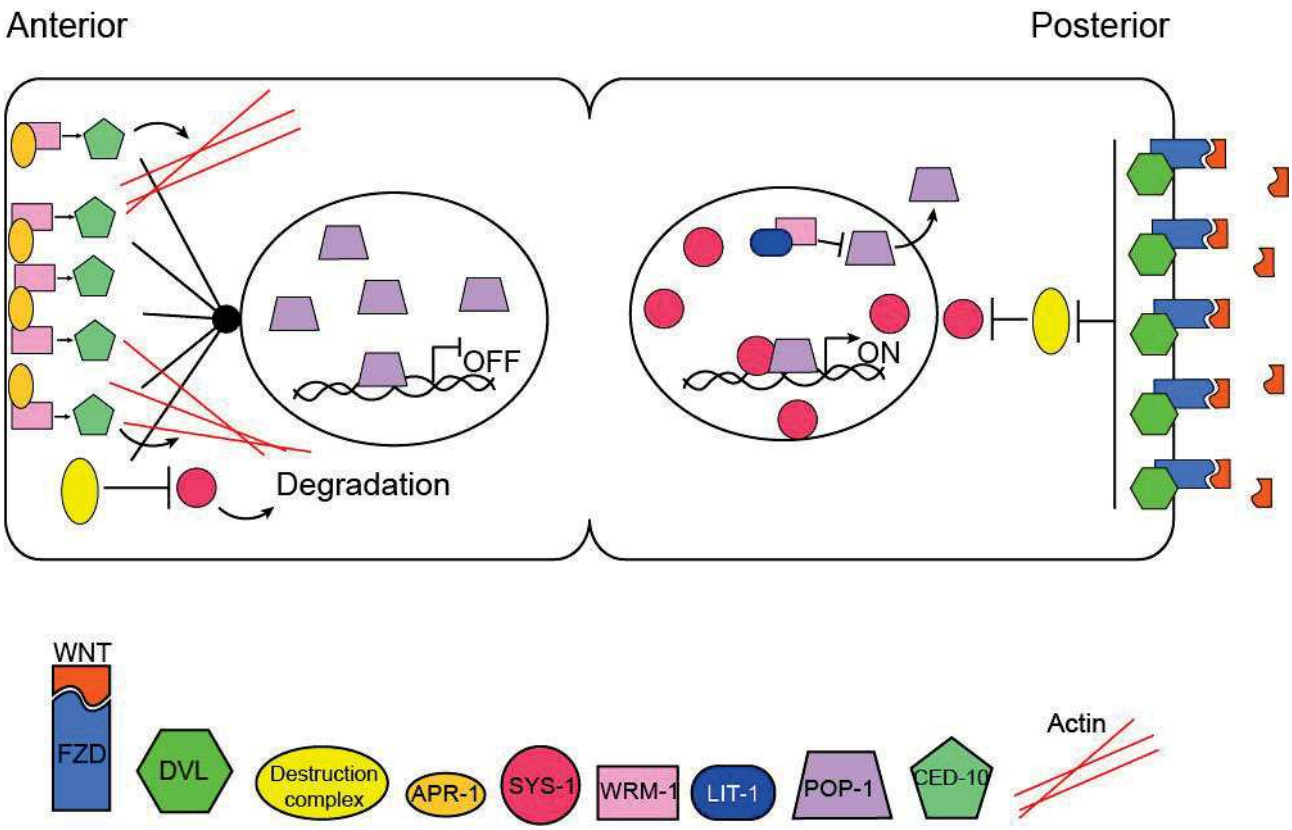

Figure 12. The Wnt/β-catenin asymmetry pathway and the non-canonical Wnt signalling pathway drive ACD as described for EMS blastomere. The two pathways share some components and work in parallel leading to different transcriptional outputs in the anterior and posterior nuclei and regulating the orientation of the mitotic spindle.

activates the expression of the endoderm genes (Bertrand, 2016; Jackson and Eisenmann, 2012). The origin of the WNT ligand determines the polarity of cell division (i.e. which cell will acquire MS identity and which E identity; Goldstein et al., 2006). In parallel, the non-canonical pathway uses the positional information provided by MOM-2 to orient the mitotic spindle through asymmetric localisation of FZD at the posterior side of the cell (Schlesinger et al., 1999). However, the Wnt pathway alone is not enough for spindle orientation and MES-1 receptor upstream to Src kinase is also involved, in a permissive way. MES-1 pathway was shown to mediate cortical recruitment of Pins (part of a spindle positioning complex, see 3.5) at the contact site between EMS and P_2. Loss of Pins or their associated Gα protein GPA-16 results in spindle orientation defects (Werts and Goldstein, 2011). Finally, LIN-5/NuMA and LET-99/DEPDC (DEP domain containing), which controls Pins and NuMA localisation, are required for EMS spindle positioning (Liro and Rose, 2016).

3.3.1.3 *The Wnt pathway in T cell asymmetric cell division*

Another well characterised cell division where the Wnt/β-catenin asymmetry pathway is involved is the T seam cell division during larval development. The mechanisms at play in EMS division are also observed in this larval blast cell (Bertrand, 2016), even though studies on the spindle orientation are missing (probably due to technical limitations such as the smaller cell size of T cells and the impossibility of *in vitro* studies like the ones performed with EMS). In T cell division the WNT ligand involved is LIN-44, produced by hypodermal cells of the worm's tail (Harterink et al., 2011; Herman et al., 1995), and the FZD receptor is LIN-17 (Herman, 1994; Sawa et al., 1996). In absence of *lin-44/WNT* the polarity of cell division (i.e. the relative position of the two different daughter cells along the AP axis) is often reversed while mutations in *lin-17/FZD* gene mostly cause loss of polarity of T cell division (i.e. symmetric cell division: same cell identity of the daughter cells) (Herman, 1994). Later, *sys-1/β-catenin*, *wrm-1/β-catenin* and *pop-1/TCF* were also found implicated in T cell ACD (reviewed by Bertrand, 2016). Two genes responding to the Wnt signalling pathway in this ACD were identified: *tlp-1*, which was shown to be genetically downstream (Zhao et al., 2002), and *psa-3*, which was shown to be directly regulated by POP-1 through POP-1 binding to its promoter (Arata et al., 2006). They are both upregulated in the posterior daughter (the signalled one) and encode transcription factors which might act as cell fate determinants. Interestingly, it was shown that Wnt signalling is not enough to activate *psa*-3 expression if the Hox gene *nob-1* and the Pbx family member *ceh-20* are mutated. Those two transcription factors also binds to *psa-3* regulatory regions and together with Wnt can cooperatively activate its transcription (Arata et al., 2006).

3.3.1.4 *The Wnt pathway in AIY neuronal lineage*

The Wnt/β-catenin asymmetry pathway also regulates ACDs which lead to terminal cell differentiation (Bertrand and Hobert, 2009; discussed by Sawa, 2010). This was described in the context of terminal differentiation of some neurons during embryogenesis where ACD of a neuroblast precursor often takes place, giving rise to two different types of neurons. Thus, the Wnt/β-catenin asymmetry pathway was shown to be required for AIY and AIN interneurons and for ASER sensory neuron terminal differentiation. For AIY generation, the activation of the pathway is required together with TTX-3 transcription factor, already expressed in the mother cell, to activate the expression of *ceh-10* terminal selector just after cell division in the posterior daughter. Interestingly, the authors observed that the asymmetries of POP-1 and SYS-1 in the nuclei of the sister cells are present just after division, when the pathway activates *ceh-10*, but are rapidly lost. Moreover, the expression of *ceh-10* and *ttx-3*, required to establish and maintain AIY identity, is sustained through autoregulatory loops, suggesting that Wnt signalling is needed transiently to drive the asymmetry between AIY and its sister SMDD neuron (Bertrand and Hobert, 2009). This study also suggested a new molecular mechanism of the Wnt/β-catenin asymmetry pathway: in the mother of AIY neuron *ttx-3* gene is expressed thanks to this pathway, however AIY mother is the anterior daughter of an ACD, in which high levels of nuclear POP-1 were traditionally considered responsible for transcriptional repression, not activation. Further investigation demonstrated that the capacity of POP-1 to activate transcription of a terminal selector in the anterior daughter is due to its interaction with other

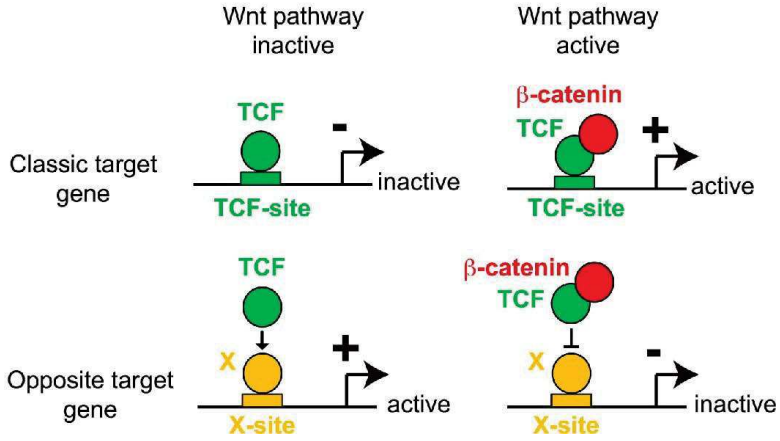

Figure 13. Model for TCF-mediated transcriptional regulation in the anterior and posterior nuclei after ACD regulated by Wnt/β-catenin asymmetry pathway in *C. elegans*. This model, proposed by V. Bertrand and colleagues, suggests that in the anterior daughter (non-signalled) high nuclear TCF represses genes that are instead activated in the posterior nucleus by TCF bound to β-catenin (those genes are defined as classical target as their regulation was the first described); on the contrary, genes that need to be activated in the anterior daughter, and not in the posterior, require the presence of additional transcription factors (depending on the cellular context) which drive transcription in presence of high TCF and cannot activate it in presence of β-catenin-bound TCF (Murgan et al., 2015).

transcription factors, in the case of AIY neuron with REF-2 (Murgan et al., 2015). Thanks to these findings, S. Murgan and V. Bertrand proposed a model (Figure 13) to explain how the Wnt/β-catenin asymmetry pathway may activate and repress different genes in the anterior and in the posterior daughters: in the former, POP-1 alone acts as a repressor while by interacting with other transcription factors it might act as an activator; in the latter, POP-1 bound to SYS-1/β-catenin is a transcriptional activator, whereas repression of genes that need to be silenced might be achieved indirectly through POP-1/SYS-1-mediated activation of a repressor or even directly through the formation of a POP-1/SYS-1/other factor complex incapable of activating transcription (Murgan and Bertrand, 2015).

3.3.2 The Wnt/PCP pathway in *Drosophila* SOP cells asymmetric cell division

In *Drosophila*, intercellular signalling mediated-ACD occurs in the sensory organ precursor (SOP) cells, during peripheral nervous system development (Werts and Goldstein, 2011) (even though this ACD was considered before an example of intrinsic ACD, Knoblich, 2008). The PCP pathway is involved in this context. This pathway relies on some Wnt signalling pathway components, such as Frizzled (Fz in *Drosophila*) receptor and Dishevelled (Dsh in *Drosophila*) signalling effector, which translate the signal into cell polarisation involving asymmetric localisation of Fz and Dsh. The signal consists in Strabismus/Van Gogh (Vang) protein localised in the membrane of neighbouring cells (Werts and Goldstein, 2011). Fz and Dsh asymmetric localisation affect polarisation of the segregating determinant Numb thanks to PCP pathway component Flamingo (Fmi), downstream to Fz. Fz and Dsh asymmetry also mediate spindle orientation in SOP cells, as Dsh was shown to physically interact with Mud/Dynein complex (Dewey et al., 2015; Gillies and Cabernard, 2011).

3.4 Mammalian cortical neurogenesis as an example of asymmetric cell division in vertebrates

The study of mammalian neurogenesis, especially cortical neurogenesis, has contributed to the understanding of ACD in vertebrates, even though the mechanisms at play are not as clear as in invertebrates. During subsequent developmental stages, switches between symmetric (proliferative stage) and asymmetric cell divisions (neurogenic stage) of neural stem cells (NSCs) occur to form the brain (Knoblich, 2008; Matsuzaki and Shitamukai, 2015). The term NSCs refers to a heterogeneous cell population sharing the capacity to self-renew and give rise to neurons and several glial cell types (astrocytes, NG2 glia, oligodendrocytes, ependymal cells) through ACD. The extent of their self-renewal capacity can vary and for this reason NSCs

are often called neural stem/progenitor cells or even just neural progenitors (Taverna et al., 2014).

During embryonic development, NSCs arise from epithelial cells of the neural plate starting from day E9 in mouse and express NSC markers while retaining some epithelial features (such as an apical and a basal domain). These neuroepithelial cells initially divide symmetrically to expand the stem cell pool. Later, at days E10 and E11, some neuroepithelial cells gain glial cell features, becoming radial glia cells, and start to divide asymmetrically to self-renew and differentiate. Differently from neuroepithelial cells, radial glia cells express astrocyte-specific glutamate receptor (GLAST) and the brain lipid binding protein (BLBP) (Knoblich, 2008). However, they are not glial cells and their name was attributed due to their radial morphology before the discovery of their self-renewing capacity. Radial glia cells are considered as neural stem/progenitor cells. Self-renewal of radial glia cells depends on the Notch signalling pathway and differentiating cells express Delta, thus allowing a crosstalk between neighbour cells that self-renew and cells that differentiate (Matsuzaki and Shitamukai, 2015). Interestingly, the function of radial glia cells as neural progenitor cells is also described in fish and is responsible of regeneration after brain injury (a capacity that however is missing in the mammalian brain) (Kroehne et al., 2011); in Zebrafish, it was also shown by live imaging experiments that NSCs can convert into differentiated neurons even without dividing (Barbosa et al., 2015).

NSCs required for cortical development localise at the apical surface of the neuroepithelium in contact with the ventricle (the ventricular zone, VZ) and the cerebrospinal fluid (CSF). They have an apicobasal polarity dependent on PAR proteins with a narrow apical membrane compared to other apicobasal polarised cells and a basal domain which includes the VZ-basolateral plasma membrane and the distal segment of the basal process. The apical domain becomes narrower during neurogenesis and has a characteristic apical plasma membrane containing receptors for signalling molecules dispersed in the CSF, a primary cilium usually with the “old” centrosome inherited from the mother cell and apical junctional complexes which also play a role in the response to signalling cues. For instance, β-catenin interacts with cadherins at these junctions (Matsuzaki and Shitamukai, 2015; Taverna et al., 2014).

The impact of the orientation of cell division in NSC is less sharp than in *Drosophila* and *C. elegans* and segregating determinants have not been identified yet. Vertical divisions of apical radial glia cells, with the cleavage furrow perpendicular to the axis of the epithelium, lead to symmetric cells division, while horizontal divisions, which in theory would lead to ACD, are rarer than expected based on the occurrence of ACD. In many cases, the orientation of the spindle is oblique and cleavage angles are variable in radial glia cells (Knoblich, 2008; Matsuzaki and Shitamukai, 2015). Thus, a simple correlation between the angle of division

and the asymmetric cell fates of the daughter cells is missing (Knoblich, 2008). What we know is that the different fates of the daughter cells depend on the distribution of the basal process (Figure 14). In symmetrically dividing radial glia cells the spindle is oriented parallel to the ventricular plane thanks to the conserved spindle positioning complex proteins, which in this case do not localise with PAR proteins, and both daughters inherit all the domains. The differential inheritance of the narrow apical domain does not determine the different fates of the daughter cells, as this domain was shown to be asymmetrically partitioned only in 10-20% of the divisions and ACD occurs more frequently. On the contrary, the asymmetric segregation of the basal process during more oblique divisions leads to the formation of one daughter cell inheriting the apical domain which differentiates and the other inheriting entirely the basal process which remains capable of self-renewal. This cell becomes a basal radial glia cell, localised in the subventricular zone (SVZ), and plays an important role in neurogenesis in primates. In NSCs lacking the expression of genes involved in spindle orientation the position of the spindle is altered *in vivo* (Matsuzaki and Shitamukai, 2015). Huntingtin, mutated in Huntington's disease, is one of the proteins required for spindle orientation. In its absence, the cleavage plane of radial glia cell division is more oblique respect to the vertical axis than in wildtype, and even horizontal divisions can be observed. This defective cleavage plane impacts on the fate of the daughter cells with a bias towards neuronal differentiation and loss of the neural progenitors (Godin et al., 2010).

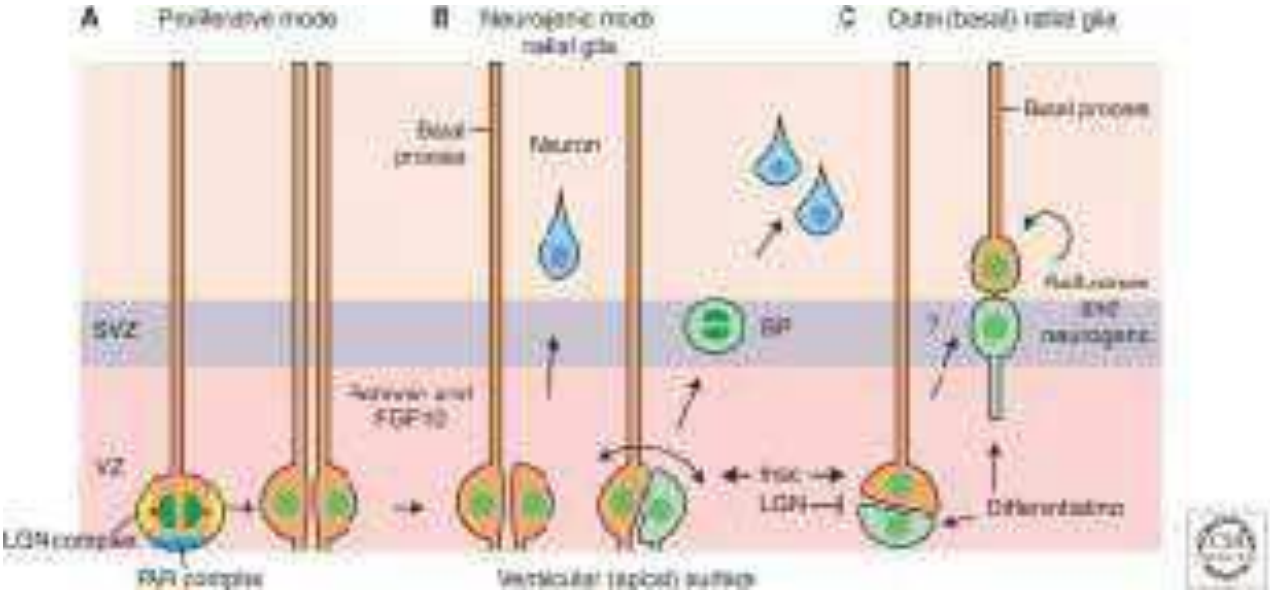

Figure 14. Division mode of radial glia cells of the VZ dictates the fate of the daughter cells. Division planes in the VZ are either vertical or oblique. There is no strict correlation between the division plane and the polarity of the division. Depending on how cellular components are segregated, in particular the basal process, the daughter cells acquire the same or different fates. Differently from other systems like *C. elegans* zygote, the LGN complex does not colocalise apically with PAR proteins (Matsuzaki and Shitamukai, 2015).

Several signalling pathways regulate neurogenesis together with Notch. Interestingly, the Wnt signalling pathway was shown to have different functions at different stages of neurogenesis. Thus, the canonical and non-canonical Wnt pathways can control self-renewal and proliferation of NSCs, but also regulate terminal differentiation of neurons. Strikingly, while in *C. elegans* the Wnt/β-catenins asymmetry pathway regulates ACD, in mouse adult neurogenesis it was shown to promote symmetric self-renewing divisions through autocrine signalling (Choe et al., 2016).

3.5 Common mechanisms for mitotic spindle orientation

Mitotic spindle orientation is a key mechanism to achieve proper ACD, especially cell intrinsic, where correct partitioning of cytoplasmic and cortical segregating determinants is required, and it was found to be regulated in many instances where cell fate choices and cell position must be coordinated. The regulation of the anchoring of astral microtubules to the cell cortex allows to orient the spindle (Morin and Bellaïche, 2011). The molecular complex which links the cell cortex to the microtubules is conserved and composed by the heterotrimeric Gαi protein (GOA-1 and GPA-16 in *C. elegans*), LGN (GPR1/2 in *C. elegans* and Pins, Partner of Inscuteable, in *Drosophila*) and NuMA (nuclear mitotic apparatus, LIN-5 in *C. elegans* and Mud, Mushroom body defect, in *Drosophila*) (Figure 15). Both cell intrinsic and cell extrinsic cues can recruit the spindle positioning complex to the cell cortex at specific positions (di Pietro et al., 2016).

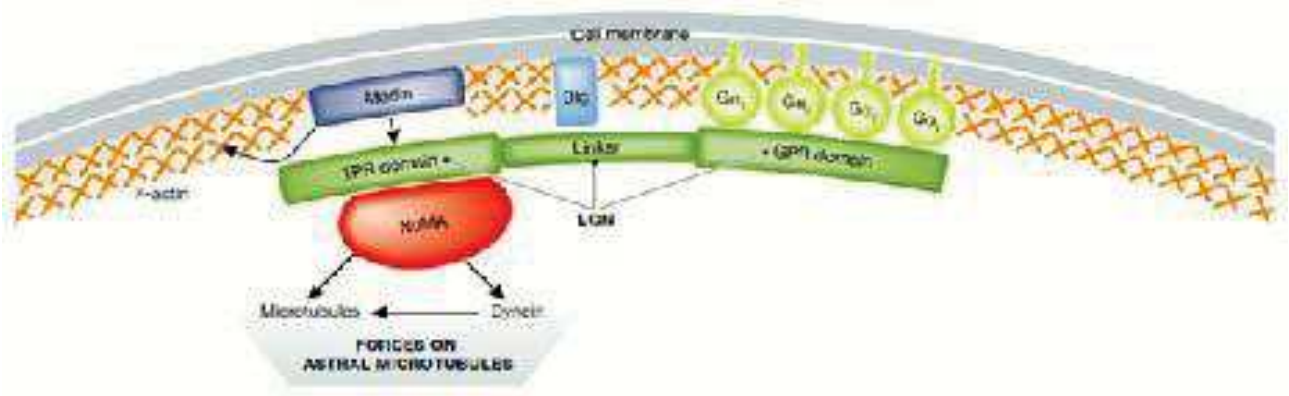

Figure 15. Representation of the conserved proteins localised at the cell cortex which regulate spindle positioning by directly acting on astral microtubules (Di Pietro et al., 2016).

When directed by cell intrinsic mechanisms, the spindle positioning complex often relies on PAR proteins, even though other mechanisms involving Dlg (Discs-large), Canoe/Afadin and Huntingtin have also been described in *Drosophila* and mouse (di Pietro et al., 2016). In *C.*

elegans zygote, the differential distribution and activity of PAR proteins lead to posterior cortical enrichment at anaphase of GPR-1 and GPR-2. These proteins interact through their GoLoco domains with the Gα proteins GOA-1 and GPA-16 which are anchored to the plasma membrane and maintained in their GDP-bound active form. Association of Pins to Gα proteins allows the interaction of Pins with LIN-5, and drives the cortical localisation of dynein-dynactin complex, which by acting directly on microtubules orients the spindle and generates pulling forces (Goldstein and Macara, 2007; Morin and Bellaïche, 2011). Phosphorylation of LIN-5 by different kinases at different residues regulates its interaction with Pins and the dynein motor (Portegijs et al., 2016). Loss of GPR-1, GPR-2, Gα proteins or LIN-5 misorients the mitotic spindle in *C. elegans* zygote (Gotta and Ahringer, 2001; Lorson et al., 2000; reviewed by Siller and Doe, 2009). Recently it was clearly shown using optogenetics that while Pins and Gα proteins are required for spindle positioning, LIN-5 is required for the generation of pulling forces through dynein activation (Fielmich et al., 2018).

The same proteins function in *Drosophila* neuroblast division to orient the mitotic spindle and segregate Numb, Prospero and Brat. In this context, Par3 (also known as Bazooka in *Drosophila*) interacts with Inscuteable (Insc) which in turns recruits Pins, leading to their interaction with Gα proteins as it occurs in *C. elegans*. Two different pathways were identified in the neuroblasts downstream to Pins: one involves Mud and is equivalent to the *C. elegans* pathway involving LIN-5, while the other requires Dlg and the microtubule plus-end-directed kinesin heavy chain Khc73. This pathway is responsible of the so called “telophase rescue” which in absence of Mud allows a correct segregation of cell fate determinants according to the spindle orientation (Morin and Bellaïche, 2011; Siller and Doe, 2009).

As anticipated above (3.2.2), *Drosophila* embryonic neuroblast ACD requires an extrinsic signal from the underlying neuroectoderm even though it is often described as a typical case of cell intrinsic ACD. This signal is not identified yet, but the intracellular response requires Tre1 GPCR which recruits Pins that in turn recruits both Par3 and Mud, thus regulating cell polarity and spindle positioning in parallel (Yoshiura et al., 2012). The ACD of the EMS blastomere in *C. elegans* (mediated by the overlapping Wnt/β-catenin asymmetry pathway and the non-canonical pathway, see 3.3.1.2) and the ACD of the *Drosophila* SOP cells (which requires the PCP pathway, see 3.3.2) are other examples of spindle orientation mediated by non-cell autonomous mechanisms. In the first case a direct interaction of the Wnt pathway components with the spindle positioning complex has not been observed yet, while for the PCP pathway Dsh was shown to interact with Mud (Cravo and van den Heuvel, 2020). Interestingly, proper orientation of the mitotic spindle in EMS and AB.ar cell divisions in *C. elegans* requires *ced-10*/Rac which is downstream to FZD and regulates actin polymerisation (Cabello et al., 2010). Indeed, a role of actin cytoskeleton on spindle orientation is becoming more and more

evident in different contexts (di Pietro et al., 2016). In *C. elegans* it was recently shown that Wnt signalling asymmetrically activates myosin in EMS blastomere, while in earlier blastomeres physical contact between cells is the regulator of myosin flow anisotropy which drives spindle orientation (Sugioka and Bowerman, 2018). Thus, from different studies it seems that the actomyosin cytoskeleton, not only microtubules and microtubules-associated proteins, contributes to spindle orientation at least in early blastomeres (Cravo and van den Heuvel, 2020). Actin cytoskeleton is responsible of cell rounding during mitosis and interestingly the shape of the cell and its contacts with other cells and the environment has been shown to also play a role in spindle orientation (di Pietro et al., 2016).

In mammals, an example of spindle orientation mediated by environment is the division of mouse skin basal progenitors during mouse skin stratification. In this context the mitotic spindle acquires an apicobasal orientation, which depends on the presence of β1-integrin and α-catenin in the basement membrane (di Pietro et al., 2016). The loss of LGN or NuMA impairs apicobasal ACD leading to planar cell division (Gillies and Cabernard, 2011; Morin and Bellaïche, 2011).

In conclusion, intracellular polarity proteins (PAR proteins) and different extracellular cues, including the Wnt pathway in *C. elegans* and PCP pathways in *Drosophila* and vertebrates, regulate spindle orientation in different contexts.

4 *C. elegans*, a powerful model to study cell reprogramming *in vivo*

C. elegans offers many advantages for studying genes involved in cell reprogramming *in vivo*. First of all, the knowledge of its somatic cell lineage (Sulston and Horvitz, 1977; Sulston et al., 1983), its transparency and the possibility to generate transgenic lines with fluorescent reporters allow researchers to follow single cells *in vivo* (Corsi et al., 2015), overcoming one of the main issues in the identification of natural reprogramming events (the existence of a lineage relationship between cells before and after, see 2.3.1; Eguchi and Kodama, 1993). These features of *C. elegans* also allow to characterise cell identities as visible by cell morphology through Differential Interference Contrast (DIC) microscopy and by the expression of fluorescent reporters for key cell identity-marker genes. Moreover, its hermaphrodite nature, its short life cycle and more recently a well-annotated genome allowed to set up genetic screens to identify and clone genes (Corsi et al., 2015) required for the occurrence of these reprogramming events. Thus, our lab could identify several genes required for Y-to-PDA transdifferentiation (Kagias et al., 2012; Richard et al., 2011; Zuryn et al., 2014).

4.1 *C. elegans* basics and life cycle

C. elegans is a nematode worm which in nature lives in the soil and eats bacteria. In the lab is usually maintained on agar plates seeded with *Escherichia coli*, OP50 strain. Its developmental growth rate depends on the environment temperature, which can vary between 12°C and 25°C to allow the worm's viability. *C. elegans* exists in two different sexes: the self-fertilising hermaphrodite (characterised by two X chromosomes) and the male (with only one X chromosome, indicated as XO). The progeny of a self-fertilised hermaphrodite is usually composed by only hermaphrodites; males can arise from self-fertilised hermaphrodites with a frequency of 0.1-0.2%, due to meiotic non-disjunctions, or due to exposure to high temperatures. When a hermaphrodite and a male mate, the progeny is composed half by hermaphrodites and half by males (Corsi et al., 2015).

The *C. elegans* life cycle lasts 3.5 days at 20°C and includes an embryonic stage, four larval stages (L1, L2, L3, L4) and adulthood (Altun and Hall, 2009; Sulston and Horvitz, 1977) (Figure 16). The embryonic development begins after fertilisation in the hermaphrodite's body and the fertilised egg is laid at gastrula stage, when the embryo is made of around 24-30 cells. The embryo hatches with 558 nuclei and enters the L1 larval stage. The other larval stages follow, separated by four molts, until the sexually mature adult has developed (Corsi et al., 2015). During larval development more cell divisions occur, many of which are required to form the reproductive organs in both sexes (Sulston and Horvitz, 1977; Sulston et al., 1980). The final

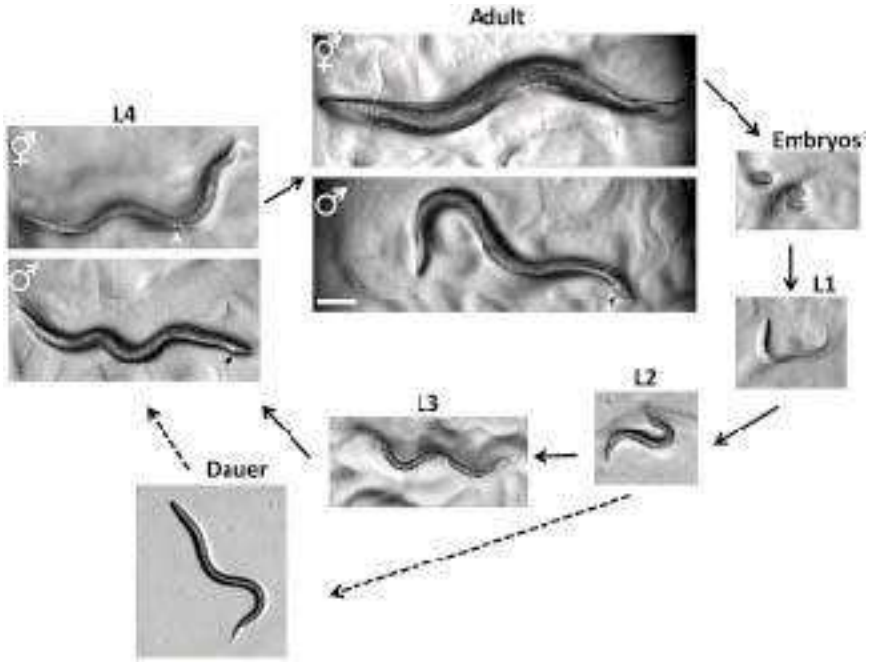

Figure 16. The *C. elegans* life cycle. Pictures of *C. elegans* at different developmental stages, from the embryo to the adult. Hermaphrodites and males become easily distinguishable from the L4 stage when the sexual dimorphism is evident (Corsi et al., 2015).

nuclei number is 959 for the hermaphrodite and 1033 for the males (1031 until the discovery of MCM neurons, Sammut et al., 2015; Lints and Hall 2009). In absence of food, *C. elegans* can arrest development and, if in L1 larval stage, it becomes a dauer with a specialised cuticle which protects it from adverse environmental conditions. When food becomes available again, *C. elegans* resumes development entering a different L4 larval stage and reaching adulthood (Corsi et al., 2015).

4.2 *C. elegans* somatic cell lineage

The knowledge of the complete somatic cell lineage of an animal is quite unique. *C. elegans* and *Panagrellus redivivus*, as already anticipated (1.1), are the only two metazoans whose somatic cell lineages are known. Their cell lineages are invariant (almost completely in *Panagrellus*), meaning they are constant among different individuals (Sternberg and Horvitz, 1982; Sulston and Horvitz, 1977; Sulston et al., 1980, 1983).

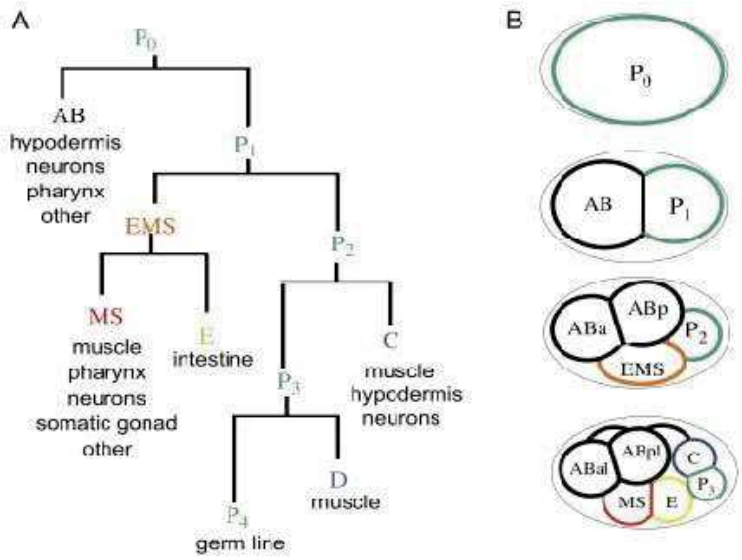

Figure 17. Schematic of the *C. elegans* somatic cell lineage (A) and the corresponding first blastomeres (B) (Rose and Gönczy, 2013).

The first cell divisions after fertilisation in *C. elegans* originate the embryonic founder cells which contribute to different cell types and tissue. The zygote is named P_0 and the first two blastomeres which arise after ACD are called AB and P_1. P_1 contributes to the germline precursor cells (P). The successive cell division of P_1 results in EMS cell and P_2 daughter cells, the former diving in MS cell and E cell (Wnt-regulated) contributing to a wide variety of somatic cells while the latter forming P_3 cell and C founder cell contributing to muscles, hypodermis and neurons. The last founder cell, D, gives rise only to muscle and is the posterior daughter of P_3 (Sulston et al., 1983) (Figure 17).

Some conventions are followed for the nomenclature of the cell lineage and for its graphical representation. Founder or blast cells are indicated by a name in capital letters. Their daughters are designated by the name of their founder or blast precursor cell followed by a sequence of lower-case letters indicating the respective positions just after cell division of all the ancestor cells in the lineage: the letter “a” is used for indicating anterior daughters, the letter “p” for posterior ones, the letter “l” for left, the letter “r” for right, the letter “d” for dorsal and the letter “v” for ventral. The lineage trees are developed from top to bottom and the left branches correspond to either anterior, or left, or dorsal cells, while the right ones to either posterior, or right, or ventral cells (Sulston and Horvitz, 1977).

4.3 Putative transdifferentiation events in *C. elegans* suggested by the somatic cell lineage

Through careful analysis of the somatic cell lineage, more putative transdifferentiation events have been found in *C. elegans*. In some cases, further observation of living worms led to the discovery of new unknown cells, which appear to originate through transdifferentiation of pre-existing cells in the less studied male (Molina-García et al., 2018; Sammut et al., 2015). Some of these events involve cell division: apart from the AMso-to-MCM described previously (Sammut et al., 2015) (see 3), more putative transdifferentiation (Td) events involving cell division have been identified by our and other labs (Rothman and Jarriault, 2019; Tursun, 2012) and some of them were studied in this Thesis:

- K-to-DVB Td: the K rectal epithelial cell gives rise to the DVB GABAergic neuron with its posterior daughter K.p after cell division (Chisholm, 1991; Sulston and Horvitz, 1977);
- Y-to-PDA Td in males: the Y rectal epithelial cell gives rise to the PDA motor neuron with its anterior daughter Y.a after cell division. Like for AMso-to-MCM and PHso1-to-PHD this process reveals sex-specific variations in Td during development (Sulston et al., 1980). However, while for Y-to-PDA, transdifferentiation occurs in both sexes through different cellular steps, AMso-to-MCM and PHso1-to-PHD Td are male-specific (Molina-García et al., 2018; Sammut et al., 2015);
- G1-to-RMH Td: the excretory pore cell G1 gives rise to the two RMH neurons in the head of the worm after cell division (Sundaram and Buechner, 2016);
- G2-to-RMF Td: the excretory pore cell G2 that after having replaced G1 divides to form G2.p, the permanent excretory pore cell, and G2.a which divides again to form the two RMF neurons (Stone et al., 2009);

- T cell-to-neuron: the seam cells T (two per side, one left and one right) function as the socket cells of the phasmid sensilla until the early L1 larva (White, 1988). Then, they both divide and give rise to ten different cells, including the permanent phasmid socket cells (PHso), hypodermal cells, neurons and one cell that undergoes apoptosis (Sulston and Horvitz, 1977);
- MSaaaapa-to-I4 neuron: I4 neuron arises from the mesodermal lineage, even though the identity of the mother MSaaaapa should be characterised to determine whether it is transdifferentiation or not (the lineage origin is not enough) (Rothman and Jarriault, 2019).

The possibility that other cells in *C. elegans*, for instance the P cells and the seam cells, could undergo transdifferentiation cannot be excluded (Tursun, 2012). The P cells are defined as neuroblasts but give rise to hypodermal cells and different types of neurons (Riddle et al., 1997); the seam cells have been described as behaving in a stem cell-like manner because of their capacity to divide during larval development and give rise to different cell types including "terminal" differentiated seam cells (Gleason and Eisenmann, 2010). However, differently from mammalian stem cells the initial seam cells have already a differentiated phenotype similar to the post-mitotic "terminal" differentiated seam cells (except not being fused and at the end of the lineage). It seems that in this context, the terms "terminal" and "post-mitotic" are used as synonyms (Altun and Hall, 2009), but the existence of terminally differentiated cells that divide is widely accepted (Zhou and Melton, 2008). Moreover, other authors defined the seam cells as "differentiated, polarised epithelial cells" (Vidal et al., 2015). All these considerations could support the occurrence of transdifferentiation also in seam cells, from epithelial cells to neurons. However, the absence of a marked specialised cell morphology/organelle, the fact of being altogether part of a transient tissue and the occurrence of several successive (and close by) rounds of division before reaching the "end-of-lineage" make more difficult to define transdifferentiation in these cellular contexts.

For this Thesis, I have been focusing on putative transdifferentiation events where the initial cell is clearly part of a permanent, specialised organ and it divides maximum once before transdifferentiation is completed: K-to-DVB, Y-to-PDA in males and G1-to-RMH. I will introduce in more details what is already known about these cells, before demonstrating whether they represent *bona fide* transdifferentiation events. For the T cells, see the Annex.

4.3.1 The K and DVB cells

The *C. elegans* K cell (AB.plpapppaa using the lineage nomenclature) is born during embryogenesis and is one of the six rectal epithelial cells forming the rectum of the worm, together with its sister K', F, U, B and Y (Chisholm, 1991; Sulston et al., 1983). The rectal cells are connected by apical junctions and form a tube which links the intestine to the environment: K and K' form the most dorsal "ring" followed ventrally by F and U, and finally by B and Y (Chisholm, 1991; Jarriault et al., 2008). K and K' appear rotated of 90° respect to the other rectal cells, being left (K) and right (K') instead of anterior (U and Y) and posterior (F and B) (Sulston et al., 1983). At the L1 stage, around 11.5 hours post hatching and 3.5 hours before the L1 molt, K divides (thus it is called blast cell) and gives rise to K.a cell, which remains a rectal cell and takes on the function of K, and K.p cell which becomes the GABAergic neuron called DVB (Figure 18) and joins the dorso-rectal ganglion (Chisholm, 1991; Mcintire et al., 1993; Sulston and Horvitz, 1977). DVB together with AVL neuron regulates defecation by controlling the intestinal, sphincter and anal depressor muscles. DVB synapses directly to the enteric muscles and shows an excitatory function. In its absence (due to laser ablation or mutant background impairing its generation) worms have defecation problems caused by decreased enteric muscle contractions (Basson and Horvitz, 1996; Mcintire et al., 1993). In agreement with its neuronal function, DVB develops visible neurites and expresses a pool of both pan-neuronal and GABAergic genes. *unc-25/GAD* (GABA biosynthetic enzyme glutamic acid decarboxylase), *unc-47/VGAT* (vesicular GABA transporter) and *unc-46/LAMP* (encoding a LAMP-like protein required for vesicular localisation of UNC-47) are all present in DVB terminally differentiated cell (Gendrel et al., 2016). Moreover, one terminal selector gene called *lim-6/LMX* is known for DVB and it regulates the expression of *unc-25* terminal differentiation gene, but not of the others (Hobert et al., 1999).

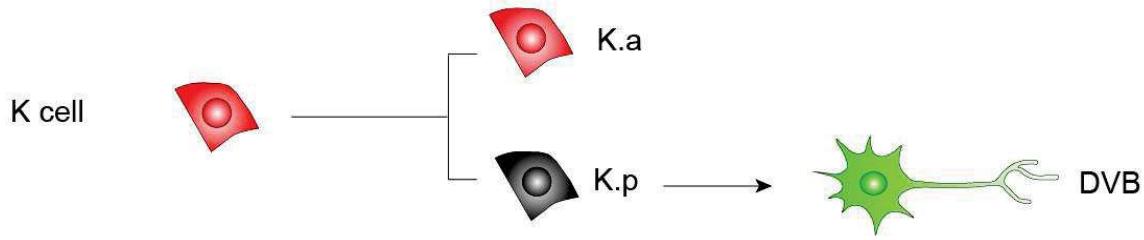

Figure 18. Lineage showing the relationship between K and DVB. Epithelial cells are in red; K.p is in black as its identity after K division is not known; DVB GABAergic neuron is in green.

4.3.2 The Y and PDA cells in males

The Y cell in the male worm (AB.prpppaaa like in the hermaphrodite) is a blast cell born during embryogenesis which differently from Y in the hermaphrodite divides forming an anterior daughter, that becomes PDA, and a posterior daughter, that further divides to give rise to ten cells contributing to the post-cloacal sensilla in the male tail (Sulston et al., 1980) (Figure 19). Apart from the capacity to divide of Y in the male, the identity of Y and PDA cells in hermaphrodite and males are not known to be different. From studies in the hermaphrodites, Y expresses epithelial and rectal markers (many in common with K) such as *ajm-1* and *dlg-1* (apical junction components, see above), *egl-5/Hox, lin-26* and *egl-26* epithelial transcription factors. On the contrary PDA expresses pan-neuronal genes such as *unc-119/UNC119*, *tag-168/RIMBP2* and *unc-33/CRMP1* together with *cog-1/NKX6-2*, *exp-1* (GABA receptor) and *ace-3/BCHE* (Jarriault et al., 2008; Richard et al., 2011).

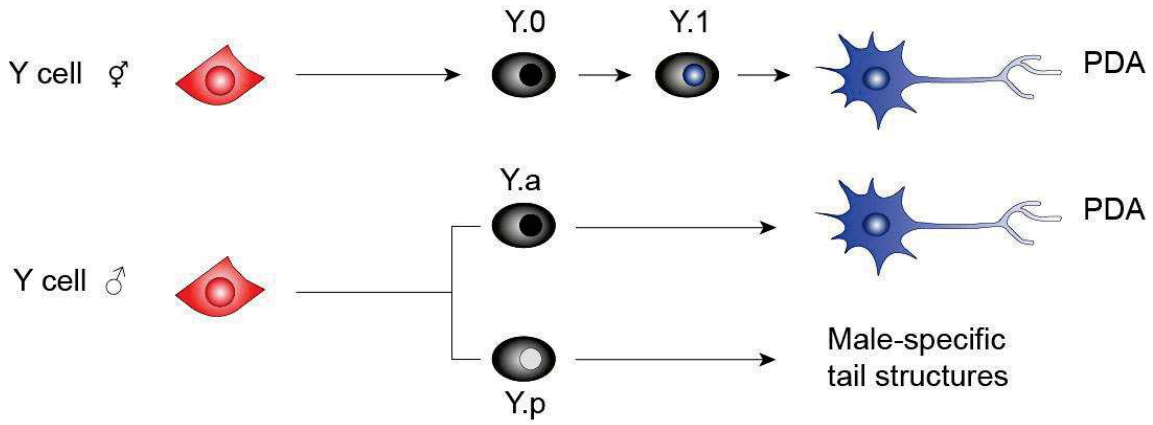

Figure 19. Lineage showing the different relationship between Y and PDA in hermaphrodites and males. Epithelial cells are in red; cells with no specific identity are in black and grey; PDA motor neuron is in blue. Y.1 nucleus is in blue as it has acquired some neuronal features.

4.3.3 The G1 and RMHL/R cells

G1 (AB.prpaaaapa) is born during embryogenesis with the contribution of EGF-Ras-ERK signalling. G1 cell is transiently the excretory pore cell of the worm's excretory system forming a tube through self-wrapping thanks to autocellular junctions (Sulston et al., 1983; Sundaram and Buechner, 2016). Interestingly, like Y and K, G1 is in contact with the environment and it secretes a luminal cuticle (Jarriault et al., 2008; Sundaram and Buechner, 2016). In mid-L1 stage, G1 starts to lose its apical junctions after F-actin dispersion, it delaminates, develops transient junctions with G2 cell (which will transiently take on the excretory pore function), and finally it divides to form two neurons called RMHL (left) and RMHR (right) (Sulston and Horvitz,

1977; Sundaram and Buechner, 2016) (Figure 20). While Ras signalling is known to be required for the loss of G1's apical junctions, the factors required for the conversion from an epithelial to a neuronal identity are completely unknown (Sundaram and Buechner, 2016).

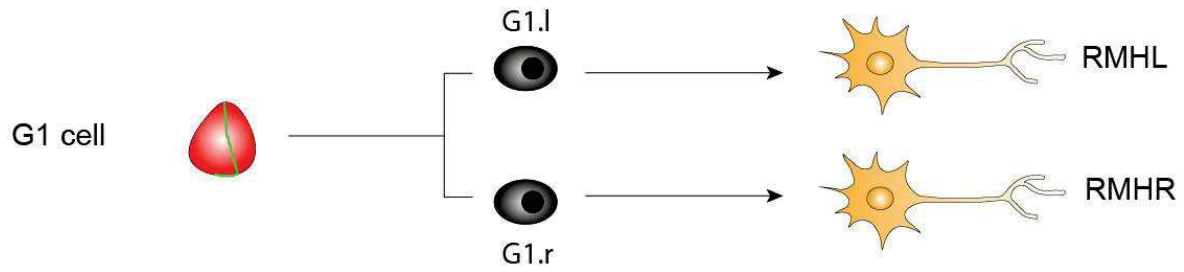

Figure 20. Lineage showing the relationship between G1 and RMH neurons. G1 epithelial cell is in red with green apical junctions; G1.l and G1.r are in black as their identity after G1 division is not known; RMH motor neurons are in yellow.

II. AIM OF THE THESIS

The different examples of reprogramming highlighted in the previous chapters demonstrate that there are some transcription factors and signalling molecules which have the capacity to drive cell reprogramming across phyla, increasing cell plasticity of the target cells and forcing them to take on a new fate. Moreover, we have seen that in some contexts cell division is present, while in other it does not occur. However, how transcription factors, signalling pathways and cell division are coordinated to drive cell reprogramming has never been directly addressed in any context.

The aim of this Thesis was to study a natural transdifferentiation event occurring in *C. elegans* which involves cell division, namely K-to-DVB, to dissect:

1) Whether cell division is required, which mechanisms are at play to regulate it and whether and how it contributes to reprogramming;
2) Whether factors required in absence of cell division in Y-to-PDA, whose mammalian orthologues are known reprogramming factors, are necessary too (Results part 1).

Moreover, to further verify the existence of conserved reprogramming factors, Y-to-PDA in males and G1-to-RMH were considered (Results part 2).

III. MATERIALS AND METHODS

1 Experimental model

Caenorhabditis elegans strains (Table 2) were maintained on agar plates containing Nematode Growth Media (NGM) seeded *with E. coli* strain OP50 (Brenner, 1974) at 20°C for all the strains except temperature-sensitive mutants (*lin-5(ev571ts)*, *wrm-1(ne1982ts), lin-18(n1051ts)*, *par-1(zu310)*). Those strains were maintained at 15°C and their eggs were shifted at 25°C to score the mutant larvae later, avoiding embryonic or early larval lethality (in some mutant backgrounds).

To characterise the phenotypes of early larval lethal alleles in larval stages, we used different knock down strategies. For *sox-2* we used either the rectal expression (under *egl-5(1.3 kb)* promoter) of a *sox-2* antisense sequence or the rectal expression (under *egl-5(6 kb)* promoter) of the nanobodyGFP system (Wang et al., 2017) in a *sox-2::gfp* KI strain. This latter strategy exploits a *Camelidae*-derived single chain antibody against GFP fused to *C. elegans* protein ZIF-1 to target GFP-tagged proteins to proteasomal degradation. For *ceh-6* we used a mutant rescued by *ceh-6* expression in all the worm but the rectal cells through a fosmid in which a part of the *ceh-6* promoter required for *ceh-6* expression in the rectum was deleted (by A. Ahier in our lab). Mutant alleles which are not lethal but lead to sterility (such as *sys-1(q544)* and *pop-1(q645)*) were maintained through a genetic balancer (Edgley et al., 2006).

To get tightly synchronized worms, we performed hatch-pulses: more than 100-200 eggs were picked on fresh plates seeded with OP50 and newly hatched larvae were transferred on new plates every hour or half an hour. Alternatively, L1-L2 worms were picked and staged according to the number of cells in the developing gonad. Less sharp synchronisations were performed by bleaching adult hermaphrodites to obtain young embryos: worms were collected from the plate with M9 buffer, pellet down at 2000 rpm for 1’ and 2 ml of bleaching solution was added for 5’. After 3 washes with M9 buffer, the eggs were seeded on fresh plates. The bleaching solution contains 20% bleach and NaOH 0.7 N in ultrapure water.

2 Method details

2.1 Plasmid construction

pSJ553 – *2nls::gfp.* *2nls* was amplified by PCR from pSJ207 with primers oCG390/oCG391 and cloned KpnI/XhoI into pPD95.75.

pSJ557 – pOD1988 (*dpy-7p::nanobodyGFP::zif-1::U54 3'UTR*) with AfeI and Xho sites. AfeI and XhoI restriction sites were added by Megawhop cloning into pOD1988. 1st Megawhop PCR made with primers OCR061/oCR062.

pSJ558 – *grl-2p::nanobodyGFP::zif-1.* *grl-2p* was amplified by PCR from gDNA with primers oCR067/oCR068 and cloned AfeI/XhoI into pSJ557.

pSJ559 – *egl-5p(6.5 kb)::nanobodyGFP::zif-1.* *nanobodyGFP::zif-1::U54 3'UTR* was amplified by PCR from pOD1988 plasmid with primers oCR073/oCR074 and cloned AscI/ApaI into pSJ671, containing *egl-5p::Δpes10*.

pSJ567 – *lin-17p::2nls::gfp.* *lin-17p* (6.5kb) was amplified by PCR from gDNA using primers oCR155/oCR156 and cloned HindIII/PstI into pSJ553.

pSJ721.14 – *let-413::gfp::pest.* For the construction of plasmid pSJ721.14, *let-413::gfp::pest*, the *Mus musculus* ornithine decarboxylase PEST sequence (Corish and Tyler-Smith, 1999) was inserted by Megawhop cloning (Miyazaki, 2011) into pML801 plasmid (a gift from Michel Labouesse). The *pest* sequence (120 bp) was obtained through annealing of oligonucleotides oCG368 and oCG369 in oligo annealing buffer containing 10mM Tris pH8, 50mM NaCl and 1mM EDTA. The annealing was performed in 50µl of buffer with 2µg per oligonucleotide in the thermocycler set for 5' at 95°C and then ramping down to about 25°C with a rate of -1.5°C/minute. After the annealing, the *pest* sequence was cloned into pJET1.2/blunt (Thermo Fisher Scientific) and subsequently amplified with primers oCG370/oCG371 and cloned into pML801 by Megawhop cloning.

pSJ739 – *lim-6int4::gfp.* *lim-6 intron 4* was cloned by Megawhop cloning into pPD95.75. *lim-6 intron 4* was amplified from gDNA with primers oCG444/oCG445.

pSJ759 – *lim-6int4(mutated)::gfp.* The sequence of the *lim-6 intron 4* with 7 out of 8 mutated TCF binding sites was ordered with BbsI and SalI restriction sites. This allowed to clone it into pSJ739, replacing the wild type sequence.

See Table 3 for primers' sequences.

2.2 Construction of *C. elegans* strains

C. elegans transgenic strains were engineered by DNA microinjection into the gonad cytoplasm of young adults (Mello et al., 1991). The mix of injected DNA was always composed by the plasmid of interest together with a co-injection marker and pBSK for a final concentration of DNA of 200-250 ng/µl in water or injection buffer (by Michael Koelle, 1994: 2% polyethylene glycol MW 6000-8000, 20 mM potassium phosphate pH 7.5, 3 mM potassium citrate pH 7.5). Transgenics F1 were cloned and the transmission of the plasmid array was evaluated in the F2 generation. Lines with a good transmission rate were usually kept.

All the other strains used for this Thesis have been obtained by crossing existing strains from our lab, other labs (obtained through CGC or directly) or from SunyBiotech for KI reporter strains which have been designed in the lab. The presence of mutations was always confirmed by PCR genotyping in case of deletions and by PCR + restriction digestion genotyping in case of point mutations (Morin et al., 2020). The only alleles that we did not genotype are *lin-17(n671)* and *hlh-14(gm34)*. Lysates of entire worms were produced for genotyping. The lysis consists in putting the worms in a solution containing ultrapure water, PCR buffer 1X and proteinase K 0.5 mM, freeze 5' at -80°C, heat at 65°C for 1 h and then at 95°C for 15 min. For all the primers used for genotyping see Table 3.

2.3 Epifluorescence Microscopy

For epifluorescence microscopy, worms were immobilized on 2% agarose pads using Tricaine 0.4% and Tetramisole 0.04% in M9 buffer. Leica DM6 B microscope was used, and images were captured with LAS X software through HAMAMATSU Digital Camera C11440.

2.4 Confocal Microscopy

To image worms at the Leica SP5 confocal microscope, worms were immobilized on 5% agarose pads with M9 buffer containing Tricaine 0.4% and Tetramisole 0.04%. Images were captured by acquiring z-step size of 0.5 µm, enough to give a nice resolution of the rectal cells. For nuclear volume quantifications images were captured with a z-step size of 0.3 µm.

2.5 Image Analysis

Standard image analyses were performed with ImageJ.

The measurement of the angle of K division was performed with ImageJ by tracing a straight line along the rectal slit of the worm and an intersecting line passing through K.a and K.p nuclei in late L1 larvae. The angle formed by the two lines was quantified using the software.

To measure K.a and K.p nuclear volumes, strains transgenic for *gaIs245* were imaged at Leica SP5 confocal microscope. The whole volume in Z containing K.a and K.p nuclei was acquired setting the z-step size at 0.3 µm. Imaris software was used to reconstruct 3D images and to analyse the volume occupied by each nucleus. Manual selection of the nuclear area was performed.

2.6 Transcription factor binding sites analysis

Both manual and automatic identification were performed.

For the manual identification of the putative POP-1/TCF binding sites the following consensus sequences was used: A/T A/T CAAA G/A (Bertrand and Hobert, 2009).

For automatic identification of putative binding sites, we used either MatInspector Software (Cartharius et al., 2005) or Jaspar and looked from vertebrate homologues when possible. Conservation of the binding sites was addressed using the multiple alignment provided by the UCSC Genome Browser. For SOX2 the binding motif used is based on the mammalian one as done by Alqadah et al., 2015: CC T/A TTGT T/C/G.

The mutation of POP-1/TCF binding site was performed by mutating the conserved CAAA nucleotides.

2.7 Statistical analyses

In the histograms, mean and standard deviation between biological replicates of the percentage of worms scored are represented. The stars summarise the statistical significance as calculated through Fisher's exact test on the merged raw data from single replicates in a contingency table. Two-tailed Fisher's exact test was used to compare reporter gene expression in mutant vs wild type worms. One-tailed Fisher's test was used to compare Td defects in mutant vs wild type worms; the choice of the one-tailed test is justified by the known 0% Td defect in wild type worms. The Student's t-test was used to analyse the significance in the difference between K.a and K.p nuclear volumes' ratio and the angle of K division in wild type vs *lin-17* and *sem*-4 mutants. F test was used to compare the variances of the angle of division between wild type vs *lin-17*, *sem-4* and *goa-1* mutants.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$ for all the tests.

Strain name	Genotype
IS800	*lin-5(ev571) II; gaIs245[col-34p::his-24::mcherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS872	*bxIs7[egl-5::gfp; lin-15(+)] I; mIIs46[dlg-1::rfp,unc-119(+)] I*
IS1041	*krIs6 II; gpr-1(ok2126) III*
IS1208	*sem-4(n1971) bxIs7[egl-5p::gfp; lin-15(+)] I; otIs173[rgef-1p::dsred2; ttx-3promB::gfp] III*
IS1210	*sem-4(n197) I; otIs117[unc-33p::GFP + unc-4(+)] IV; gaIs245[col-34p::his-24::mcherry; unc-119(+)] V*
IS1299	*gaIs245[col-34p::his-24::mcherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS1370	*lin-17(n671) I; gaIs245[col-34p::his-24::mcherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS1374	*fpIs17[hmr-1::gfp] IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS1332	*egl-5(n945) III; gaIs245[col-34p::his-24::mcherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS1432	*wrm-1(ne1982) III; gaIs245[col-34p::his-24::mcherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS2968	*sem-4(n1971) I; gaIs245[col-34p::his-24::mcherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3083	*fpIs110[lin-26peABCD+i::gfp; rol-6(su1006)] IV*
IS3094	*flh-1(bc374) IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3095	*flh-2(bc375) III; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3096	*flh-3(gk1049) IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3097	*unc-86(n846) III; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3101	*fpEx1062[let-413a::gfp::PEST; myo-2p::gfp]*
IS3107	*fpIs110[lin-26peABCD+i::gfp; rol-6(su1006)] IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V*
IS3113	*egl-27(ok1670) II; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*

IS3119	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; fpEx1062[let-413a::gfp::PEST; myo-2p::gfp]*
IS3120	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X; fpEx955[GFP::ceh-6 13kb locus in Strataclone (sens1)- 5' part. prom. OFF [from ATG : -2846pb to -102pb (2744pb)-oligo ceh-6 MIDR/ceh-6PROMmF] (10ng/µl); odr-1::rfp (50ng)]*
IS3122	*ceh-6(gk665) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V ; oxIs12[unc-47p::gfp; lin-15(+)] X; fpEx955[GFP::ceh-6 13kb locus in Strataclone (sens1)- 5' part. prom. OFF [from ATG : -2846pb to -102pb (2744pb)-oligo ceh-6 MIDR/ceh-6PROMmF] (10ng); odr-1::rfp (50ng/µl)]*
IS3125/6	*hlh-16(fp12) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3142	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X; fpEx788[egl-5(1,3kb)p::sox-2 full antisense::U543'UTR (10ng/µl); rol-6(su1006)]*
IS3146	*lin-40(ku285) V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3155	*bxIs7[egl-5::gfp; lin-15(+)] I; krIs6[unc-47p::Dsred2; lin-15(+)] II; unc-86(n846) III; ceh-18(mg57) X*
IS3156	*wdr-5.1(ok1417) III; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3158	*him-5(e1490) V; fpEx1066[exp-1p::NZyfp (60ng/µl); unc-17p::CZyfp (60ng/µl); odr-1::dsred (50ng/µl)]*
IS3173	*fpIs17[hmr-1::gfp] IV*
IS3179	*qnEx59[dct-5p::mCherry; unc-119(+)]*
IS3181	*hlh-16(fp12) I; egl-27(ok1670) II; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3182	*fpIs17[hmr-1::gfp] IV; qnEx59[dct-5p::mCherry; unc-119(+)]*
IS3259	*otIs313[sem-2(fosmid)::yfp; rol-6(su1006)] II; fpIs17[hmr-1::gfp] IV; qnEx59[dct-5p::mCherry; unc-119(+)]*
IS3277	*fpIs17[hmr-1::gfp] IV; otIs534[cho-1(fosmid)::SL2::NLS::YFP::H2B]; qnEx59[dct-5p::mCherry; unc-119(+)]*
IS3295	*fpIs88[hlh-16::gfp; rol-6(su1006)] III; him-8(e1489) IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V*
IS3297	*wyIs75[unc-47p::DsRed; exp-1p::gfp; odr-1p::rfp] III; him-5(e1490) V*
IS3319	*hlh-16(fp12) I; otIs313[sem-2(fosmid)::yfp; rol-6(su1006)] II; fpIs17[hmr-1::gfp] IV; qnEx59[dct-5p::mCherry; unc-119(+)]*

IS3320	*hlh-16(fp12) I; wyIs75[Punc-47::DsRed; Pexp-1::GFP; Podr-1::RFP] III; him-5(e1490) V*
IS3321	*sem-4(n1971) I; wyIs75[unc-47p::DsRed; exp-1p::gfp; odr-1p::rfp] III; him-5(e1490) V*
IS3326	*sem-4(n1971) I; otIs313[sem-2(fosmid)::yfp; rol-6(su1006)] II; fpIs17[hmr-1::gfp] IV; qnEx59[dct-5p::mCherry; unc-119(+)]*
IS3327	*edIs6[unc-119::gfp; rol-6(su1006)] IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V*
IS3328	*lin-17(n671) I; edIs6[unc-119::gfp; rol-6(su1006)] IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V*
IS3329	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; otIs118[unc-33::GFP; unc-4(+)] X?*
IS3330	*lin-17(n671) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; otIs118[unc-33::GFP; unc-4(+)] X?*
IS3335	*lin-17(n671) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; juIs8 [unc-25::gfp; lin-15(+)]*
IS3336	*egl-5(n945) III; syIs63[cog-1::gfp;unc-119(+)] IV; him-5(e1490) V; fpEx30[exp-1p::mCherry (10ng/µl); myo-2::gfp (10 ng/µl)]*
IS3339	*jcIs1[ajm-1::GFP; rol-6(su1006)] IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V*
IS3343	*egl-27(ok1670) II; otIs534[cho-1(fosmid)::SL2::NLS::YFP::H2B]; qnEx59[dct-5p::mCherry; unc-119(+)]*
IS3344	*hlh-16(fp12) I; otIs534[cho-1(fosmid)::SL2::NLS::YFP::H2B]; qnEx59[dct-5p::mCherry; unc-119(+)]*
IS3349	*lin-17(n671) I; fpIs110[lin-26peABCD+i::gfp; rol-6(su1006)] IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V*
IS3357	*lin-17(n671) I; jcIs1[ajm-1::GFP; rol-6(su1006)] IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V*
IS3368	*lin-56(n2728) II; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3379	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; fpEx1111[lim-6int4::gfp(50ng/µl); coel::dsRed(30ng/µl)]*
IS3383	*lin-17(n671) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; fpEx1062[let-413a::gfp::PEST; myo-2p::gfp]*
IS3385	*sin-3(tm1276) I; wyIs75[Punc-47::DsRed; Pexp-1::GFP; Podr-1::RFP] III; him-5(e1490) V*
IS3387	*sem-4(n1971) I; fpIs17[hmr-1::gfp] IV; otIs534[cho-1(fosmid)::SL2::NLS::YFP::H2B]; qnEx59[dct-5p::mCherry; unc-119(+)]*

IS3388	*egl-5(n945) III; fpIs17[hmr-1::gfp] IV; otIs534[cho-1(fosmid)::SL2::NLS::YFP::H2B]; qnEx59[dct-5p::mCherry; unc-119(+)]*
IS3420	*lin-17(n67) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; fpEx1111[lim-6int4::gfp(50ng/µl); coel::dsRed(30ng/µl)]*
IS3426	*wyIs75[unc-47p::DsRed; exp-1p::gfp; odr-1p::rfp]II ; him-5(e1490) V; fpEx788[egl-5(1,3kb)p::sox-2 full antisense::U543'UTR (10ng/µl); rol-6(su1006)]*
IS3433	*wyIs75[unc-47p::DsRed; exp-1p::gfp; odr-1p::rfp] III; vang-1(tm1422) X*
IS3452	*unc-73(e936) dpy-5(e61) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3464	*dsh-1(ok1445) II; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3466	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; sox-2(syb737[GFP::linker::sox-2]) X*
IS3469	*egl-27(ok1670) II; wyIs75[unc-47p::DsRed; exp-1p::gfp; odr-1p::rfp] III; him-5(e1490) V*
IS3471	*fpEx1153[grl-2p::EpiDeg (20ng/ul); rol-6(su1006) (50ng/ul); pBSK (150ng/ul)]*
IS3473	*wyIs75[Punc-47::DsRed; Pexp-1::GFP; Podr-1::RFP] III; him-5(e1490) V; ref-2(fp9) X*
IS3476	*fpEx1154[egl-5p(6.5kb)::EpiDeg (20ng/ul);coel::gfp (40ng/ul); pBSK (150ng/ul)]*
IS3477	*fpEx1155[egl-5p(6.5kb)::EpiDeg (20ng/ul);coel::gfp (40ng/ul); pBSK (150ng/ul)]*
IS3478	*fpEx1156[egl-5p(6.5kb)::EpiDeg (20ng/ul);coel::gfp (40ng/ul); pBSK (150ng/ul)]*
IS3485	*egl-20(n585) IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3486	*lin-44(n1792) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
ML679	*ced-10(n3246) IV; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3490	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; lim-6(nr2073) oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3491	*par-1(zu310) gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3511	*fmi-1(rh308) gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3512	*dsh-1(ok1445) mig-5(tm2639) II; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3516	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; sox-2(syb737[GFP::linker::sox-2]) X; fpEx1154[egl-5p(6.5kb)::EpiDeg (20ng/ul);coel::gfp (40ng/ul); pBSK (150ng/ul)]*

IS3517	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; sox-2(syb737[GFP::linker::sox-2]) X; fpEx1156[egl-5p(6.5kb)::EpiDeg (20ng/ul);coel::gfp (40ng/ul); pBSK (150ng/ul)]*
IS3521	*wyIs75[unc-47p::DsRed; exp-1p::gfp; odr-1p::rfp] III; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; sox-2(syb737[GFP::linker::sox-2]) X; fpEx1156[egl-5p(6.5kb)::EpiDeg (20ng/ul);coel::gfp (40ng/ul)]*
IS3530	*gpr-1(ok2126) III; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3536	*edIs6[unc-119::gfp; rol-6] IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; lim-6(nr2073) X*
IS3538	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; lim-6(nr2073) X otIs118[unc-33::GFP; unc-4(+)] X?*
IS3583	*lin-17(n671) bxIs7[egl-5::gfp; lin-15(+)] I; otIs173[rgef-1::Dsred2; ttx-3promB::GFP] III*
IS3596	*pop-1(q645) I/hT2[bli-4(e937) let-?(q782) qIs48] (I;III); gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3598	*goa-1(sa734) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3600	*pop-1(q645) I/hT2[bli-4(e937) let-?(q782) qIs48] (I;III); gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; [lim-6int4::gfp(50ng/µl);coel::dsRed(30ng/µl)]*
IS3604	*otIs173[rgef-1p::Dsred2 + ttx-3promB::GFP] III; oxIs12[unc-47p::gfp; lin-15(+)] X*
IS3611	*gpa-16(ok2349) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3618	*pop-1(q645) I/hT2[bli-4(e937) let-?(q782) qIs48] (I;III); lim-6(syb971[lim-6::linker::gfp]) X*
IS3619	*lin-5(ev571) II; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; fpIs101[col-34p::PH::gfp::3'UTRunc-54; odr-1p::dsRed] X*
IS3633	*sem-4(n1971) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; lim-6(syb971[lim-6::linker::gfp]) X*
IS3634	*sem-4(n1971) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; fpEx1062[let-413a::gfp::PEST; myo-2p::gfp]*
IS3666	*ham-1(n1438) IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3667	*cam-1(gm122) II; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3668	*krIs6[unc-47::DsRed2; lin-15(+)] II; bar-1(ga80) X*
IS3669	*lin-17(n671) I; [col-34p::his-24::mCherry; unc-119(+)] V; lim-6(syb971[lim-6::linker::gfp]) X*

IS3676	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; lin-18(n1051) oxIs12[unc-47::gfp; lin-15(+)] X*
IS3677	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; lim-6(syb971[lim-6::linker::gfp]) X*
IS3681	*sem-4(n1971) I; edIs6[unc-119::gfp; rol-6(su1006)] IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V*
IS3682	*sem-4(n1971) I; jcIs1[ajm-1::gfpP; rol-6(dm)] IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V*
IS3683	*sem-4(n1971) I; fpIs110[lin-26peABCD+i::gfp; rol-6(su1006)] IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V*
IS3696	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; fpEx1232[lin-17p(6.5kb)::2nls::gfp(20ng/ul); odr-1::dsRed(50ng/ul); pBSK(150ng/ul)]*
IS3702	*lin-17(n671) ceh-6(syb972[3xFlag::gfp::linker::ceh-6]) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V*
IS3717	*gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; juIs8[unc-25::gfp; lin-15+]*
IS3718	*sys-1(q544) I/ hT2[bli-4(e937) let-?(q782) qIs48] (I;III); gaIs245[col-34p::HIS-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3719	*sem-4(n1378) I; gaIs245[col-34p::HIS-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3720	*sem-4(n1971) I; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; juIs8[unc-25::gfp; lin-15+]*
IS3722	*krIs6[unc-47::DsRed2; lin-15(+)] II; wrm-1(ne1982) III; gaIs245[col-34p::HIS-24::mCherry; unc-119(+)] V; fpEx1156[egl-5p(6.5kb)::EpiDeg (20ng/ul);coel::gfp (40ng/ul); pBSK (150ng/ul)]*
IS3723	*krIs6[unc-47::DsRed2; lin-15(+)] II; wrm-1(ne1982) III; gaIs245[col-34p::HIS-24::mCherry; unc-119(+)] V; sox-2(syb737[GFP::linker::sox-2]) X; fpEx1156[egl-5p(6.5kb)::EpiDeg (20ng/ul);coel::gfp (40ng/ul); pBSK (150ng/ul)]*
IS3729	*krIs6[unc-47::DsRed2; lin-15(+)] II; wrm-1(ne1982) III; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; sox-2(syb737[GFP::linker::sox-2]) X; fpEx1156[egl-5p(6.5kb)::EpiDeg (20ng/ul);coel::gfp (40ng/ul); pBSK (150ng/ul)]*
IS3731	*sem-4(n1378) I; wrm-1(ne1982) III; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3796	*lin-22(n372) IV; gaIs245[col-34p::his-24::mCherry; unc-119(+)] V; oxIs12[unc-47::gfp; lin-15(+)] X*
IS3797	*lin-22(n372) IV; wyIs75[unc-47p::DsRed; exp-1p::gfp; odr-1p::rfp] III; him-5(e1490) V*
IS3799	*fpIs17[hmr-1::gfp] IV; ref-2(fp9) X; otIs534[cho-1(fosmid)::SL2::NLS::YFP::H2B]; qnEx59[dct-5p::mCherry; unc-119(+)]*

IS3801	*ceh-6(syb972[3xFlag::gfp::linker::ceh-6]) I; fpIs17[hmr-1::gfp] IV; qnEx59[dct-5p::mCherry; unc-119(+)]*
IS3803	*fpIs17[hmr-1::gfp] IV; sox-2(syb737[GFP::linker::sox-2]) X; qnEx59[dct-5p::mCherry; unc-119(+)]*
IS3804	*lin-39(n709) III; otIs534[cho-1(fosmid)::SL2::NLS::YFP::H2B]; qnEx59[dct-5p::mCherry; unc-119(+)]*
IS3805	*ceh-6(gk665) I; syIs63[cog-1::gfp;unc-119(+)] IV; him-5(e1490) V; fpEx955[GFP::ceh-6 13kb locus in Strataclone (sens1)- 5' part. prom. OFF [from ATG : -2846pb to -102pb (2744pb)-oligo ceh-6 MIDR/ceh-6PROMmF] (10ng); odr-1::rfp (50ng/µl)]*

Table 2. List of strains used for this Thesis.

Oligo name	Sequence	Use
BDL94/ref-2seqF	GCAAGTCCAACTGACCCGTG	Genotyping *ref-2(fp9),* digest w/ HaeIII
BDL95/ref-2seqR	CTGGTCATACTGCCCGGAATC	Genotyping *ref-2(fp9),* digest w/ HaeIII
EB5F	TCCAGTCTCTTCAGGTCAGTGATCT	Genotyping *egl-27(ok1670)*
EB5R	CGAGATTTCCAAATTCTTACCCGACTG	Genotyping *egl-27(ok1670)*
EB6R	GTGTAATTGACAGCGATGATGATGAAGG	Genotyping *egl-27(ok1670)*
EB110F	AGAAGACCGCCCCTCTTTTGA	Genotyping *ceh-6(syb972)*
EB110R	GGCTGCCTCCATCTCGTTCT	Genotyping *ceh-6(syb972)*
LIN5 FW 01	GACAAGACCAAGTTATCGGC	Genotyping *lin-5(ev571),* digest w/ BglII
LIN5 RV 01	CCCATTGACTGAAATTCTTCG	Genotyping *lin-5(ev571),* digest w/ BglII
mcm93F	GGGCTTCAAGACAGTAAGATCCAACAATTCCTAACAAATAAAAAAAATCCTA	Genotyping *hlh-16(fp12),* digest w/ AvrII
mcm93R	GCCATGTTTAATTCCATTTGAAGCTTGTGATTTGC	Genotyping *hlh-16(fp12),* digest w/ AvrII

mcm124F	GAACTACAACTACTTTGGTCAACCATTGGG CCCTGCCACGTTTCCCCCAT	Genotyping *egl-5(n945),* digest w/ NcoI
mcm124R	CGTAAGATAGCATATAGGGTCAGACG	Genotyping *egl-5(n945),* digest w/ NcoI
mcm125F	CCGCGCCATTGACACCGATTTGGTAC	Genotyping *sem-4(n1971),* digest w/ Acc65I
mcm125R	CCTAACAAGCTAGCCTTTTCAGTTACAAAA ACATCTCTCTTAACTGGGTA	Genotyping *sem-4(n1971),* digest w/ Acc65I
mcm283F	GGTTCCTCGCAAAGTGCTCATCG	Genotyping *lin-56(n2728)*
mcm283R	CCTCAAGCCTCCTTCTTGCCACCTC	Genotyping *lin-56(n2728)*
mcm284R	GTCAGGCGACTATCCGGAGTTCCAAC	Genotyping *lin-56(n2728)*
oCG347 rev début GFP	CCACTGACAGAAAATTTGTGCCC	Genotyping *sem-4 (syb1287)* and sequencing
oCG368 sens PEST	CTTAGCCATGGCTTCCCGCCGGCGGTGGC GGCGCAGGATGATGGCACGCTGCCCATGT CTTGTGCCCAGGAGAGCGGGATGGACCGT CACCCTGCAGCCTGTGCTTCTGCTAGGATC AAT	PEST sequence fw
oCG369 rev PEST	ATTGATCCTAGCAGAAGCACAGGCTGCAG GGTGACGGTCCATCCCGCTCTCCTGGGCA CAAGACATGGGCAGCGTGCCATCATCCTG CGCCGCCACCGCCGGCGGGAAGCCATGG CTAAG	PEST sequence rv
oCG370 sens MW PEST	GGATTACACATGGCATGGATGAACTATACA AACTTAGCCATGGCTTCCCGCCGGCGGTG GC	Cloning *pest* sequence into pML801
oCG371 ev MW PEST	GGTAGCGACCGGCGCTCAGTTGGAATTCT ACGAATGCTACACATTGATCCTAGCAGAAG CACAGGCTG	Cloning *pest* sequence into pML802

oCG372 sens flh1 bc374	GTCTACCGTCTGGCTGCATAG	Genotyping *flh-1(bc374)*
oCG373 rev flh1 bc374	CTGTTCGGTTCTCCTTTCCACA	Genotyping *flh-1(bc374)*
oCG374 rev wt flh1 bc374	CGCTAGACATATCAATTCCGCTCG	Genotyping *flh-1(bc374)*
oCG375 sens flh2 bc375	CAATTCTCCGATGGTACCACTTCC	Genotyping *flh-2(bc375)*
oCG376 rev flh2 bc375	CCGTGTGTCCAATTGCTGATTAG	Genotyping *flh-2(bc375)*
oCG377 rev wt flh2 bc375	GGTTGTTGTAACAGTAATCCGGC	Genotyping *flh-2(bc375)*
oCG378 sens flh3 gk1049	CAGTGGATCATTGTTTGTTCCTCAG	Genotyping *flh-3(gk1049)*
oCG379 rev flh3 gk1049	GTGATAGGGGAACATTGATTTTGGATG	Genotyping *flh-3(gk1049)*
oCG380 rev wt flh3 gk1049	GCCCATTCCATAGTTGCATTTCC	Genotyping *flh-3(gk1049)*
oCG390 for NLS1 kpnI	AGGGTACCGAGCTCAGAAAAAATGACAGC	Cloning *2nls* into pPD95.75
oCG391 rev GFP XhoI	GGGTATCTCGAGAAGCATTGAACACCATAA CAGAAAG	Cloning *2nls* into pPD95.75
oCG411 sens egr-1 ku285	GCCCCAAAAGCCTGAAAAAGCCCAAAATTT CTCAATTTCCA	Genotyping *egr-1(ku285)*, digest w/ Hpy188III
oCG412 rev egr-1 ku285	GACGTCTCCGCAGAAGCTTCGGTGGC	Genotyping *egr-1(ku285)*, digest w/ Hpy188III
oCG444 lim-6 3int sens	GGATACGCTAACAACTTGGAAATGAAATAG GCGCCCTTCTTGAGATTGCG	Cloning *lim-6 intron 4* into pPD95.75
oCG445 lim-6 3int rev	CGACCTGCAGGCATGCAAGCTAAAGATTG ACATATTGGAGACATCTGCC	Cloning *lim-6 intron 4* into pPD95.75

oCG461 sens sox-2 CRISPR	GGTTGTCTTTTGCAGTGTCCGG	Genotyping *sox-2(syb737)*
oCG462 rev sox-2 CRISPR	CAGAGCCATTTTCTTCCGCTGTC	Genotyping *sox-2(syb737)*
oCG463 sens sem-4 CRISPR	GACGACGAATCTTCGATGTGGC	Genotyping *sem-4 (syb1287)*
oCG464 rev sem-4 CRISPR	GGGGGAAAGAGGGAAAATTAGCTG	Genotyping *sem-4 (syb1287)*
oCR017 ceh-6 fw	GGCGGATGCAAGATTTTACG	Genotyping *ceh-6(gk665)*
oCR018 ceh-6 rv wt	GGATGACGACGAAGGTATGAG	Genotyping *ceh-6(gk665)*
oCR019 ceh-6 rev gk665	CTGTGACAATGTTCCCGGAG	Genotyping *ceh-6(gk665)*
oCR029 fw ceh-18(mg57)	CCCACACCAGTTTCCACAAATGGC	Genotyping *ceh-18(mg57)*
oCR030 rv ceh-18(mg57)	AGGCTAGAAAGTTCTACGGG	Genotyping *ceh-18(mg57)*
oCR036 rv ceh-18 wt	GCTCGCCGCCTCAATTCTTGAT	Genotyping *ceh-18(mg57)*
oCR058 ok737 fw	CCAGCTGATATTTGGGCAGCTC	Genotyping *ceh-13(ok737)*
oCR059 ok737 rv	CATACCAGTTCCCATGGTTTTCAAGTTG	Genotyping *ceh-13(ok737)*
oCR060 ok737 rv wt	CAGCCCCCTTGGAATTACTTTGG	Genotyping *ceh-13(ok737)*
oCR061 fw AfeI EpiDeg	GAGGGTACCAGAGCTCAAGCGCTATTCAC CTGGCACCGACTAC	Cloning AfeI and XhoI sites into pOD1988
oCR062 rv XhoI EpiDeg	CCAGACTCCACCAGTTGGACTTGATCCATC TCGAGTTATCTGGAACAAAATGTAAG	Cloning AfeI and XhoI sites into pOD1988
oCR067 AfeI grl-2p	GCCAGCGCTATGAACTTACCACACCTGC	Cloning *grl-2p* into pSJ557

oCR068 grl-2p XhoI	GCCCTCGAGACTTGGTATAATTATGAAACT AACG	Cloning *grl-2p* into pSJ557
oCR073 AscI nanob	ATAAAAGGCGCGCCAAAAAATGGATCAAGT CCAACTGGT	Cloning *nanobodyGFP::zif-1* into pSJ671
oCR074 U54 ApaI	GTAATAGGGCCCTTAACCCTCACTAAAGGG AACAAAA	Cloning *nanobodyGFP::zif-1* into pSJ671
oCR075 tm1422 fw	GGGCCAGAAGATTGCACCAC	Genotyping *vang-1(tm1422)*
oCR076 tm1422 rv wt	GCATGCTGAAGCCGAAACGT	Genotyping *vang-1(tm1422)*
oCR077 tm1422 rv	CGCAATCGGTAGAATTGAAAATTTCGG	Genotyping *vang-1(tm1422)*
oCR078 tm2639 fw	CATGGACGCGAGTGGATCATC	Genotyping *mig-5(tm2639)*
oCR079 tm2639 rv wt	CCATATCTGGGACTGAGCGTG	Genotyping *mig-5(tm2639)*
oCR080 tm2639 rv	CATTTATGGAGCCGCCATGCA	Genotyping *mig-5(tm2639)*
oCR084 ok1445 fw	CTCTTGCATCGGATTCGGAGC	Genotyping *dsh-1(ok1445)*
oCR085 ok1445 rv wt	CGAAGGTACGGCGGATCAAAT	Genotyping *dsh-1(ok1445)*
oCR086 ok1445 rv	CCCTTCCATCTCCTTCTGGC	Genotyping *dsh-1(ok1445)*
oCR087 nr2073 fw	GTAATGCGCGAAGCTTCCTG	Genotyping *lim-6(nr2073)*
oCR088 nr2073rv wt	GGGAGCCTATAGGTCAGCTCT	Genotyping *lim-6(nr2073)*
oCR089 nr2073 rv	CCTCCGCTTGGAAGGACAAAA	Genotyping *lim-6(nr2073)*

oCR092 rh308 fw	GTGATAATGCTCGTATTGTCTATTCCATTGATTCCTAT	Genotyping *fmi-1(rh308),* digest w/ AseI
oCR093 rh308 rv	GTGGATGAGATCCGCCGTCAG	Genotyping *fmi-1(rh308),* digest w/ AseI
oCR094bis	CCTCTTAAAAACTTACCTCTCAAATTTGAACTTATTCAAGC	Genotyping *egl-20(n585),* digest w/ HindIII
oCR095 n585 rv	GAACATTGGCATTTGTGGGTTCAAAC	Genotyping *egl-20(n585),* digest w/ HindIII
oCR096 n1792 fw	CTTCAAAACTGTGCGAATCGTTTGAGATTTCAGCCCT	Genotyping *lin-44(n1792),* digest w/ AvrII
oCR097 n1792 rv	CCTTTTGACCCTACCCGCCGAAC	Genotyping *lin-44(n1792),* digest w/ AvrII
oCR105 zu310 fw	CCCACATTCATCCATCGATCTTTCATAAT	Genotyping *par-1(zu310),* digest w/ SspI
oCR106 zu310 rv	GTCTCTGCTGTTCAATATTTGCATTCG	Genotyping *par-1(zu310),* digest w/ SspI
oCR107 ok2126fw wt	CTGAACTGCCTGCTGCCAGA	Genotyping *gpr-1(ok2126)*
oCR108 ok2126rv	CACGAAAGTCATCAACGTATGTAGTAAAG	Genotyping *gpr-1(ok2126)*
oCR109 ok2126fw mu	CCAAGGCTCGACGGTTTGC	Genotyping *gpr-1(ok2126)*
oCR113 ga80 fw	GCATAGTGAGTTCTGGAATTGCTCGAACTGTGTTATACTGCCC	Genotyping *bar-1(ga80),* digest w/ BclI
oCR114 ga80 rv	CATCCATGGCCGACTATGAGCCGATCCCCACTCTTTCTGAT	Genotyping *bar-1(ga80),* digest w/ BclI
oCR122 q645 fw	CGATGGATTTCGACCGGCACC	Genotyping *pop-1(q645),* digest w/ ClaI
oCR123 q645 rv	GATATAAAAATACACAAAAATGATGGCCGACGAAGAGCTCATCGA	Genotyping *pop-1(q645),* digest w/ ClaI

oCR128 n1378 fw	CAACACCGAATCCAAAAAACGAAAATCCAC TGCTTGGCATG	Genotyping *sem-4(n1378),* digest w/ SphI
oCR129 n1378 rv	CCACGAGTTGTGAATGCGCGTCCAC	Genotyping *sem-4(n1378),* digest w/ SphI
oCR138 syb971fw	GACATTCGAAGCTCTGATGATG	Genotyping *lim-6(syb971)*
oCR139 syb971rv wt	GTGCAAAGATTAGAGCTCTGAC	Genotyping *lim-6(syb971)*
oCR140 syb971rv mu	GGGTATCTCGAGAAGCATTG	Genotyping *lim-6(syb971)*
oCR144 n1051 fw	CACTACAGAGTTATGGCAAACATCGACTAC CTCTCGTTCCCAT	Genotyping *lin-18(n1051),* digest w/ NcoI
oCR145 n1051 rv	CCTGTCGCAATTTCACTTTCAACGGCTC	Genotyping *lin-18(n1051),* digest w/ NcoI
oCR146 n1438 fw wt	GCCTCCCGAGTGACCATCT	Genotyping *ham-1(n1438)*
oCR147 n1438 rv	GCATGAAGCCCATGTAAGTGATCGA	Genotyping *ham-1(n1438)*
oCR148 n1438 fw mu	CAGCAGCTTTCATTGTTTTTTCCAC	Genotyping *ham-1(n1438)*
oCR149 gm122 fw	GACCACGATTTACTTCGGCAACG	Genotyping *cam-1(gm122),* digest w/ BclI
oCR150 gm122 rv	CATCATATGTATAAAGTTTGCGAATCGGATT CTAATGAT	Genotyping *cam-1(gm122),* digest w/ BclI
oCR151 q544 fw	CCTGTTGGCGGAGGAGGTTGATCATGTGG	Genotyping *sys-1(q544),*digest w/ AflIII
oCR152 q544 rv	GGCAAAAAGATCCTCACATGAAACACTGCG CAAATCACGT	Genotyping *sys-1(q544),*digest w/ AflIII
oCR153 n671	CCGCATTTTTCGTAGATCACACC	Sequencing *lin-17(n671)*
oCR154 n671	CGAGCACATTCCACAGAAGATG	Sequencing *lin-17(n671)*

oCR155 lin-17p fw	CTGAAGCTTACACTTTGTTCGCTC	Cloning *lin-17p* reporter
oCR156 lin-17p rv	CGGCTGCAGTTTGGAGAAGGAGCCAGTCTCTC	Cloning *lin-17p* reporter
oCR157 wrm-1 fw	GATGTTCTTCCGACTGAATGC	Sequencing and genotyping *wrm-1(ne1982ts)*
oCR158 wrm-1 rv	CTTGTGCTCCACCCATTTG	Sequencing and genotyping *wrm-1(ne1982ts)*
oCR173 ok646 fw	GTGAGGCGGCAGTGTCATTG	Genotyping *ztf-11(ok646)*
oCR174 ok646 rv wt	GGTCTTGGAAGAACCGAGCAAC	Genotyping *ztf-11(ok646)*
oCR175 ok646 rv mu	CGCCTGCCGCATACCTAAATTG	Genotyping *ztf-11(ok646)*
oCR201 n372 fw	CGGCTTAATTTAATTTTCAACATTTTCAGAAAAACAAACCTCCCATG	Genotyping *lin-22(n372)*, digest w/ NcoI
oCR202 n372 rv	GAAGTGCAAAACCACAATTTGGCG	Genotyping *lin-22(n372)*, digest w/ NcoI
oCR205 n709 fw	CCGAGTATTGGATATTGAGGACCTCCC	Genotyping *lin-39(n709)*, digest w/ XbaI
oCR206 n709 rv	CTCTTTTCAATCAATTCTTATCAATCACTTTCACTCGTTTTCACTCTA	Genotyping *lin-39(n709)*, digest w/ XbaI
oCR207 e1490 fw	CTATGTTAGTAATTTTTAAAAACATGGAATTTTACTGATTATTTCTCA	Genotyping *him-5(e1490)*, digest w/ DdeI
oCR208 e1490 rv	CTCTAAATCATCGTCGGTGCTTAAATC	Genotyping *him-5(e1490)*, digest w/ DdeI
sz80-Fwdr-5.1(ok1417)	CAATTTCGCCATCAAATCCGACTG	Genotyping *wdr-5.1(ok1417)*
sz81-Rwdr-5.1(ok1417)	GATGTTCTGAACTGGATGACAATCG	Genotyping *wdr-5.1(ok1417)*

sz141-Rwdr-5.1(ok1417)	CTTGTTCCGAGATACTTTCCACA	Genotyping *wdr-5.1(ok1417)*

Table 3. List of oligonucleotides used for this Thesis. I designed oCRXXX primers.

IV. RESULTS

1 K-to-DVB: a transdifferentiation event involving mitosis empowered by integrating signalling inputs with conserved reprogramming factors

1.1 K-to-DVB is a transdifferentiation event: K is a rectal epithelial cell and DVB is a GABAergic neuron

The somatic cell lineage of *C. elegans* suggests that the formation of the DVB neuron after division of the K rectal cell might be a transdifferentiation event. To confirm this hypothesis, we analysed the identity of K and DVB based on cellular morphology and expression of cell identity marker genes (Table 4). In addition, the respective functions of K and DVB in their tissues are known from previous studies (see Introduction, 4.3.1).

The K cell in L1 larvae has a rectal cell morphology as seen by electron microscopy (EM) and by DIC microscopy (Figure 21). It has a hypodermal nucleus, large and flat and with a visible nucleolus (as already observed by Sulston and Horvitz, 1977), and forms apical junctions with

C.elegans gene	Orthologues	K	DVB
Epithelial markers			
dlg-1	Discs-large	+	-
ajm-1	Apical junction molecule	+	-
hmr-1	E-cadherin	+	-
let-413	SCRIB	+	-
lin-26	Zinc-finger transcription factor	+	-
Rectal markers			
col-34	Cuticle collagen	+	-
egl-5	HOX transcription factor	+	-
sox-2	SOX transcription factor	+	-
ceh-6	POU transcription factor	+	-
Pan-neuronal markers			
rgef-1	RAS guanyl releasing protein	-	+
unc-33	CRMP	-	+
unc-119	UNC119	-	+
Gabaergic markers			
unc-47	VGAT	-	+
unc-25	GAD	-	+

Table 4. K is a rectal epithelial cell and DVB a GABAergic neuron. Summary of the epithelial, rectal, pan-neuronal and GABAergic genes expressed either in K or DVB.

its sister cell K'. As seen by EM, K and K' are indistinguishable in L1 larval stage (Figure 21A), even though K' does not divide and remains a rectal cell throughout the life of the worm. The

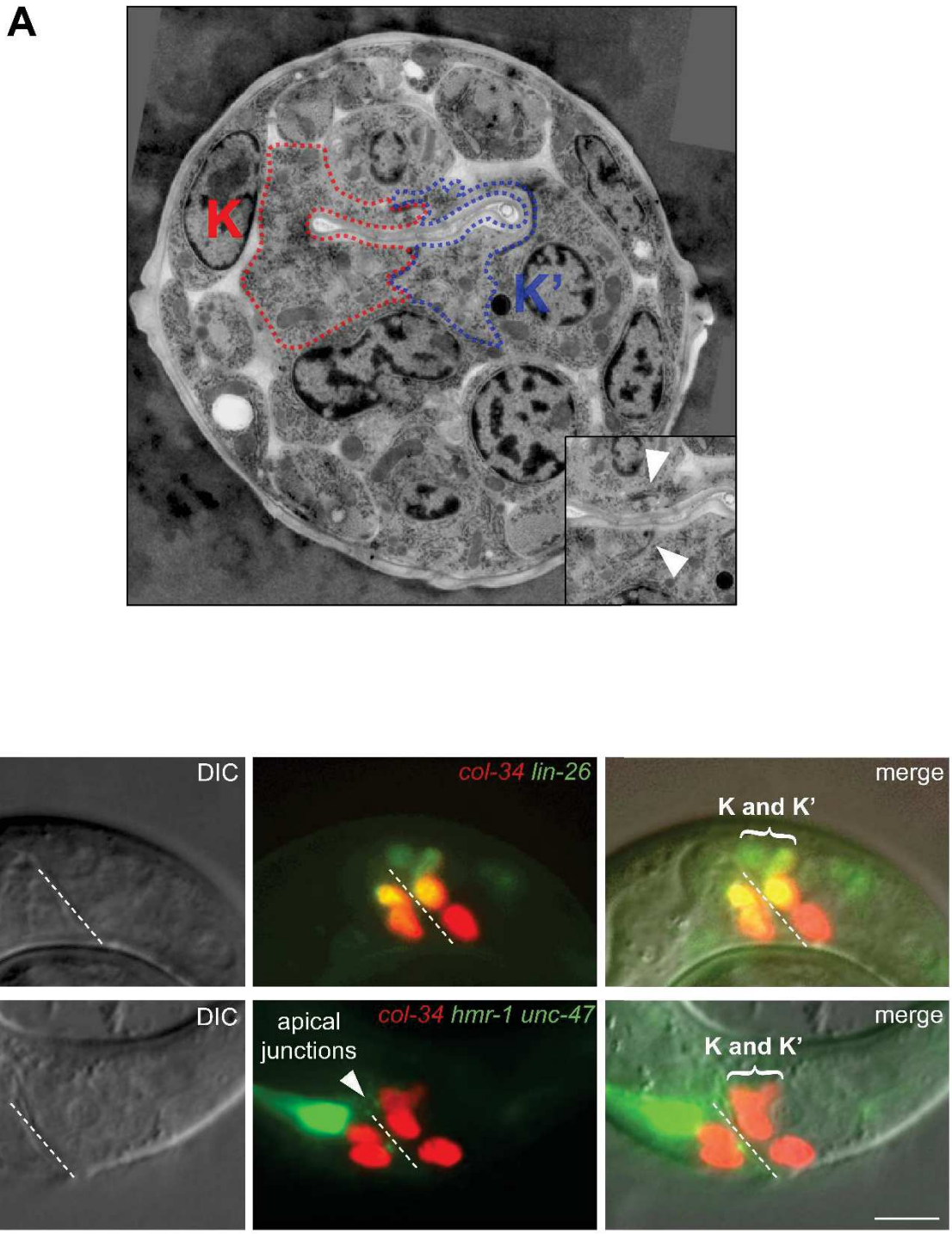

Figure 21. K is a rectal epithelial cell in L1 larvae. A) EM picture of a transversal section of a L1 worm showing K (left) and K' (right) rectal epithelial cells connected by apical junctions and forming the rectal lumen (picture from Sophie Jarriault and Yannick Schwab). **B)** DIC and fluorescence microscopy pictures of L1 worms: the rectal cells are marked with the rectal marker *col-34* (red) and express the epithelial transcription factor *lin-26* (above), and the E-cadherin *hmr-1* but do not express the GABAergic marker *unc-47/VGAT* (below). *hmr-1* and *unc-47* reporters are both with *gfp* but they can be recognised by subcellular localisation and cells that are positive (epithelial vs neurons). Scale bar = 5 µm.

expression in K of epithelial and rectal genes was observed using transgenic reporter strains through fluorescent microscopy. Epithelial genes such as the *lin-26* transcription factor (Labouesse et al., 1994), the Scribble orthologue *let-413* and the apical junction components *hmr-1/E-cadherin*, *dlg-1/Discs-large* and *ajm-1* are expressed in K. Moreover, K also expresses the rectal genes *col-34* (a collagen, whose reporter was used in this study to identify the rectal cells), *egl-5/HOX, sem-4/SALL*, *sox-2/SOX* and *ceh-6/POU* (defined by their expression in all the other rectal cells) (Figure 21B, Table 4). Finally, the importance of K (and its sister K') as a functional cell of the rectum is suggested by laser ablation experiments performed by Sulston, which demonstrated that the rectum is blocked in absence of K and K' (Sulston et al., 1983).

DVB is known to be a GABAergic neuron thanks to several studies both on its gene expression and its function. We confirmed the neuronal identity of DVB by checking the expression of both pan-neuronal and GABAergic markers (Table 4, Figure 22). We found that the pan-neuronal genes *rgef-1/RASGRP3*, *unc-33/CRMP* and *unc-119/UNC119* are expressed in DVB (Figure 22A, Table 4); we also observed expression of terminal differentiation genes *unc-25/GAD* and *unc-47/VGAT* (Figure 22, Table 4) thus confirming previous observations (Gendrel et al., 2016). Moreover, the morphology of DVB is completely different from the one of its mother cell as seen by DIC: the DVB nucleus is neuronal-like, smaller than K.a one and similar to DVA and DVC nuclei with which DVB forms the dorso-rectal ganglion (Figure 22, DIC). Finally, the use of transcriptional reporters (with *gfp* controlled by specific promoters but not fused to a coding sequence) allows to visualise by fluorescence microscopy the DVB neurite that elongates anteroventrally.

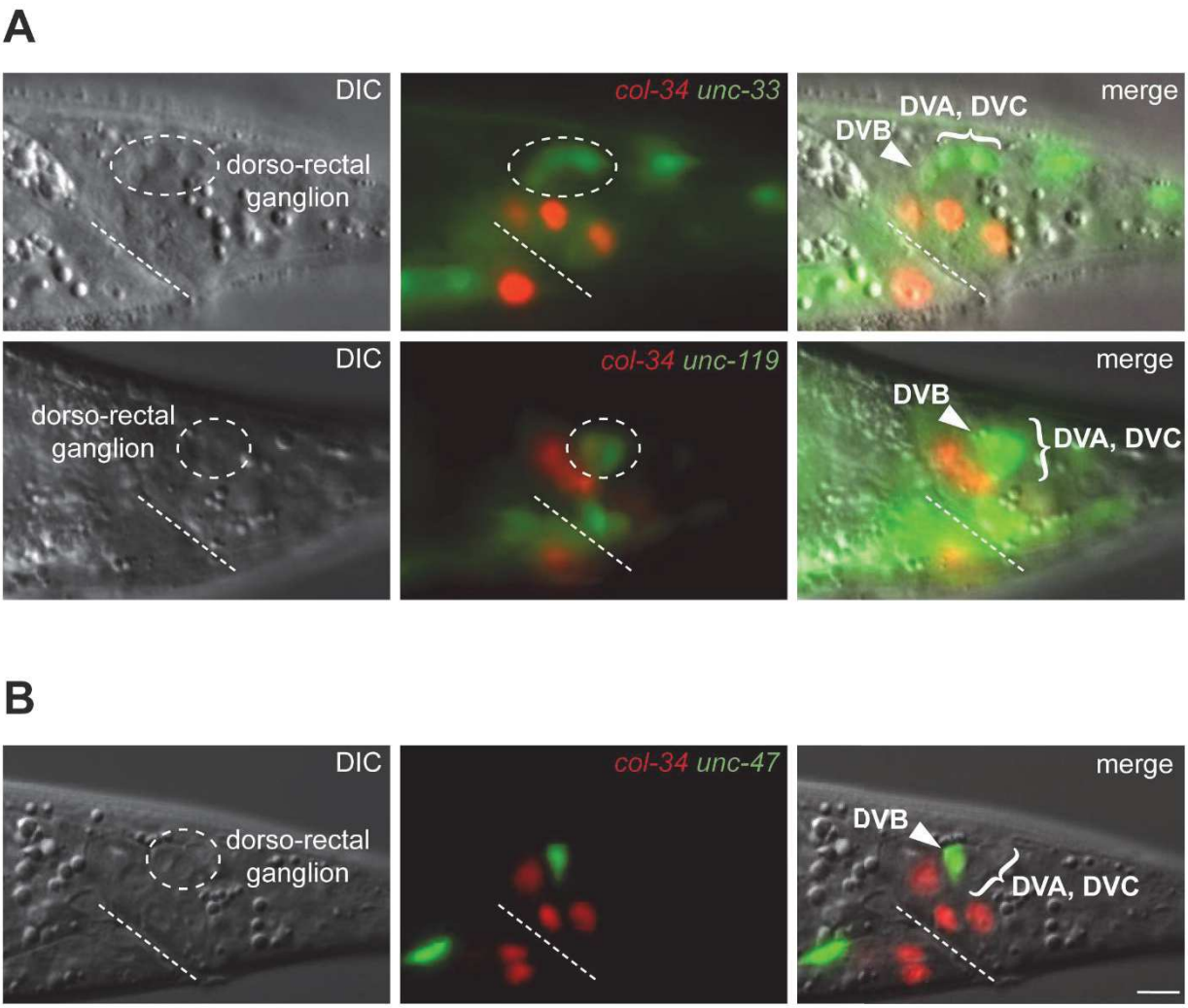

Figure 22. DVB is a GABAergic neuron. A) DIC and fluorescence microscopy pictures of the tails of L3/L4 worms showing the dorso-rectal ganglion, the rectal cells (in red) and neurons marked with pan-neuronal genes (in green) *unc-33/CRMP* (above) and *unc-119/UNC119* (below). In the dorso-rectal ganglion three neuronal nuclei are visible and marked with the pan-neuronal markers: DVA, DVB (faintly marked with *col-34* as well) and DVC. **B)** DIC and fluorescence microscopy pictures of the tail of a L4 worm showing the dorso-rectal ganglion, the rectal cells (in red) and DVB in the dorso-rectal ganglion marked with the GABAergic marker *unc-47/VGAT*. Scale bar = 5 µm.

Overall, these morphological, gene expression and functional assays demonstrate that K and DVB are two different types of differentiated cells playing a role in their respective tissues. Together with the knowledge of the lineage relationship between K and DVB, these results show that K-to-DVB is a *bona fide* transdifferentiation event.

1.2 A cell division is required for K-to-DVB; it is stereotyped, oriented and asymmetric and does not directly produce a neuron

K gives rise to DVB after mitosis. Thus, we first investigated whether K division *per se* is required for DVB formation and afterwards we characterised the dynamics of K-to-DVB encompassing K division.

1.2.1 K division is required for DVB formation

To assess the role of cell division in DVB formation we took advantage of a mutant background which impairs K cell division. We found that *lin-5/NuMa* mutation at restrictive temperature (25°C) prevents K cell division by affecting cytokinesis, even though DNA replication seems to occur as visualised through chromatin marker *his-24::mCherry* (Figure 23A). This phenotype is expected considering the function of LIN-5/NuMA in the regulation of the mitotic spindle (see 3.5, Morin and Bellaïche, 2011). We observed that in *lin-5/NuMA* mutants the percentage of worms lacking DVB increases with the temperature (from 20 to 25°C) and that it correlates with the percentage of worms not completing K division (Figure 23B). Thus, we performed a score-recover-score experiment to check cell division in L1 (looking also for misoriented K divisions, as described in the next paragraph in wild types) and DVB formation in L4 in the same animals and this approach confirmed what was suggested by correlation: DVB does not form in absence of cell division; conversely, when K divides, DVB always develops (Table 5). No misoriented K division were observed in this background (see below for criteria).

A

***lin-5(ev571)*, 20°C**		**K division**		
		wt orientation	**misoriented**	**no division**
DVB presence	Yes	41	0	0
	No	0	0	17

B

***lin-5(ev571)*, 22°C**		**K division**		
		wt orientation	**misoriented**	**no division**
DVB presence	Yes	13	0	0
	No	0	0	13

Table 5. Formation of DVB depends on the occurrence of K division. A) Summary of the results obtained with a score-recover-score of *lin-5/NuMA* mutants about occurrence and orientation of K division in L1s and presence of DVB based on *unc-47* expression in the same animals in L4s, grown at 20°C. **B)** Same as in A), but worms grown at 22°C. The score-recover-score experiments was not performed at the restrictive temperature of 25°C because in that condition K does not divide in >90% of *lin-5/NuMA* mutant worms (no relationship between orientation of K division and DVB formation to be checked).

These results demonstrate that K division is required for the development of the DVB neuron in the worm.

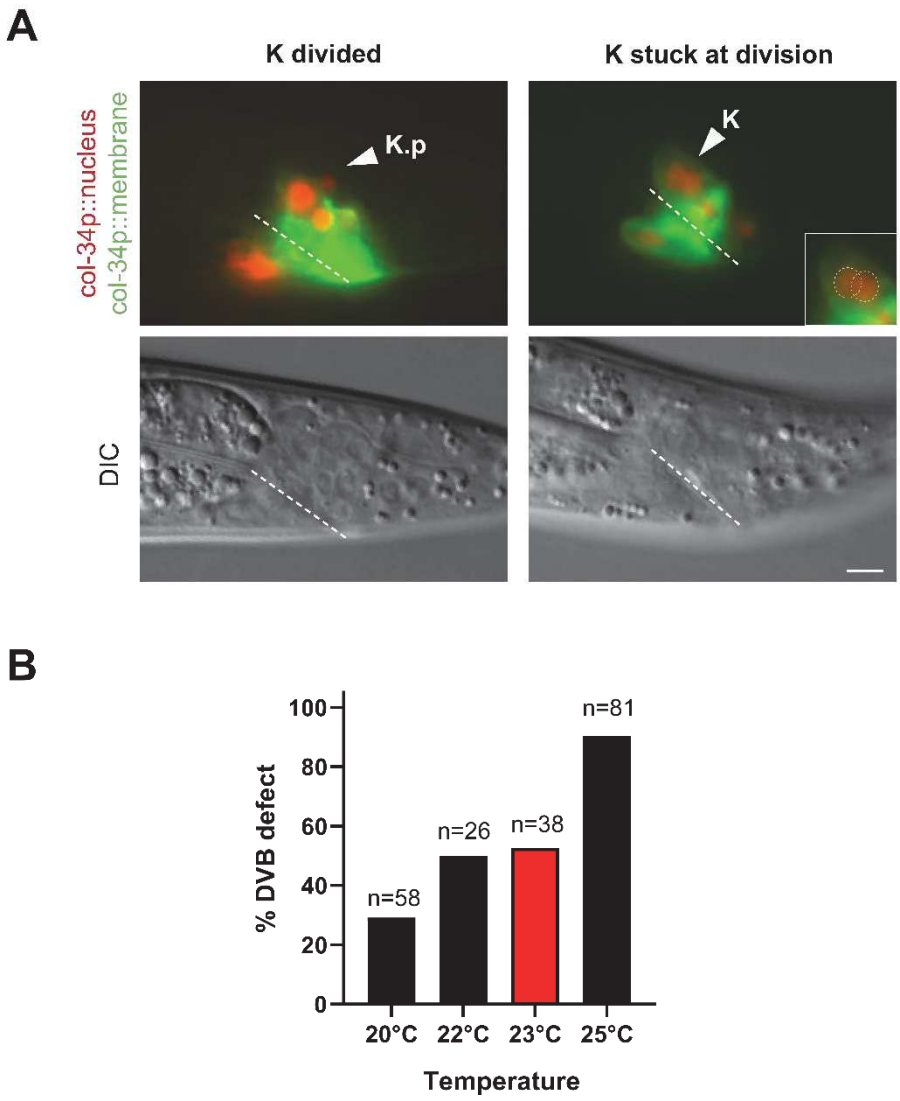

Figure 23. K division is required for DVB formation. A) Pictures of *lin-5(ev571ts)* mutants in which K division has (left) or has not (right) occurred. Absence of K cytokinesis in the worm on the right is showed by the presence of a unique cytoplasmic membrane around two nuclei. Scale bar = 5 µm. **B)** Histograms summarising the percentage of absence of DVB in *lin-5* mutant worms at different restrictive temperatures. Black bars: transgenic strain for a GABAergic reporter; red bar: transgenic strain for the rectal membranes and nuclei reporters (DVB presence scored based on occurrence of cell division, lack of *col-34* expression, and nuclear morphology).

1.2.2 K divides without losing cell-cell junctions in late L1 and DVB differentiates from K.p in L2 larval stage

Given the importance of K division for DVB formation, we next aimed to characterise it starting from the description of the timeline of cellular events. In order to visualise the rectal cells' nuclei and apical junctions, we used a rectal reporter consisting of the *mCherry* gene fused to histone *his-24* gene controlled by *col-34* rectal promoter (*gaIs245[col-34p::his-24::mCherry]* array) and *hmr-1/E-cadherin* reporter (*fpIs17[hmr-1::gfp]*). At the same time we used an *unc-47/VGAT* reporter (*oxIs12[unc-47p::gfp]*) to monitor the differentiation of DVB (see Figure 21B, bottom panel).

We observed that K divides in less than 10 minutes, at around 11.5 hours (h) post hatching (PH) at 20°C. Using time-lapse spinning disk microscopy, we saw that K.p buds off from the K cell posteriorly (in agreement with the name that it was given), above the F rectal cell, without disrupting K apicobasal polarity. We did not observe any rounding of the K cell as often associated with cell division (Cadart et al., 2014), nor loss of adherence to its K' cell partner, allowing the maintenance of the integrity of the rectum during K division (although we cannot exclude that we did not observe it because of limitations in time resolution). After the L1/L2 molt, which corresponds to 15 h PH, we started to observe *unc-47/VGAT* expression in K.p in few young L2 larvae, and 16 to 17 h PH we were able to detect *unc-47/VGAT* expression in all the L2s scored. On the other hand, K and K.a never express *unc-47/VGAT* (Figure 24). Since *unc-47/VGAT* is a terminal differentiation gene for GABAergic identity, I will henceforth use "K.p" when referring to K posterior daughter until 16h PH, and "DVB" after this time point. *unc-47/VGAT* reporters have been used in this study to identify DVB in all the cases unless otherwise specified.

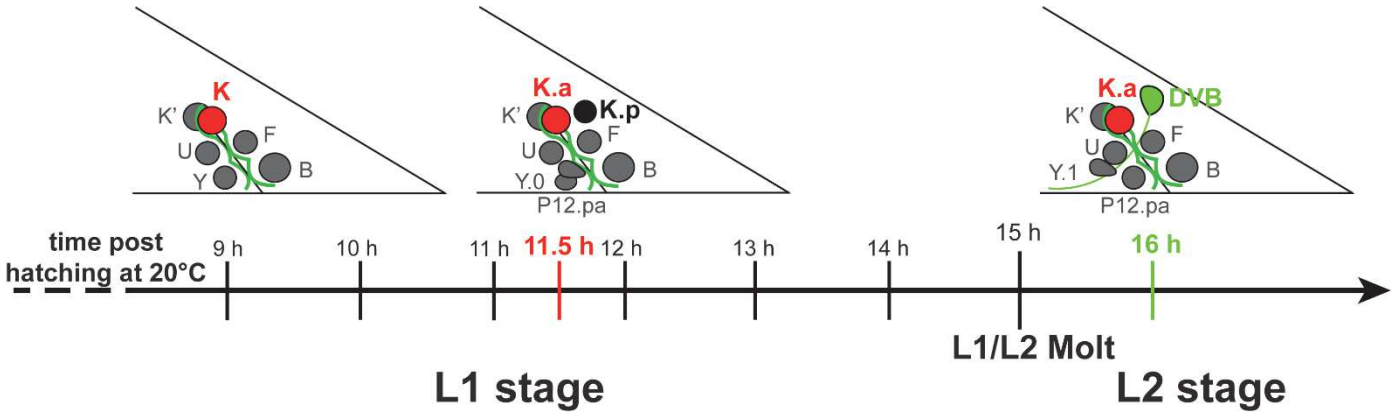

Figure 24. K divides 11.5 h PH and DVB differentiates by 16 h PH. Timeline of K division and DVB differentiation. K and K.a nuclei are represented in red (like they appear in our images) and K.p in black (identity not defined). DVB appears in light green. The apical junctions are represented in dark green, connect rectal cells and are maintained in K during its division.

These observations suggest that K divides with an anteroposterior orientation while maintaining its apical junctions and thus its position in the rectum. Complete differentiation of DVB from the posterior daughter occurs in a few hours in L2 stage, differently from PDA differentiation which takes longer and it is completed in the L3 stage (Jarriault et al., 2008).

1.2.3 K division is oriented and asymmetric

To gain further insights into the features of K cell division, we analysed its orientation and its asymmetry using a quantitative approach. To quantify the orientation of K division we measured the angle formed by the K.a and K.p nuclei alignment with the rectal slit, maximum 1 h after cytokinesis. This analysis showed that the orientation of K division is stereotyped among animals, forming an angle of 52.0±10.3° (Figure 25A). We next assessed the asymmetry of K division: for this purpose, the nuclear volumes of K.a and K.p were used as a readout of ACD as done for instance in a previous work on T, B , F and U cells' ACDs (Herman, 1994). The quantification of the nuclear volumes of K.a and K.p 1 h after K division, using the *col-34p::his-24::mCherry* reporter as proxy, revealed an asymmetry in K.a and K.p nuclear volumes with K.a nucleus being 1.7-fold bigger than K.p one (Figure 25B).

This quantitative characterisation shows that K division is stereotyped, oriented in space and asymmetric.

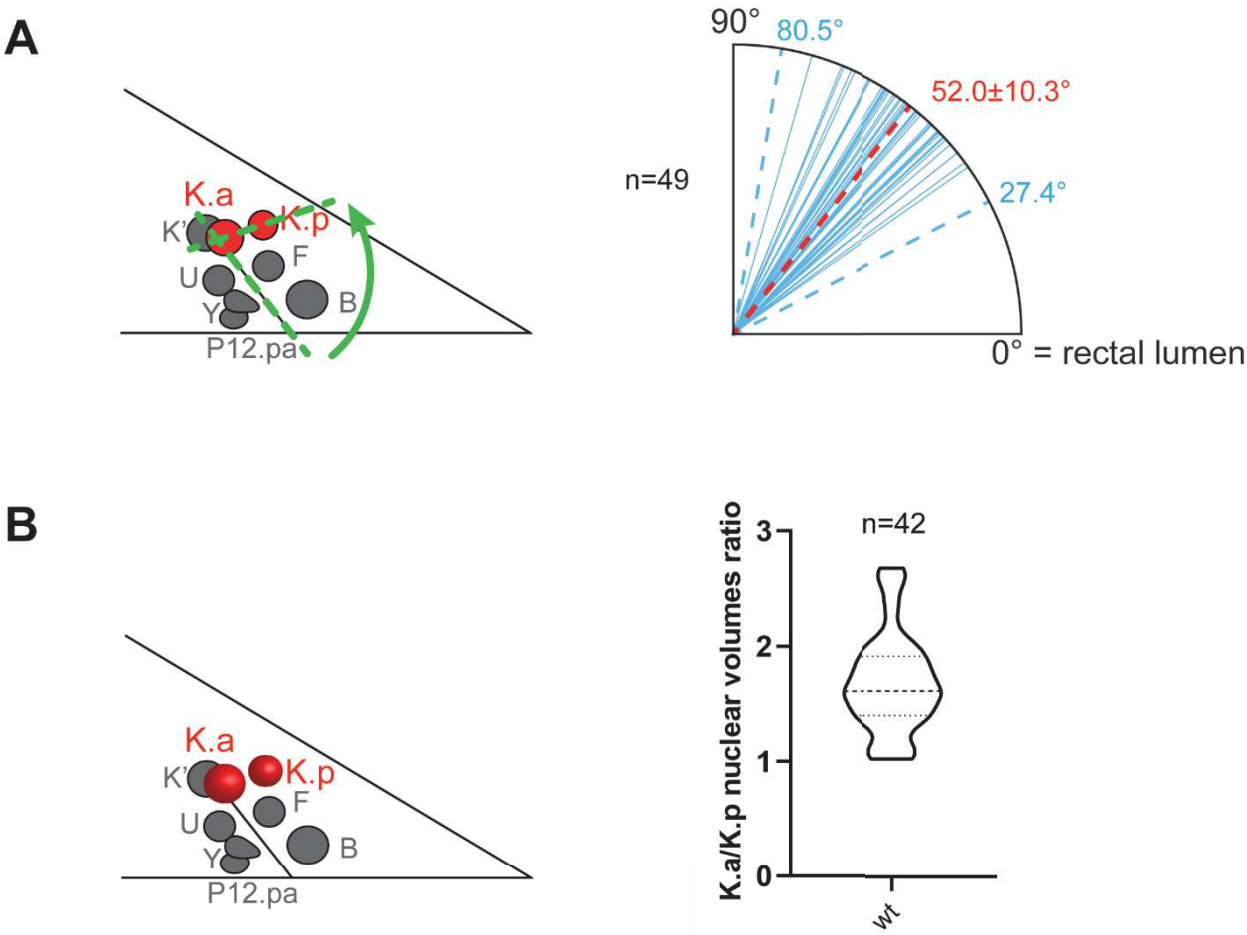

Figure 25. K division is oriented and asymmetric. A) Orientation of K division in wild type worms quantified as the angle formed by the rectal slit with K.a and K.p nuclei alignment maximum 1 h after K division. In red, mean and standard deviation; in light blue, measured angles with dashed lines for max and min. **B)** Asymmetry of K division quantified as the ratio between K.a and K.p nuclear volumes maximum 1 h after K division. K.a nucleus is almost twice bigger than K.p nucleus.

1.2.4 ACD of K cell does not produce a neuronal K.p daughter cell

Since the K cell divides in an asymmetric manner, we examined whether cell division directly produces a neuronal daughter. We focused our attention on K.p (and K.a) identity shortly after division. We observed that the basolateral marker LET-413/Scribble is present in K.p at the same level as in K and K.a in all the animals. This presence is confirmed when a destabilised version of LET-413::GFP protein is used (see MATERIALS AND METHODS) (Figure 26A, left panel). The proportion of animals showing LET-413::GFP protein in K.p starts to decrease after 16 h PH when the DVB terminal differentiation marker *unc-47/VGAT* is expressed (Figure 26B). Similarly, the epithelial transcription factor *lin-26* is expressed in K.a and K.p immediately after the division (Figure 26A, middle panel), but it rapidly disappears from K.p: in about 20% of the worms scored, *gfp* reporter expression starts to be lost in K.p 1 h after K division (Figure 26C). These results are supported by data previously obtained with LIN-26 antibody staining

(Labouesse et al., 1996). In addition, rectal markers such as *col-34* (collagen), *egl-5/HOX*, *sox-2/SOX* and *ceh-6/POU* are also expressed in K.p shortly after K division, but then they disappear in a few hours (Figure 26A and Table 6). Conversely, apical markers like AJM-1 and HMR-1 are not inherited by K.p while they remain in K.a, as expected based on the orientation

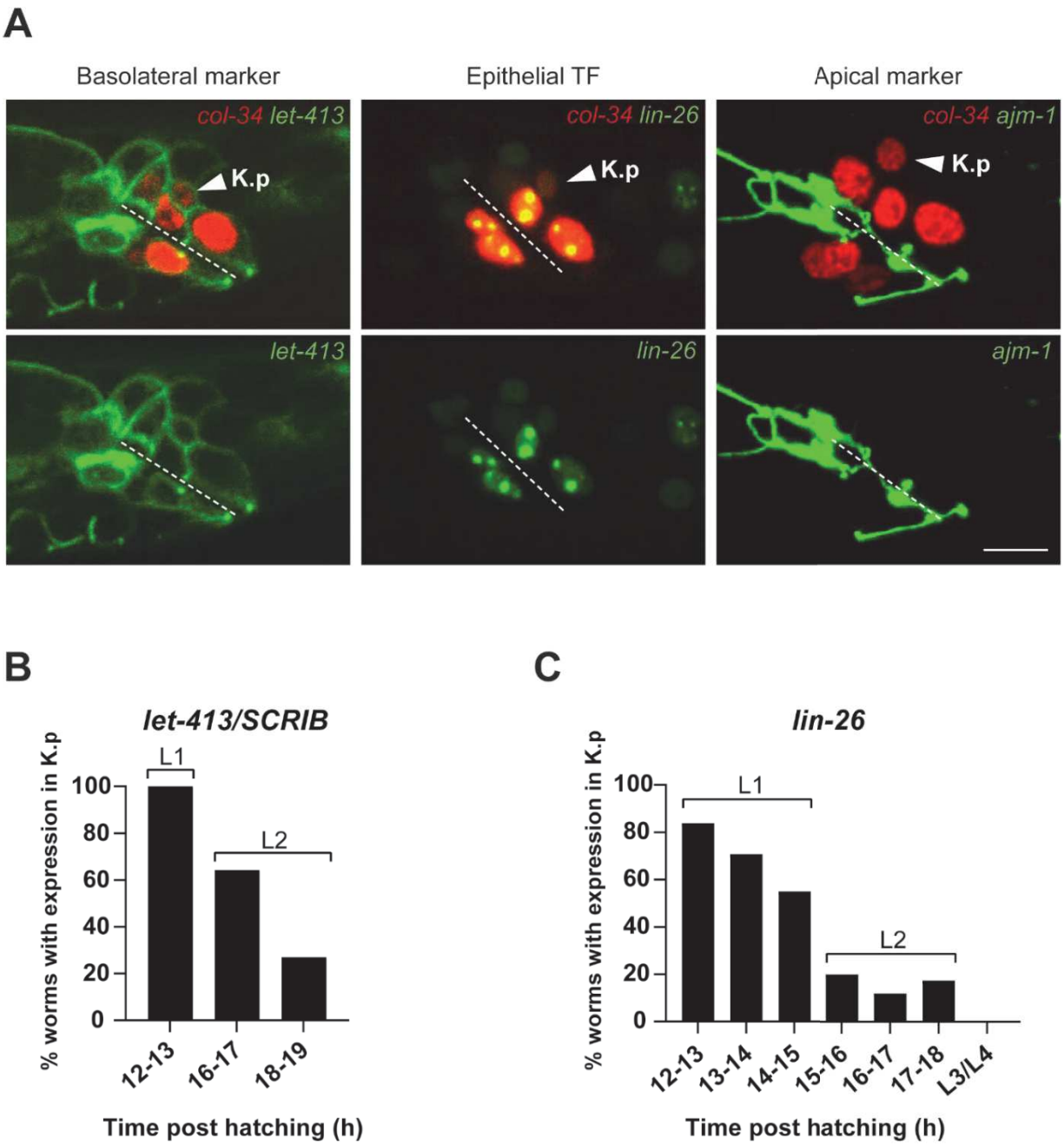

Figure 26. K.p retains some, but not all, epithelial features for some time after K division. **A)** Confocal microscopy pictures of wild type worms 1 h after K division showing the presence and expression respectively of the basolateral marker LET-413/SCRIB and of *lin-26* transcription factor (left and middle) and the absence of apical junction protein AJM-1 in K.p. Scale bar = 5 µm. **B)** Histograms showing the dynamic loss of LET-413/SCRIB fused to PEST sequence in K.p at different times after K division (16 h = DVB formed). **C)** Histograms showing the dynamic loss of *lin-26* expression in K.p at different times after K division.

of K division (Figure 26A, right panel). Thus, K divides asymmetrically resulting in the differential partitioning of apical junctions between the two daughters and in the generation of an apparently rectal K.p cell with epithelial features. Finally, we checked whether neuronal markers are present in K.p after K division: we found that none of the pan-neuronal genes *unc-33/CRMP* (n=40) and *unc-119/UNC119* (n=28) and GABAergic terminal differentiation gene *unc-25/GAD* (like *unc-47/VGAT*) we assessed are expressed, but rather they appear later (Table 6) overlapping for some hours at least with the epithelial marker *let-413/SCRIB*.

C.elegans gene	Orthologues	K	K.a	K.p	DVB
Epithelial markers					
dlg-1	Discs-large	+	+	-	-
ajm-1	Apical junction molecule	+	+	-	-
hmr-1	E-cadherin	+	+	-	-
let-413	SCRIB	+	+	+	-
lin-26	Zinc-finger transcription factor	+	+	+	-
Rectal markers					
col-34	Cuticle collagen	+	+	(+)	-
egl-5	HOX transcription factor	+	+	+	-
sox-2	SOX transcription factor	+	+	+	-
ceh-6	POU transcription factor	+	+	+	-
Pan-neuronal markers					
rgef-1	RAS guanyl releasing protein	-	-	-	+
unc-33	CRMP	-	-	-	+
unc-119	UNC119	-	-	-	+
Gabaergic markers					
unc-47	VGAT	-	-	-	+
unc-25	GAD	-	-	-	+

Table 6. K.a is a rectal epithelial cell like its mother cell K, while K.p retains rectal and some epithelial features at birth. Summary of the epithelial, rectal, pan-neuronal and GABAergic genes expressed in K, K.a, K.p and DVB.

Altogether, these data show that K division is required for DVB formation and it is oriented and asymmetric. K ACD gives rise to two daughter cells: K.a, with a large nucleus and which retains all the rectal epithelial features; K.p, with a smaller nucleus and which retains rectal and, partially, epithelial features while still lacking the expression of neuronal identity genes. This result suggest that successive steps are required to convert K.p into a GABAergic motor neuron.

1.3 K-to-DVB requires the Wnt/β-catenin asymmetry pathway

ACD coupled to mitotic spindle orientation leads to binary diversification of cell fates in several instances in *C. elegans*, from early embryonic development to larval stages, and the Wnt signalling pathway is known to orient the mitotic spindle (Schlesinger et al., 1999) and to regulate several ACDs in *C. elegans* (Mizumoto and Sawa, 2007; Sawa and Korswagen, 2013) (see 3.3.1). Thus, we tested the involvement of the Wnt signalling pathway components in K-to-DVB at different steps.

1.3.1 *lin-44/*WNT, *lin-17/FZD* and downstream intracellular components of the Wnt/β-catenin asymmetry pathway are required for DVB formation

To assess whether the Wnt/β-catenin asymmetry pathway is required for K-to-DVB, we screened the mutants for the most important Wnt pathway components for defects in DVB formation (Figure 27). First, we analysed the phenotypes of two WNT ligand mutants, *lin-44* and *egl-20,* expressed in the tail and in the rectal area (Harterink et al., 2011): in absence of

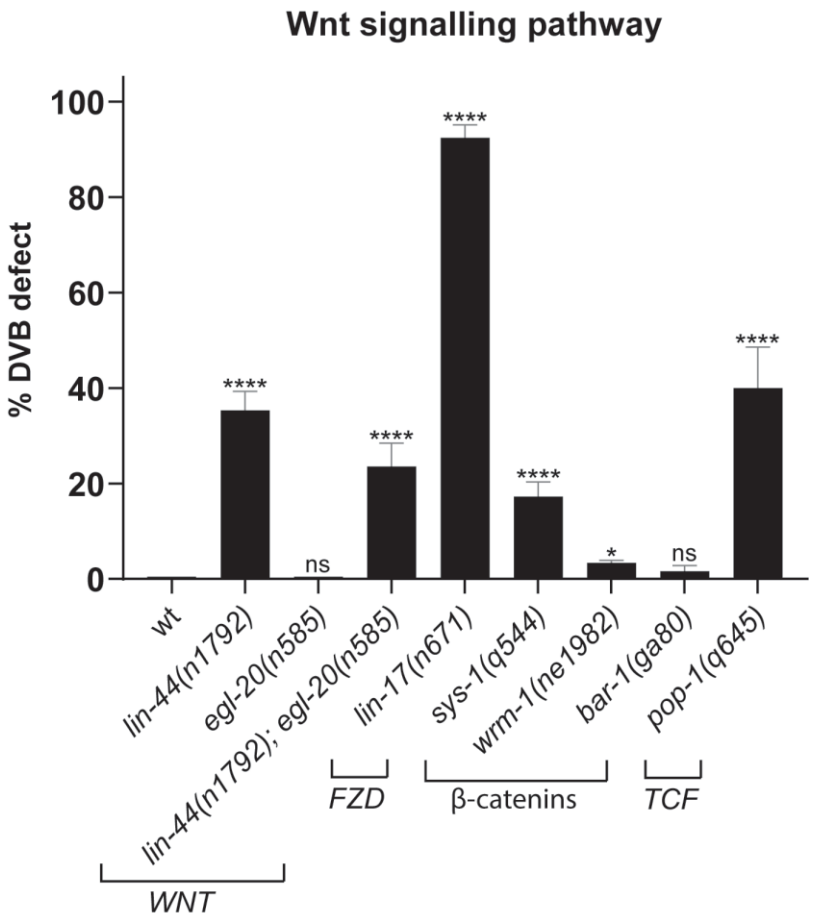

Figure 27. Wnt signalling pathway is required for K-to-DVB. Histograms summarising the percentages of worms without DVB in mutant backgrounds for different Wnt signalling pathway genes. DVB presence based on *unc*-47 expression and visible neurite. N≥3; mean and SD shown.

lin-44 32% of the worms lack DVB, while *egl-20* is dispensable for DVB formation. Moreover, double mutants *lin-44; egl-20* do not show a higher penetrance of “No DVB” phenotype. Next, we chose to test *lin-17/FZD* as WNT receptor gene, being expressed in the tail area and required for ACD of T blast cell and B, F and U (rectal) blast cells in the male (Herman, 1994; Sawa et al., 1996; Sternberg and Horvitz, 1988). We found that in *lin-17/FZD* mutants DVB is absent in more than 90% of the worms. In addition, we tested the involvement of all the three β-catenins which play a role in the Wnt signalling pathways in *C. elegans* (Sawa and Korswagen, 2013). Our observations demonstrate that whereas *bar-1* of the conserved canonical Wnt pathway does not play an important role, both *sys*-1 and *wrm-1* of the Wnt/β-catenin asymmetry pathway are required for K-to-DVB. 17% of *sys-1* mutants lack DVB, while in *wrm*-1 mutant background 3.4% of the worms are affected. Finally, we scored the phenotype of *pop-1/TCF* mutants and found that DVB is absent in 40% of the worms in this background. The low penetrance of the “No DVB” phenotype in *pop-1/TCF* and *sys-1/β-catenin* mutants (compared to *lin-17/FZD*) could be due to maternal contribution, as these strains are maintained with a genetic balancer. Moreover, in absence of *sys-1, bar-1/β-catenin* might partially rescue its function as described in other contexts (Green et al., 2008). Linked to this, the *pop*-1 allele *q645* affects the interaction of POP-1 protein with SYS-1/β-catenin, but not with BAR-1/β-catenin (Kidd et al., 2005). For *wrm-1(ne1982ts)* mutants, the low defect might be due to the hypomorphic nature of this mutation even at restrictive temperatures, as discussed in previous studies on T and V5.p cells (Takeshita and Sawa, 2005).

These results demonstrate that the Wnt signalling pathway, especially genes of the Wnt/β-catenin asymmetry pathway, are required for DVB formation.

1.3.2 K-to-DVB does not require the non-canonical PCP pathway nor other WNT-dependent pathways

To obtain a complete view of the regulation of K-to-DVB by WNTs, we decided to extend our analysis to components of the non-canonical Wnt pathways (which either depend on WNT ligands or share some downstream components with the canonical Wnt signalling pathway).

First, we tested the *C. elegans* orthologues of the PCP pathway components by checking the presence of DVB in their mutants (even though PCP pathway regulates planar cell divisions in other organisms and it has not been described in this role in *C. elegans*. Moreover, K division does not appear planar, but rather apicobasal). By scoring *vang-1/Vangl* and *fmi-1/Flamingo* mutants we could not observed any impact on DVB formation (Figure 28A) except for abnormal neurites in *fmi-1/Flamingo* mutants, consistently with a role of PCP pathway components in axon guidance and neurite formation and not in cell polarity in *C. elegans* (Cravo and van den

Heuvel, 2020). We next assessed a possible role of other WNT receptors, namely *lin-18/RYK* and *cam-1/ROR*. *lin-18/RYK* is known to be involved in cell migration and neurite growth (Sawa and Korswagen, 2013), but it was also shown to act in parallel to *lin-17/FZD* in vulval cells specification (Inoue et al., 2004) by regulating POP-1/TCF localisation (Deshpande et al., 2005). *cam-1/ROR* is a transmembrane RTK with a WNT binding domain similar to FZD's one and was shown to negatively regulate the canonical Wnt pathway and to control the migration of different cell types (Green et al., 2008; reviewed by Sawa and Korswagen, 2013). We did not observe any significant impact on DVB formation in either mutants (Figure 28B). As mentioned above, *lin-18/RYK* was shown to act in parallel to *lin-17/FZD* in other cell types (Inoue et al., 2004); however, we did not further test the impact of the *lin-17/lin-18* double mutant on K-to-DVB, since in our case the absence of DVB is already very high in the *lin-17/FZD* single mutant and *lin-18/RYK* has no impact at all. Finally, we tested whether *ced-10/Rac* is required for K-to-DVB as it was shown to be involved in EMS ACD downstream to the non-canonical Wnt signalling pathway (Cabello et al., 2010); however, DVB formation does not seem significantly affected in this background.

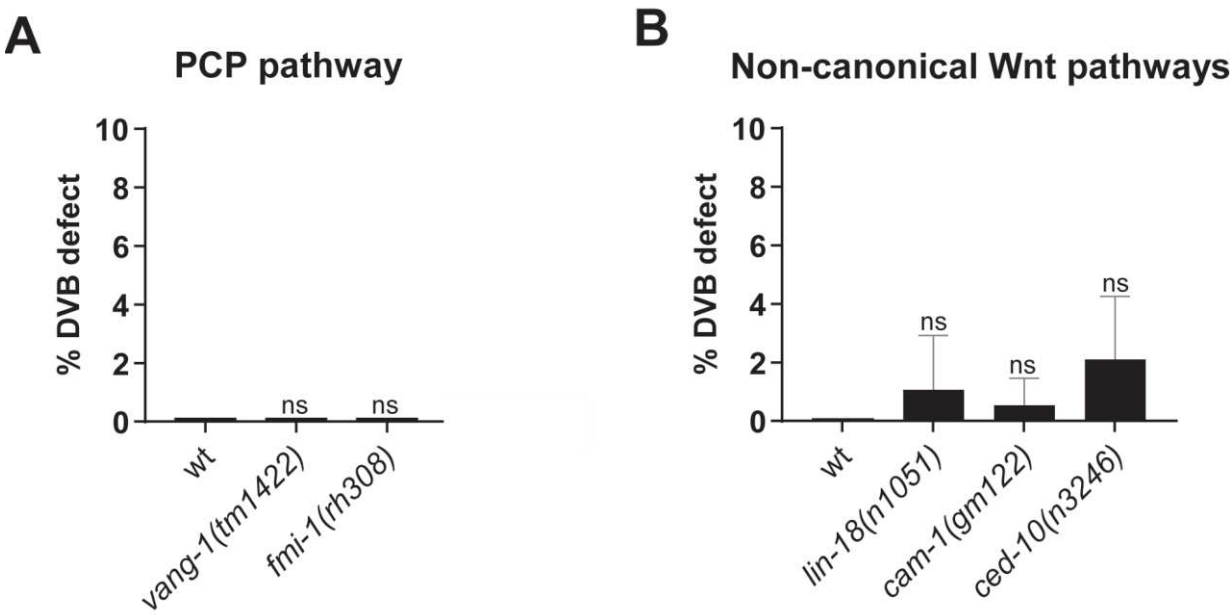

Figure 28. The non-canonical Wnt pathways are not required for K-to-DVB. A) Histograms showing the percentage of "No DVB" worms in mutant backgrounds for genes of the PCP pathway. **B)** Histograms showing the percentage of "No DVB" worms in mutant backgrounds for non-canonical Wnt-dependent pathways (*lin*-18 and *cam*-1) or their downstream effectors (*ced-10*). DVB presence based on *unc*-47 expression and visible neurite. N=3; mean and SD shown.

These results demonstrate that while the Wnt/β-catenin asymmetry pathway is involved in K-to-DVB, other WNT-dependent pathways are not required. Thus, we decided to focus on the roles of the Wnt/β-catenin asymmetry pathway during K-to-DVB.

1.3.3 *lin-17/FZD* is required for the asymmetry and the orientation of K division

First, we wanted to assess whether the Wnt signalling pathway regulates the asymmetry of K cell division, as this might be one of the steps at which the pathway is required for DVB formation. As observed for T, B, F and U cells, the asymmetry of K division is lost in the loss of function mutants *lin-17(n671)*: the measurement of K.a and K.p nuclear volumes shortly after K division demonstrated that K.p nucleus is almost as big as K.a nucleus, with a hypodermal appearance (Figure 29A). Moreover, in the lab previous observations of the localisation of LIN-17/FZD just before K division showed that LIN-17/FZD is transiently asymmetrically localised to the posterior membrane, where K.p buds off. In agreement with a link between FZD localisation and spindle positioning, the orientation of K division appears misoriented in about 8.9% of the worms in the *lin-17/FZD* mutant background compared to the range of angles observed in wild type (we think that this low percentage of misorientation is due to the parallel influence of physical constrains on orientation of K division). Moreover, the average angle of K division in *lin-17/FZD* mutants, which is significantly different from the wild type, suggests that in this background K.p is often born more ventrally than in wild type worms (being around 43.4° instead of 52.0°), even if in a few worms observed K.p was born more dorsally (Figure 29B and Figure 25A).

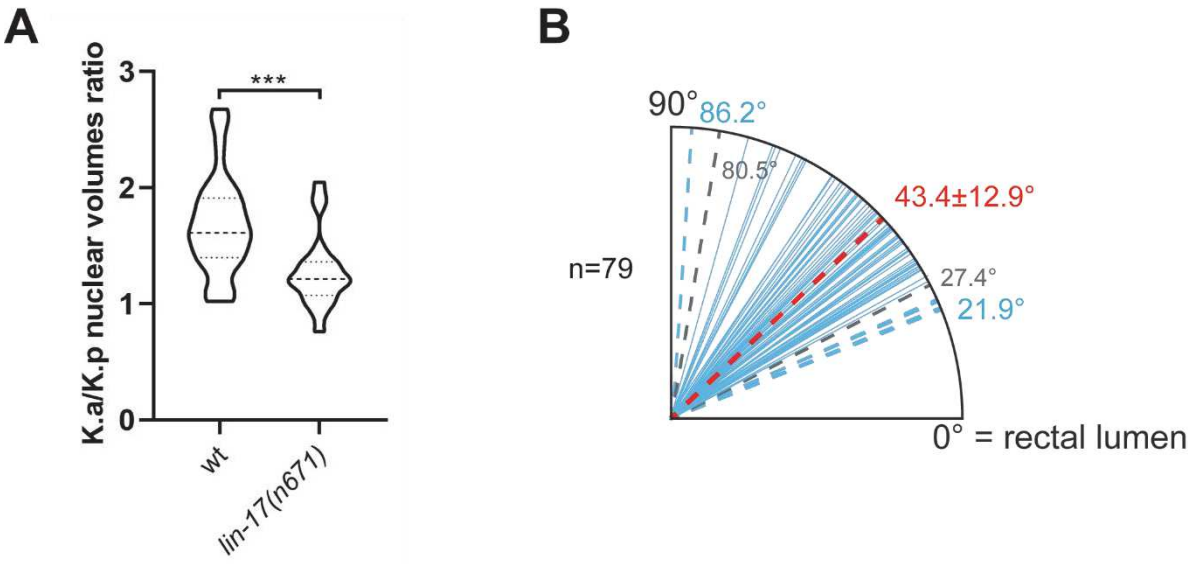

Figure 29. *lin-17/FZD* is required for the asymmetry and the orientation of K division. A) Representation of K.a/K.p nuclear volumes ratio in *lin-17/FZD* mutants, decreased almost to 1 as measured 1 h after K division. **B)** Representation of the angles of K division in *lin-17(n671)* mutants. In red, average value and standard deviation in *lin-17/FZD* mutants; in light blue, measured angles in *lin-17/FZD* mutants with dashed lines for angles out of the wild type range; in grey, wild type minimum and maximum values.

Thus, the phenotypes observed in *lin-17/FZD* mutants demonstrate that the Wnt signalling pathway is required for K ACD and capable of orienting K mitotic spindle. In the *lin-17/FZD*

mutants where the angle of K division is outside of the wild type range, K.p may be born either more ventrally (more often) or dorsally. Even though there is no correlation between the percentage of worms with K division misorientation and the worms without DVB, the division defects observed in *lin-17/FZD* mutants might be in part responsible of the absence of DVB.

1.3.3.1 *Orientation of K division per se is not required for DVB formation*

We decided to directly test whether the misorientation of K division alone might have an impact on K-to-DVB. The asymmetrical partitioning of the apical junctions between K.a and K.p could suggest that it is the posterior K daughter lacking the junctions (and maybe unidentified cell fate determinants) that can take on the neuronal fate. If this hypothesis were correct, the orientation of the spindle would be key to ensure the asymmetric segregation of these proteins. This occurrence would allow to define K division as an ACD where both cell extrinsic (WNT) and cell intrinsic mechanisms (based on K intrinsic cell polarity) are at play.

We took advantage of mutants known to randomise the cell division axis, but not directly involved in the Wnt signalling pathway (Gotta and Ahringer, 2001). Since all temperature conditions tested on the *lin-5(ev571ts)* (NuMA) mutants resulted in either DVB formation or absence of cytokinesis, precluding its use for our purpose (see 1.2.1), we examined the

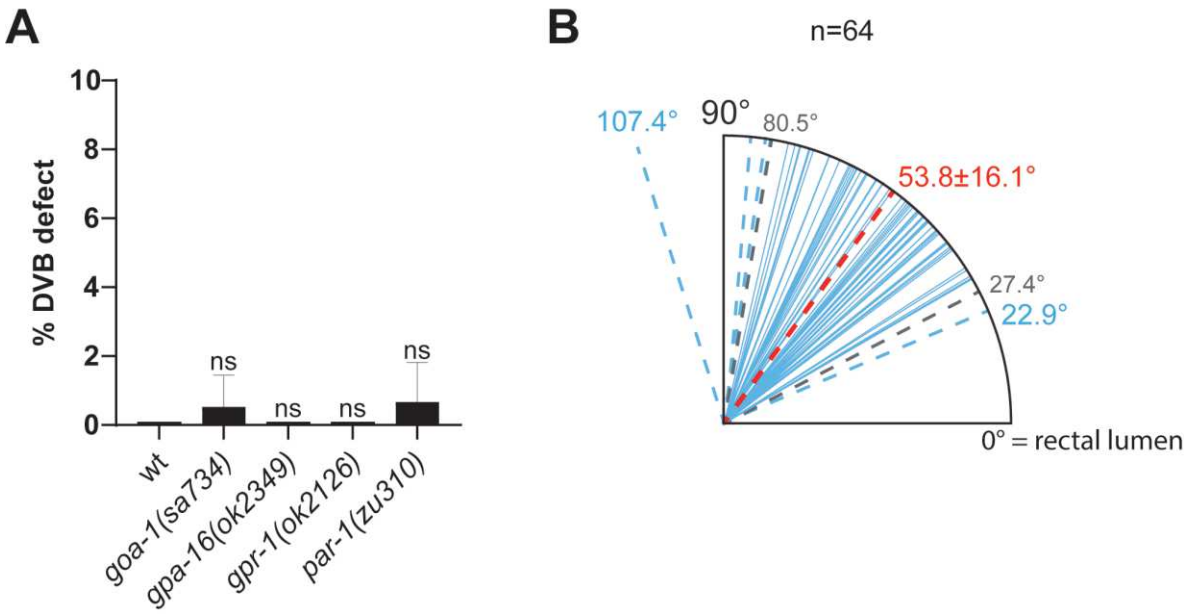

Figure 30. DVB formation is not affected when spindle orientation factors or *par* genes are mutated. A) Histograms showing the percentage of worms without DVB in mutants for the Gα genes and *gpr-1/LGN* involved in spindle orientation in *C. elegans* zygote and the *par* gene *par-1*. The low penetrance of DVB absence is due to an impairment in K cytokinesis. N=3; mean and SD shown. **B)** Representation of the angles of K division in *goa-1(sa734)* mutants. In red, average value and standard deviation in *goa-1* mutants; in light blue, measured angles in *goa-1* mutants with dashed lines for angles out of the wild type range; in grey, wild type minimum and maximum values.

division angle in a *goa-1(sa734)* (Gα) null mutants which display a very low 0.9% "No DVB" defect (due to absence of K division) (Figure 30A). We found that the average angle of division in *goa*-1 mutant is 53.8°, not significantly different from the wild type one (52.0°) but with a significantly higher variance (Figure 30B and Figure 25A). Since we observed 7-8% of the animals exhibiting an abnormal division angle in late L1/early L2 compared to the wild type range (a number reminiscent of the out-of-range misorientation in *lin-17/FZD* mutants), we also used a score-recover-score strategy to assess how these angles impacted DVB formation. We found that misoriented K division did not translate into any DVB defect (Table 7), in accordance with the lack of correlation between misoriented K division and DVB absence in *lin-17/FZD* mutants. We also tested whether we could find any "No DVB" defect in *gpa-16* mutants (the other Gα) or in *gpr-1/LGN* mutants: however, as for *goa-1*, we could not find worms lacking DVB in these backgrounds. Finally, in support to a lack of cell intrinsic mechanisms regulating K ACD together with Wnt, we found that the posterior *par* gene *par-1* is not required for K-to-DVB (Figure 30).

***goa-1(sa734)*, 20°C**		**K division**		
		wt orientation	**misoriented**	**no division**
DVB presence	Yes	58	4	0
	No	0	0	0

Table 7. Wild type orientation of K division is not required for DVB formation. Summary of the results obtained with a score-recover-score of *goa*-1 mutants about occurrence and orientation of K division in L1s and presence of DVB in the same animals in L4s, grown at 20°C (N=2). Even when K division is misoriented compared to wild type worms, DVB is always formed.

Thus, these results suggest that orientation of K division *per* se is not required for DVB formation. However, due to the low number of worms with orientation of K division affected in both *lin-17/FZD* and *goa-1* mutants, we think that other factors like physical constraints could impact on the orientation of K division.

1.3.4 WNT ligands might orient the polarity of K division

After having described the role of *lin-17/FZD*, we further focused on the function of the WNT ligands, which have been shown to have an instructive role for the polarisation of several ACDs - i.e. for deciding which daughter cell will activate the Wnt signalling pathway and adopt a specific fate - depending on the position which it comes from (see Introduction 3.3.1). By carefully observing the position of DVB with respect to its sister cell, we discovered that the polarity of K division is inverted in 11% of *lin-44/WNT* mutants, and strikingly it is inverted in 58% of *lin-44; egl-20* double mutants. In fact, while some "No DVB" defects are observed in

the double mutants, the majority of the defects are due to an inversion of polarity. Thus, in this double mutant background the absence of DVB is less penetrant than in *lin-44/WNT* single mutants, but the suppression of "No DVB" observed consists in most cases in the anterior daughter cell taking on the DVB fate (Figure 31). Considering that other three WNT ligands are present in the worm, including one, *cwn-1*, expressed just anteriorly to the rectum (Harterink et al., 2011), we suggest that in absence of a posterior signal (mainly LIN-44 but also EGL-20 in absence of LIN-44), anteriorly expressed WNT ligands can re-set the polarisation of K division by reverting it.

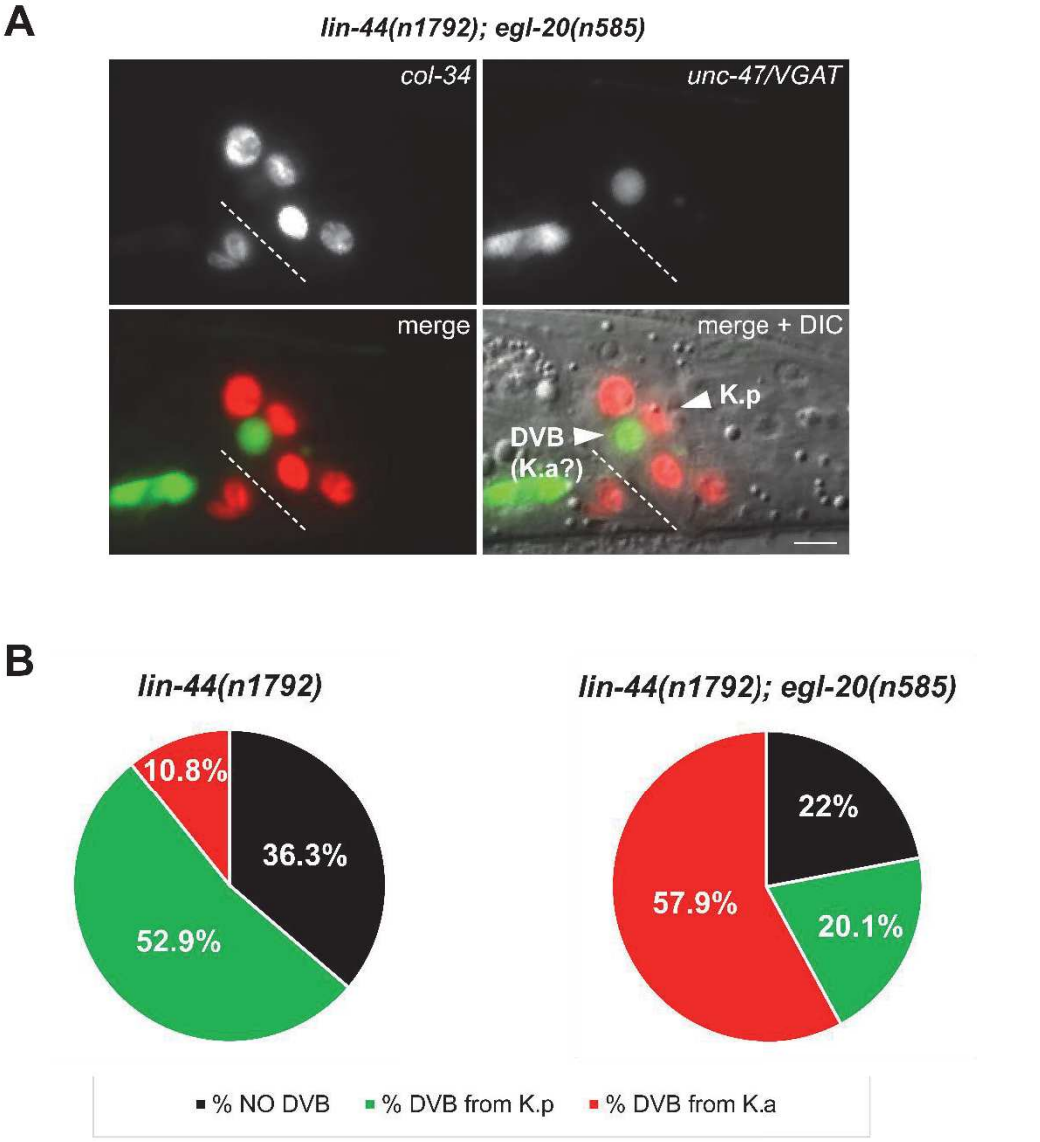

Figure 31. WNT ligands orient the polarity of K division. A) Fluorescence microscopy pictures of a double mutant *lin-44(n1792); egl-20(n585)* L4 worm where DVB appears to be formed from K anterior daughter. *col-34* highlights the rectal cells' nuclei and *unc-47/VGAT* is expressed in GABAergic neurons including DVB. Scale bar = 5 µm. **B)** Representation of the percentages of the indicated phenotypes in *lin-44/WNT* single mutant and in the double mutant strain showed in A). The double mutant strain has less "No DVB" defect, but DVB is mostly formed by K.a.

These observations reinforce the results described in the previous paragraph: the stereotyped orientation of the mitotic spindle is not directly necessary for DVB formation but rather it is a consequence of WNT ligand-dependent LIN-17/FZD position and activity, that probably directs spindle orientation through a non-canonical Wnt pathway and independently determines which daughter cell will activate the signalling cascade and become DVB through the Wnt/β-catenin asymmetry pathway.

1.3.5 The Wnt signalling pathway is required after K division to erase K.p epithelial identity

Since K division occurs in all the Wnt/β-catenin asymmetry pathway members tested, but no DVB is found, we examined the identity of the K.p daughter in *lin-17/FZD* mutant (which showed the highest "No DVB" defect, Figure 27) and whether the Wnt pathway can impact not only the polarity of the division but also the process after division. All the epithelial markers examined remain expressed in the cell that should have become a DVB: this was the case for continued *lin-26* and *let-413* expression, and even for *ajm-1* expression. Although this K apical junction protein is not inherited by K posterior daughter, AJM-1 was found in K.p in *lin-17/FZD* mutants suggesting that *ajm-1* gene was either not silenced or was re-expressed. Similarly, we found *col-34* (collagen), *egl-5/HOX*, *sox-2/SOX* and *ceh-6/POU* among the rectal markers retained in K.p in these mutants at L3/L4 larval stages. On the contrary, DVB neuronal markers, including pan-neuronal (*rgef-1/RASGRP3*, *unc-33/CRMP*, *unc-119/UNC119*) and GABAergic markers, are never expressed except in the few mutant worms where DVB develops (Table 8, Figure 32) in agreement with what observed by scoring DVB with *unc-47* reporter (Figure 27). Interestingly, by scoring double reporter strains, we could observe mutually exclusive expression of the rectal marker *col-34* with respect to pan-neuronal markers *unc-33/CRMP* and *unc-119/UNC119*, and of the rectal marker *egl-5/HOX* with respect to the pan-neuronal marker *rgef-1/RASGRP3* (Figure 41). Moreover, *col-34* reporter allows to see nuclear size and shape by driving the expression of the linker histone *his-24* gene fused to the *mCherry* gene: in every worm where we could see a rectal epithelial K.p based on *col-34* expression and nuclear morphology, we could not detect pan-neuronal expression (Figure 41); in the few worms where K.p nucleus appears neuronal-like and with faint mCherry the neuronal genes were brightly expressed.

Altogether, these findings suggest that in absence of *lin-17/FZD*, K.p retains a rectal epithelial identity and fails to express neuronal genes.

***C.elegans* gene**	**wt**	***lin-17(n671)***
Epithelial markers		
lin-26	-	+
let-413	-	+
ajm-1	-	+
Rectal markers		
col-34	-	+
egl-5	-	+
sox-2	-	+
ceh-6	-	+
Pan-neuronal markers		
rgef-1	+	-
unc-33	+	-
unc-119	+	-
Gabaergic markers		
unc-47	+	-
unc-25	+	-

Table 8. K.p remains rectal epithelial in *lin-17/FZD* mutants. Summary of the epithelial, rectal, pan-neuronal and GABAergic genes expressed in K.p in wild type vs *lin-17/FZD* mutant worms.

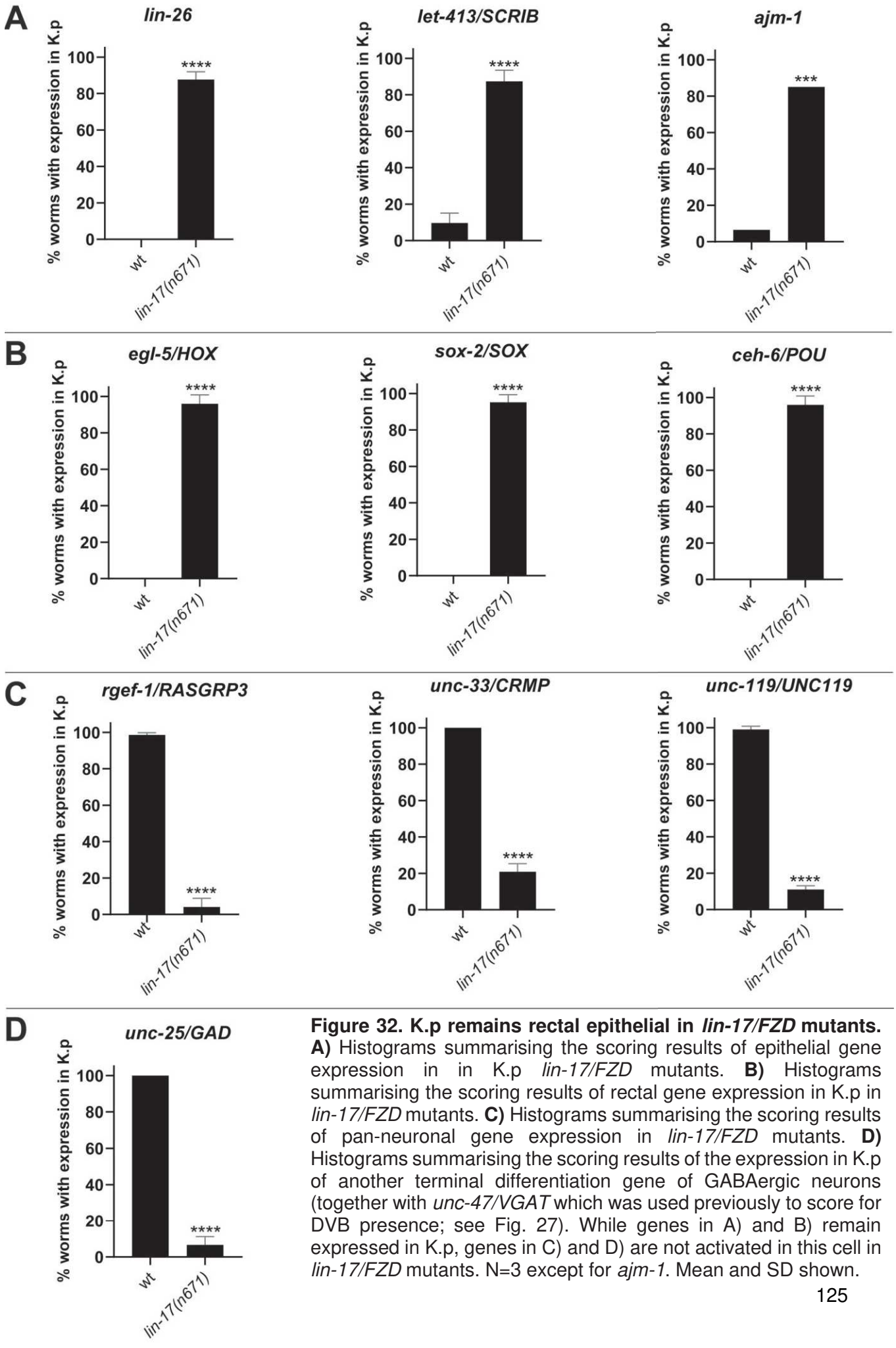

Figure 32. K.p remains rectal epithelial in *lin-17/FZD* mutants. **A)** Histograms summarising the scoring results of epithelial gene expression in in K.p *lin-17/FZD* mutants. **B)** Histograms summarising the scoring results of rectal gene expression in K.p in *lin-17/FZD* mutants. **C)** Histograms summarising the scoring results of pan-neuronal gene expression in *lin-17/FZD* mutants. **D)** Histograms summarising the scoring results of the expression in K.p of another terminal differentiation gene of GABAergic neurons (together with *unc-47/VGAT* which was used previously to score for DVB presence; see Fig. 27). While genes in A) and B) remain expressed in K.p, genes in C) and D) are not activated in this cell in *lin-17/FZD* mutants. N=3 except for *ajm-1*. Mean and SD shown.

1.3.6 The Wnt signalling pathway might directly regulate the onset of *lim-6* expression through POP-1 binding sites in *lim-6 intron 4*

Terminal selectors are transcription factors which regulate the expression of terminal differentiation genes in the nervous system (Hobert, 2008b). The only known terminal selector in the DVB neuron is *lim-6/LMX* (Gendrel et al., 2016; Hobert et al., 1999). Interestingly, we found that *lim-6/LMX* is expressed in K.p earlier than pan-neuronal markers, being visible since 1 h after K division (Figure 33). This implies that its expression overlaps with the expression of some epithelial and rectal genes in K.p. Thus, K.p has still epithelial and rectal features shortly after its birth, however a neuronal marker can be already detected. Furthermore, it is important to bear in mind that the loss of function of *lim-6/LMX* gene does not preclude the acquisition of a neuronal identity in K.p, as seen with *unc-47/VGAT* reporter (Figure 34A), even if some terminal differentiation genes are not expressed in K.p in this background (Hobert et al., 1999).

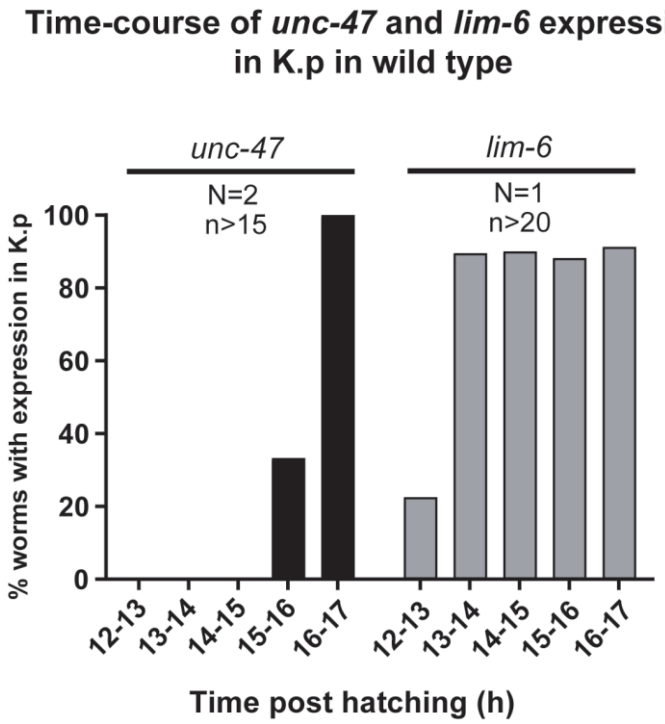

Figure 33. Time-course of *unc-47/VGAT* and *lim-6/LMX* expression in K.p. K divides 11.5 h post hatching and while *unc-47* terminal differentiation gene is expressed after 15-16 h post hatching, *lim-6* terminal selector transcription factors is already expressed 1 hour after K division.

The early expression of *lim-6/LMX* led us to hypothesise that it might be directly downstream to the Wnt signalling pathway, as *ceh-10* terminal selector is in AIY neurons (Bertrand and Hobert, 2009). To test this possibility, first we checked the expression of a *lim-6::gfp* knock-in reporter (recapitulating the endogenous behaviour) in *lin-17/FZD* (null) and in *pop-1/TCF* (lf) mutant backgrounds and we found that it is downregulated (Figure 34B). Like for the lowest

penetrance of “No DVB” defect in *pop-1/TCF* mutants compared to *lin-17/FZD* mutants, the slight downregulation of *lim-6/LMX* expression in *pop-1/TCF* mutants might be due to the loss of function but not null allele used (*q645*, maintaining the capacity of POP-1/TCF of binding to BAR-1/β-catenin, Kidd et al., 2005), or to maternal contribution through the genetic balancer.

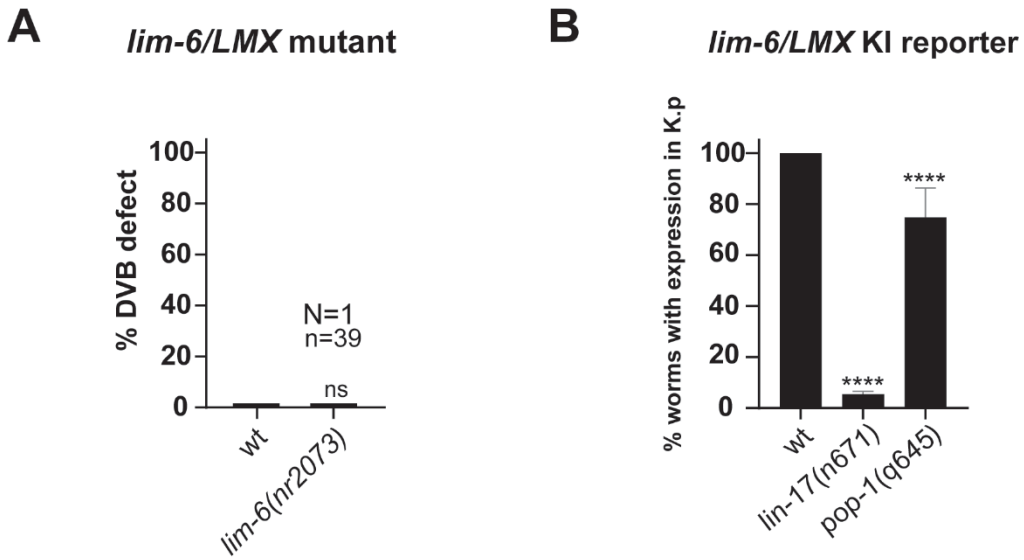

Figure 34. *lim-6/LMX* mutation does not affect DVB formation but its expression is affected in Wnt signalling pathway mutants. A) Histograms showing that in *lim-6/LMX* mutants the acquisition of a DVB identity is not completely affected, in agreement with the role of terminal selector genes and with what previously observed (Hobert el al., 1999). DVB presence based on *unc-47* expression and visible neurite. **B)** Histograms showing the expression of a *lim-6/LMX gfp* KI reporter in K.p in *lin-17/FZD* and *pop-1/TCF* mutants, at late larval stages. N=3; mean and SD shown.

Next, we focused on a regulatory region in the *lim-6/LMX* locus which was shown to be sufficient for driving its expression in DVB and only three other neurons in the worm (namely PVT, RIS and AVL neurons; Hobert et al., 1999). This *cis*-regulatory region corresponds to *lim-6 intron 4* (*intron 3* with the previous annotation (Figure 35A)), which we cloned upstream to *gfp* to characterise its putative regulation downstream to Wnt signalling. Interestingly, by perturbing the Wnt signalling pathway in a *lin-17/FZD* or in a *pop-1/TCF* mutant background, expression of the reporter is absent in K.p in a high percentage of worms (Figure 35B). This effect is stronger in *pop-1/TCF* mutant compared to what observed with the KI reporter strain. Thus, we looked for putative TCF binding sites in the *lim-6 intron 4* sequence: we found 8 conserved, with four of them overlapping with SOX2 binding sites, TCF and SOX2 being both HMG-box proteins (Pevny and Lovell-Badge, 1997; Rueyling et al., 1995). When we mutated 7 out of 8 putative TCF binding sites (the first one not being very conserved, see MATERIALS AND METHODS) we found that GFP is absent in L4s with the transgene (Figure 35C, 3 different lines). However, by scoring younger, L2 worms from the same lines, the expression of the *gfp* in most animals suggests that the Wnt signalling pathway might play an important

role in *lim-6/LMX* maintenance rather than in its initiation, at least through this *cis*-regulatory region (Figure 35D). This result appears to be the opposite of what V. Bertrand and O. Hobert observed for *ceh-10* activation in AIY neurons (Bertrand and Hobert, 2009). However, we need to highlight the fact that we are just looking at one regulatory region in this case, which might be important for the maintenance of *lim-6/LMX* expression, but this result does not rule out that the promoter region or other regulatory sequences could be required for initial *lim-6/LMX* activation dependent on Wnt signalling.

Overall, these data demonstrate that the Wnt/β-catenin asymmetry pathway regulates the asymmetry and the polarity of K ACD, is required to erase the rectal epithelial identity of K.p and to give rise to DVB, also through regulation of a terminal selector gene. Moreover, they show that this pathway might impact on K mitotic spindle positioning even though this alone does not dictate whether K.p will be able to convert into a DVB neuron. This conclusion agrees with recent work from V. Bertrand's lab suggesting for the division of AIY mother, that the defects in cell fates do not appear to be a direct consequence of defects in spindle orientation (Kaur et al., 2020).

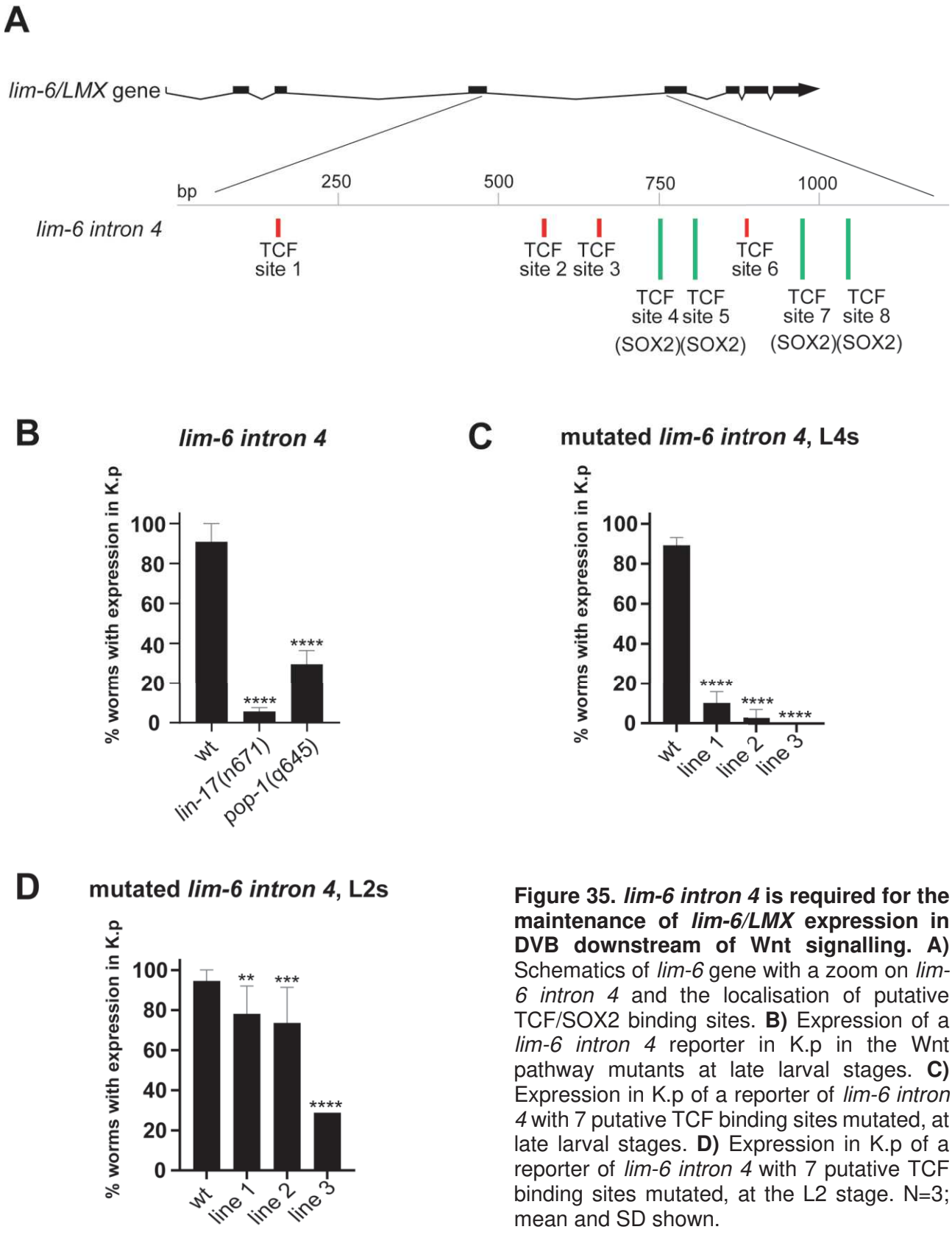

Figure 35. *lim-6 intron 4* is required for the maintenance of *lim-6/LMX* expression in DVB downstream of Wnt signalling. A) Schematics of *lim-6* gene with a zoom on *lim-6 intron 4* and the localisation of putative TCF/SOX2 binding sites. **B)** Expression of a *lim-6 intron 4* reporter in K.p in the Wnt pathway mutants at late larval stages. **C)** Expression in K.p of a reporter of *lim-6 intron 4* with 7 putative TCF binding sites mutated, at late larval stages. **D)** Expression in K.p of a reporter of *lim-6 intron 4* with 7 putative TCF binding sites mutated, at the L2 stage. N=3; mean and SD shown.

1.4 Y-to-PDA reprogramming factors *egl-5/HOX, sox-2/SOX*, *ceh-6/POU* and *sem-4/SALL* are required for K-to-DVB, while *egl-27/MTA* is not

Our lab had previously shown that *sem-4/SALL, sox-2/SOX, ceh-6/POU* and *egl-27/MTA* are necessary for the erasure of Y rectal identity during Y-to-PDA transdifferentiation and that *egl-5/HOX* gene is a likely downstream effector during Y dedifferentiation (Kagias et al., 2012). Since we found that cell division and the Wnt signalling pathway impact on the generation of the DVB neuron from K, we were wondering whether there could be a need for additional mechanisms, and whether they could be shared mechanisms with Y-to-PDA, regardless of the different cellular steps required in the two processes. Indeed, most of the factors expressed in Y and involved in Y-to-PDA initiation, i.e. *ceh-6/POU*, *sox-2/SOX*, *sem-4/SALL* and *egl-5/HOX*, are also expressed in K (Bürglin and Ruvkun, 2001; Ferreira et al., 1999; Jarriault et al., 2008; Vidal et al., 2015). Thus, we tested the involvement of those genes in K-to-DVB.

1.4.1 *egl-5/HOX, sox-2/SOX* and *ceh-6/POU* are required for K division and for DVB formation

For testing the involvement of *egl-5/HOX, sox-2/SOX* and *ceh-6/POU* we used different approaches, as the mutation of these genes differently affects the worm's development and viability. For *egl-5/HOX* we used a viable null mutant (Chisholm, 1991); *sox-2/SOX* and *ceh-6/POU* mutations are early larval lethal before transdifferentiation occurs, thus we used other strategies to deplete or downregulate them in the rectal cells (see MATERIALS AND METHODS). By scoring those strains for DVB formation, we discovered that absence of *egl-5/HOX* leads to a "No DVB" phenotype in more than 80% of the worms, while *ceh-6(gk665)* and *sox-2/SOX* knockdown showed lower but significant defects (about 10% and 20% respectively), probably due to experimental limitations (Figure 36, left). Indeed, genetic mosaic analysis of *sox-2/SOX* null mutants performed by O. Hobert's lab showed that DVB is absent in 100% of the worms in this background (Vidal et al., 2015). We also tested the paralogues of *sox-2/SOX* (not shown) and *ceh-6/POU* (Figure 37), but none of them displayed any significant transdifferentiation defect, confirming that those two are the core components required for reprogramming. The defect in the *unc-86; ceh-18* double mutant shows high variability between biological replicates and could be a consequence of the difficulty in the identification of DVB in these worms which are globally highly affected (extra *col-34+* cells have been also seen in a variable fraction of them).

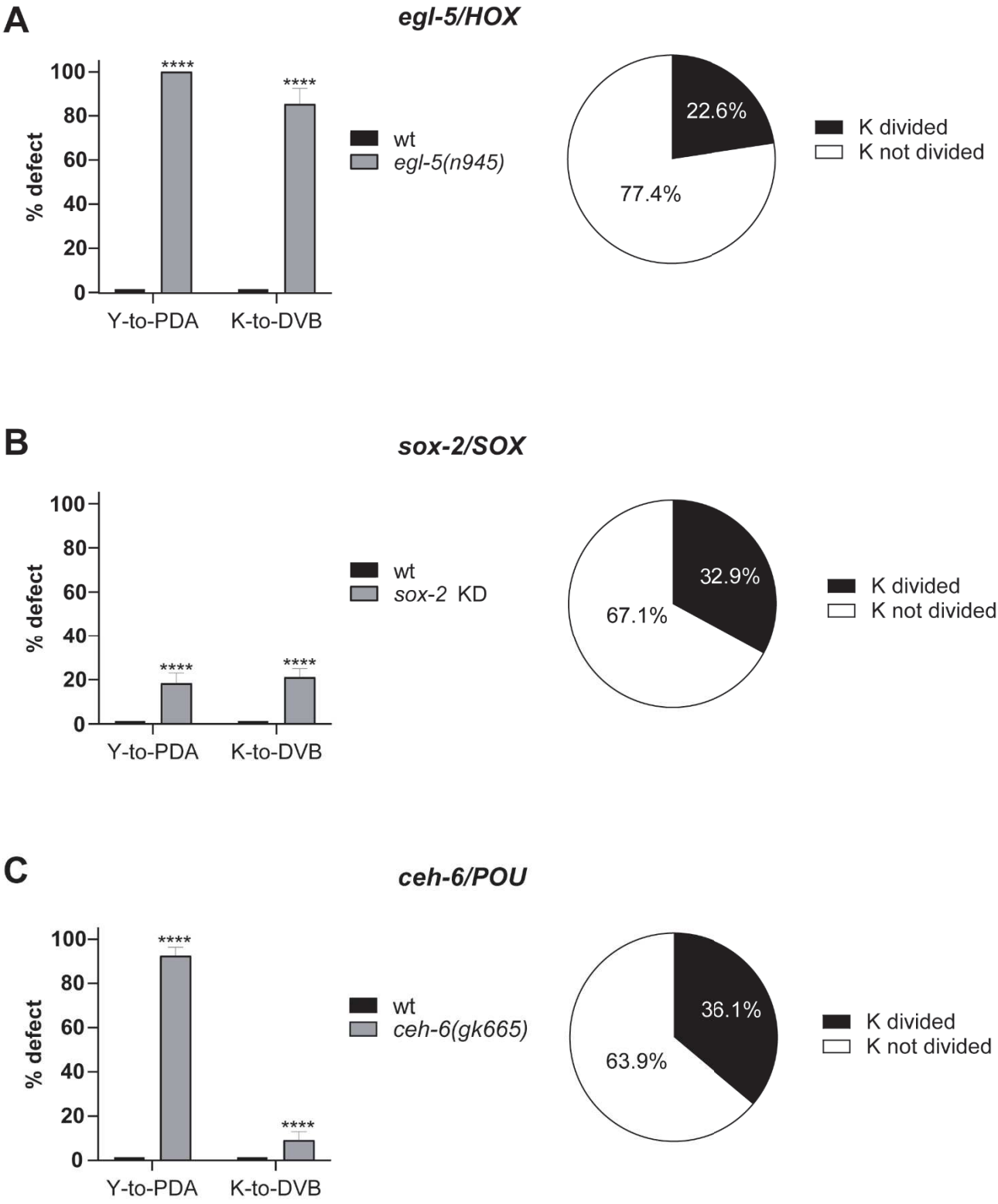

Figure 36. *egl-5/HOX*, *sox-2/SOX* and *ceh-6/POU* are required for K division and for DVB formation. A) Histograms showing the Y-to-PDA and K-to-DVB defects (left) and the percentages of worms without DVB in which K division has either occurred or not (right) in *egl-5/HOX* mutants. **B)** Histograms showing the Y-to-PDA and K-to-DVB defects (left) and the percentages of worms without DVB in which K division has either occurred or not (right) in *sox-2/SOX* KD strains. **C)** Histograms showing the Y-to-PDA and K-to-DVB defects (left) and the percentages of worms without DVB in which K division has either occurred or not (right) in *ceh-6/POU* mutants. PDA presence based on *cog-1* or *exp-1* expression and DVB presence based on *unc-47* expression. Neurite presence also considered. N≥3; mean and SD shown.

To understand better how these three genes work, we focused on K division. We could not assess the orientation and asymmetry of K division in *egl-5/HOX*, *sox-2/SOX* and *ceh-6/POU* mutant and KD backgrounds due to technical limitations (in *egl-5/HOX* the rectal marker *col-34* used for these measurements is downregulated). However, we could observe that absence of DVB is due to K not dividing in 3/4 of the worms in *egl-5/HOX* mutant and in 2/3 of the cases in *sox-2/SOX* and *ceh-6/POU* KD/mutants (Figure 36, right). When K does divide, K.p appears to retain a rectal identity as visualised by sustained *col-34* (collagen) expression.

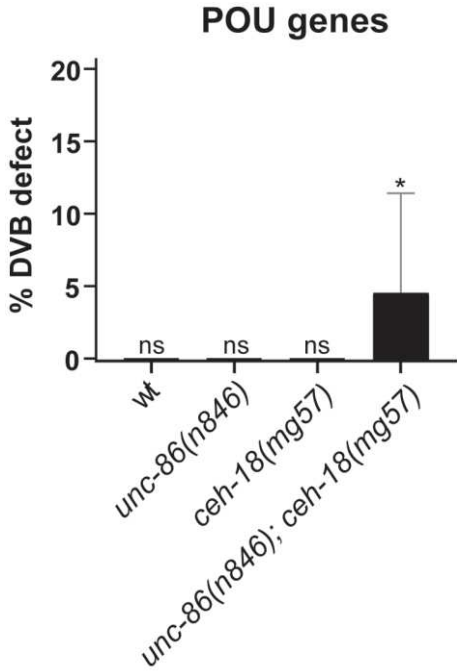

Figure 37. *ceh-6/POU* paralogues are not required for K-to-DVB. Histograms showing the percentage of worms lacking DVB in mutant backgrounds for the other *C. elegans* POU genes alone or in combination. DVB presence based on *unc-47* expression and visible neurite. N=3; mean and SD shown.

Thus, these genes seem to have two roles during K-to-DVB: i) allow the occurrence of K division; and ii) allow the K.p daughter to undergo an identity change, very reminiscent of their role during Y-to-PDA transdifferentiation (Jarriault et al., 2008; Kagias et al., 2012).

1.4.2 Neither *egl-27/MTA* nor its paralogue *lin-40/MTA* are key for K-to-DVB

Another gene shown to be involved in the initiation of Y-to-PDA together with *egl-5/HOX*, *sox-2/SOX* and *ceh-6/POU* is *egl-27/MTA* (Kagias et al., 2012), a putative component of chromatin modifier complexes whose mammalian counterpart is found in NuRD and NODE repressive complexes (Liang et al., 2008). We thus assessed if *egl-27/MTA* and its paralogue *lin-40/MTA* (also known as *egr-1*) are required for K-to-DVB. In null mutants for *egl-27/MTA* long isoform (*egl-27(ok1670)*) we found a very low “No DVB” defect of 3% (but K division always occurred) compared to the >80% observed for PDA; this low defect might not be explained by the activity

of its paralogue in K-to-DVB as *lin-40* mutants show no defect at all (Figure 38). However, we cannot exclude a redundancy of these two genes because we could not characterise the double mutant as it is not viable.

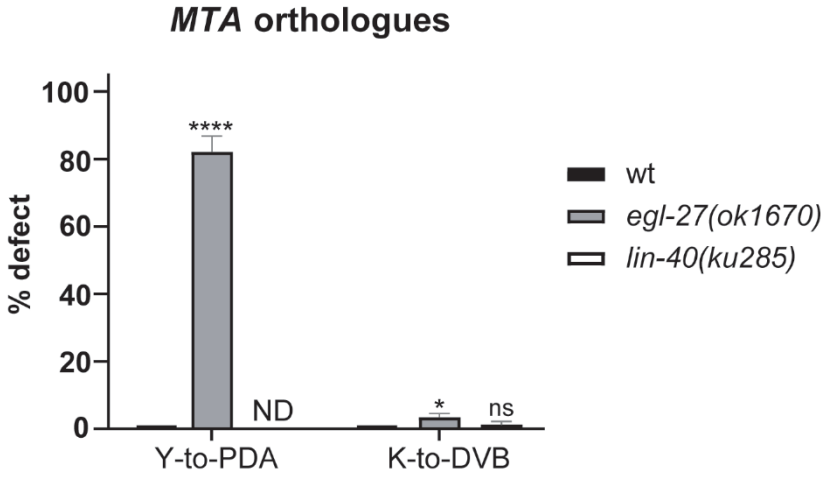

Figure 38. *MTA* orthologues are not required for DVB formation as they are for PDA. Histograms showing the Y-to-PDA and K-to-DVB defects in mutants for the two *C. elegans MTA* orthologues, *egl-27* which is known to be required for PDA formation and its paralogue *lin-40*. PDA presence based on *exp-1* expression and DVB presence based on *unc-47* expression. Neurite presence also considered. N=3; mean and SD shown.

1.4.3 *sem-4/SALL* is required after K division for DVB formation

Finally we tested *sem-4/SALL*, whose a viable null mutant allele is available (Basson and Horvitz, 1996). In *sem-4(n1971)* null mutants, DVB does not form in more than 90% of the animals as showed by absence of *unc-47/VGAT* expression (Figure 40A). Nevertheless, K divides and K.p cell is present in all the *sem-4/SALL* null worms, even when it does not become a DVB (Basson and Horvitz, 1996). From Figure 39 it is also clear how different K.p nucleus appears in worms where it forms DVB compared to worms where it fails to, as visualised by the *col-34* reporter we use (*gaIs245[col-34p::histone::mCherry]* array): in the former, *col-34* rectal reporter has become faint; in the latter, *col-34* is still brightly expressed in K.p in L4 worms. This observation suggests that K.p remains rectal in *sem-4* null mutant. Interestingly, we found a 100% correlation between the absence of *unc-47/VGAT* expression and the presence of rectal marker *col-34* expression.

We next quantitatively characterised K division, as done for *lin-17/FZD* mutants. In *sem-4/SALL* null worms, K division appears slightly misoriented in 11.1% of the animals (Figure 40B), with an average and variance significantly different compared to wild type. As the percentage of misoriented K divisions does not correlate with the number of worms lacking

DVB (as observed for *lin-17/FZD*), this is not likely to be the cause of the very high “No DVB” defect of *sem-4* null worms.

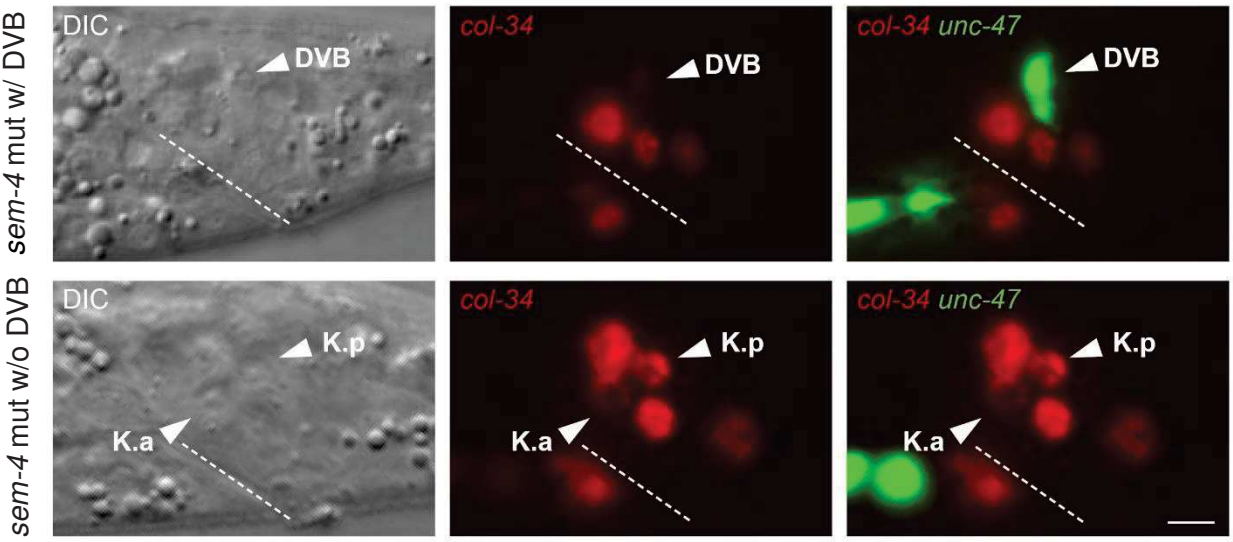

Figure 39. K divides in *sem-4/SALL* null mutant independently of DVB formation. (Top) DIC and fluorescence microscopy images of a *sem-4(n1971)* L4 worm which developed a DVB from K.p. This is observed in less than 10% of *sem-4/SALL* null mutants. (Bottom) DIC and fluorescence microscopy images of a *sem-4(n1971)* L4 worm which did not develop a DVB from K.p. However, K.p was born like in all the *sem-4(n1971)* worms observed and it expressed *col-34*. Scale bar = 5 µm.

We further analysed K.p identity by checking its nuclear size and the expression of rectal epithelial and neuronal marker genes. We found that in *sem-4* mutants K.p exhibits a larger nucleus than in wild types (Figure 40C), reminiscent of an epithelial identity, in agreement with previous DIC observations reporting that in 10 out of 12 *sem-4(n1971)* L3 animals K.p has a hypodermal-like nuclear size that is similar to K.a (Basson and Horvitz, 1996).

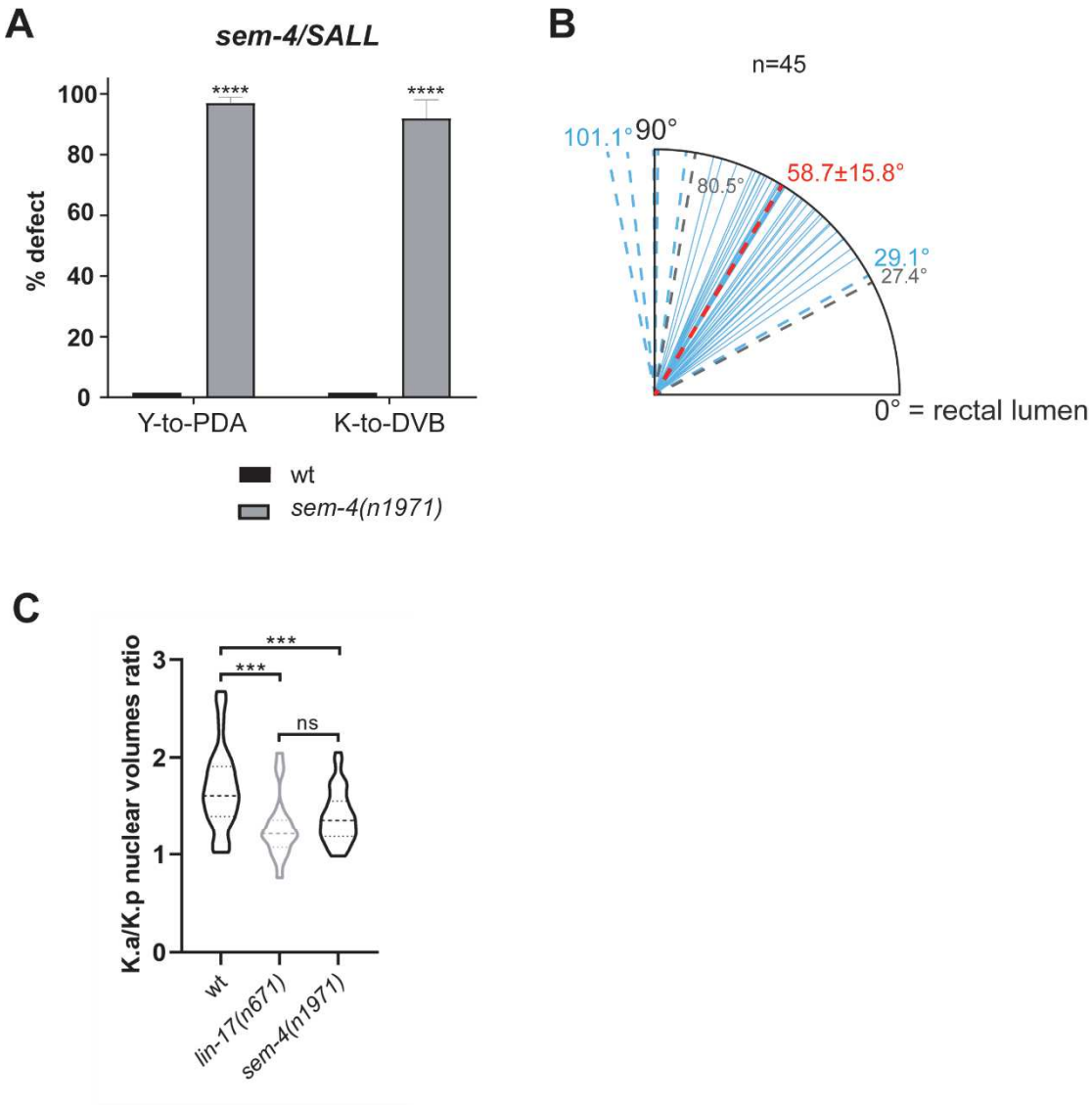

Figure 40. Lack of *sem-4/SALL* gene affects DVB formation, orientation of K division at a less extent and asymmetry of K.a and K.p nuclear volumes. A) Histograms showing the percentages of worms lacking either PDA or DVB in *sem-4/SALL* mutant background. N=3; mean and SD shown. **B)** Representation of the angles of K division in *sem-4/SALL* mutants. In red, average value and standard deviation in *sem-4* mutants; in light blue, measured angles in *sem-4* mutants with dashed lines for angles out of the wild type range and for the minimum; in grey, wild type minimum and maximum values. **C)** Representation of K.a/K.p nuclear volumes ratio, decreased almost to 1 as measured 1 h after K division in *sem-4* like in *lin-17* mutants.

In addition, we found that epithelial (*lin-26* transcription factor and *let-413/Scribble*) and rectal markers (*col-34* collagen, *egl-5/HOX* and *sox-2/SOX*) are expressed in K.p in L3 and L4 *sem-4(n1971)* mutant larvae, when in wild type animals K.p has already become DVB; interestingly, we also observed clusters of AJM-1 (apical junction) protein, suggesting that the gene was not silenced or was re-expressed as observed in *lin-17/FZD* mutants. Conversely and like in *lin-17/FZD*, DVB neuronal markers, including pan-neuronal (*rgef-1/RASGRP3*, *unc-33/CRMP* and

unc-119/UNC119), the terminal selector *lim-6/LMX* and GABAergic markers, are never expressed in those mutant K.p cells which fails to become DVB (Figure 42, Table 9) and an anti-correlation between rectal marker and neuronal-marker expression is observed in double reporter strains (Figure 41).

We conclude that in absence of *sem-4/SALL* K.p cannot tear down its rectal epithelial identity which is retained indefinitely.

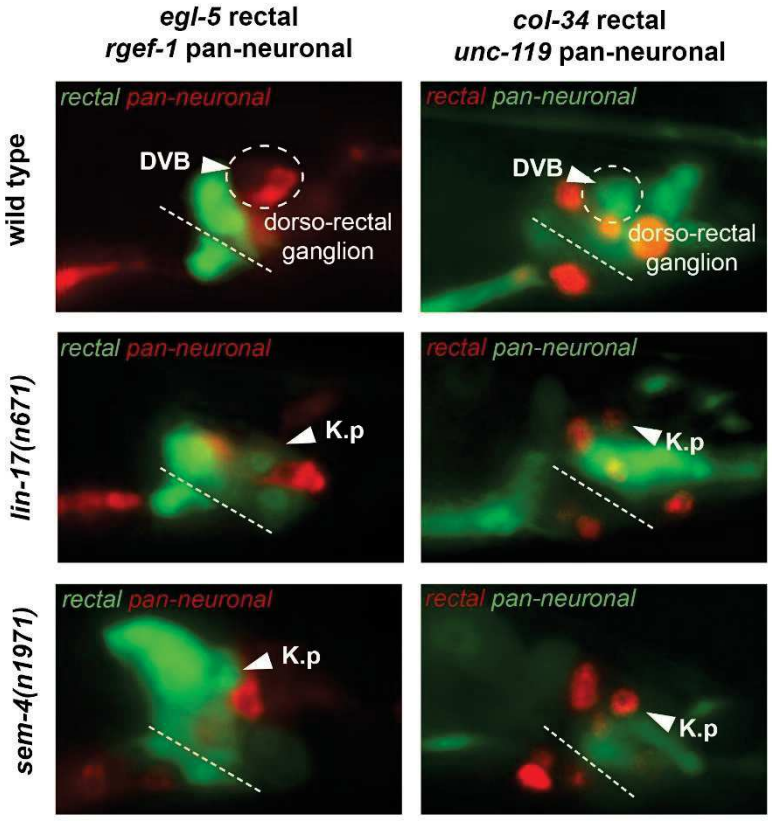

Figure 41. Anti-correlation of rectal and pan-neuronal gene expression in K.p in wild type, *lin-17/FZD* and *sem-4/SALL* mutant backgrounds. While in wild type L4 worms K.p has lost the expression of rectal genes *egl-5* and *col-34* and gained the expression of pan-neuronal genes *rgef-1* and *unc-119*, in *lin-17* and *sem-4* mutants K.p expresses *egl-5* and *col-34* and does not express the pan-neuronal genes.

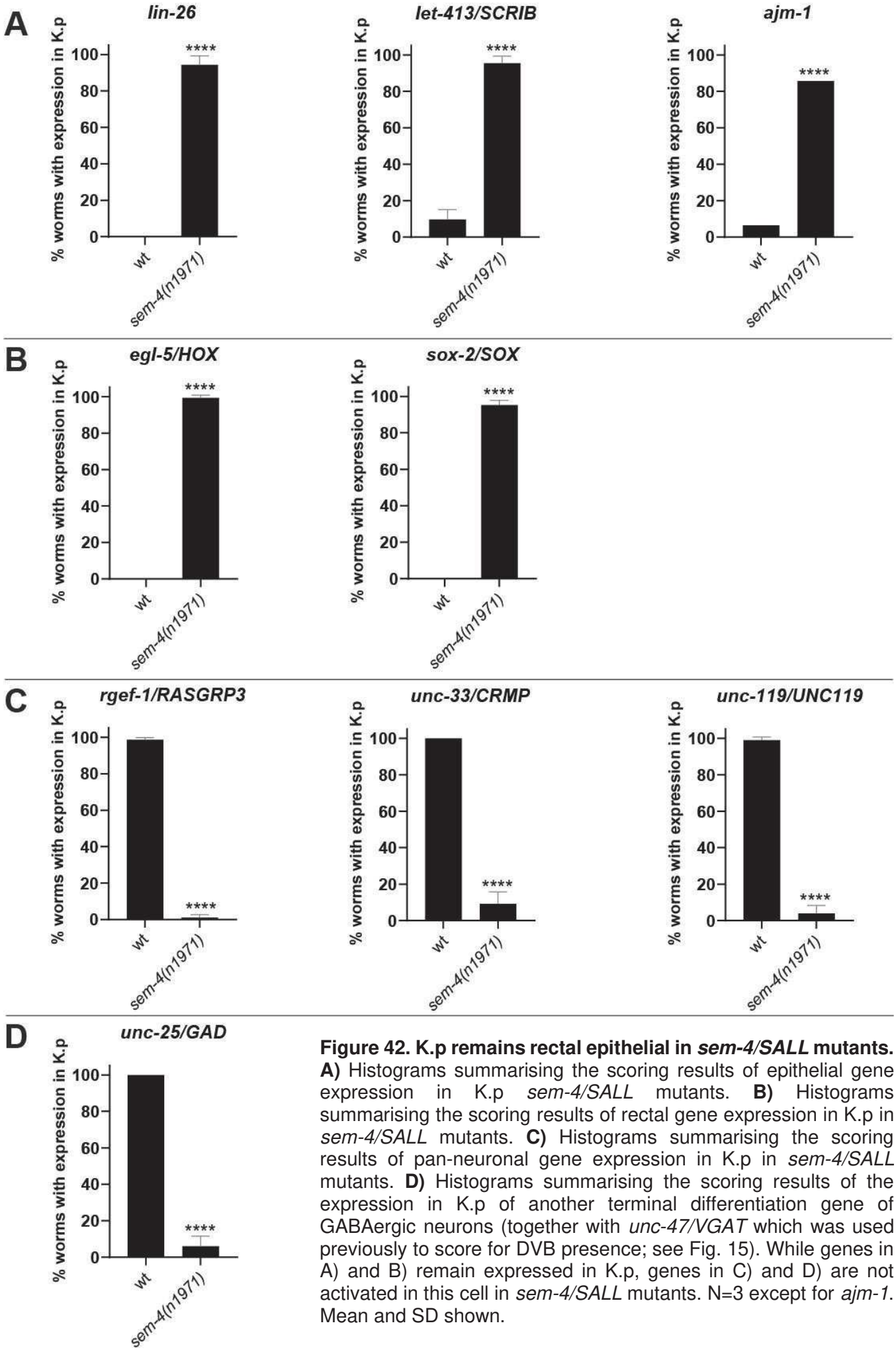

Figure 42. K.p remains rectal epithelial in *sem-4/SALL* mutants. **A)** Histograms summarising the scoring results of epithelial gene expression in K.p *sem-4/SALL* mutants. **B)** Histograms summarising the scoring results of rectal gene expression in K.p in *sem-4/SALL* mutants. **C)** Histograms summarising the scoring results of pan-neuronal gene expression in K.p in *sem-4/SALL* mutants. **D)** Histograms summarising the scoring results of the expression in K.p of another terminal differentiation gene of GABAergic neurons (together with *unc-47/VGAT* which was used previously to score for DVB presence; see Fig. 15). While genes in A) and B) remain expressed in K.p, genes in C) and D) are not activated in this cell in *sem-4/SALL* mutants. N=3 except for *ajm-1*. Mean and SD shown.

C.elegans gene	wt	*sem-4(n1971)*
Epithelial markers		
lin-26	-	+
let-413	-	+
ajm-1	-	+
Rectal markers		
col-34	-	+
egl-5	-	+
sox-2	-	+
ceh-6	-	ND
Pan-neuronal markers		
rgef-1	+	-
unc-33	+	-
unc-119	+	-
Gabaergic markers		
unc-47	+	-
unc-25	+	-
lim-6	+	-

Table 9. K.p remains rectal epithelial in *sem-4/SALL* mutants. Summary of the epithelial, rectal, pan-neuronal and GABAergic genes expressed in K.p in wild type vs *sem-4/SALL* mutant worms.

Overall, we have demonstrated that the NODE-like complex transcription factors *sem-4/SALL*, *egl-5/HOX*, *sox-2/SOX* and *ceh-6/POU* necessary for the initiation of Y-to-PDA (Kagias et al., 2012) are required for K-to-DVB, and that some even act at two different steps; conversely, the putative chromatin modifier *egl-27/MTA* is not. We speculate that the presence of cell division might account for *egl-27/MTA* dispensability. For *ceh-6/POU* the lower penetrance in K-to-DVB than in Y-to-PDA (Figure 36) might have several explanations: the rescuing construct leading to *ceh-6/POU* expression in all the worm's cells but the rectal cells might be slightly differently active in K versus Y, leading to a rescue of the "No DVB" defect; another possibility is that the high penetrance of the "No PDA" defect is due to the reporter used, *cog-1/NKX*, a transcription factor, not a terminal differentiation gene like *unc-47/VGAT* used for DVB. Indeed, in the lab we have found a different penetrance of "No PDA" defect when PDA is scored with either *cog-1* or *exp-1* reporters. Finally, it is also possible that lack of *ceh-6/POU* impacts less – but still impacts - on K-to-DVB.

1.5 Wnt signalling and *sem-4/SALL* act in parallel pathways for DVB formation

From the characterisation of K division and K.p identity in *lin-17/FZD* and *sem-4/SALL* mutants it appears that in both backgrounds K.p is sometimes born misoriented, and in more than 90% of the animals has a hypodermal-like nucleus and it remains completely rectal epithelial. Since the K.p phenotypes and their penetrance in those two mutants are so similar, we wanted to assess whether those two genes are part of the same pathway or they rather function in two different parallel (non-redundant) pathways. To address this question, first we dissected the regulatory relationship between *sem-4/SALL* and *lin-17/FZD* and afterwards we performed double mutant analysis with a *sem-4/SALL* mutant in combination with a Wnt pathway mutant.

1.5.1 *sem-4/SALL* is not downstream to *lin-17/FZD, while lin-17/FZD* is probably indirectly downstream to *sem-4/SALL*

To determine whether *sem-4/SALL* is downstream to the Wnt signalling pathway (like *SALL4* in human cells Böhm et al., 2006), we analysed the expression of a *sem-4::gfp* KI reporter in the *lin-17/FZD* null background. *sem-4/SALL*, which is expressed in all the rectal cells, in K.p and remains in DVB, was not affected in *lin-17(n671)* animals neither in K.p nor in the other rectal cells (Figure 43A). The opposite analysis was performed by checking the expression of a *lin-17/FZD* transcriptional nuclear reporter in *sem-4/SALL* null mutant. The use of a *gfp* KI strain to score the expression of *lin-17/FZD* in K.p would have not been ideal as the LIN-

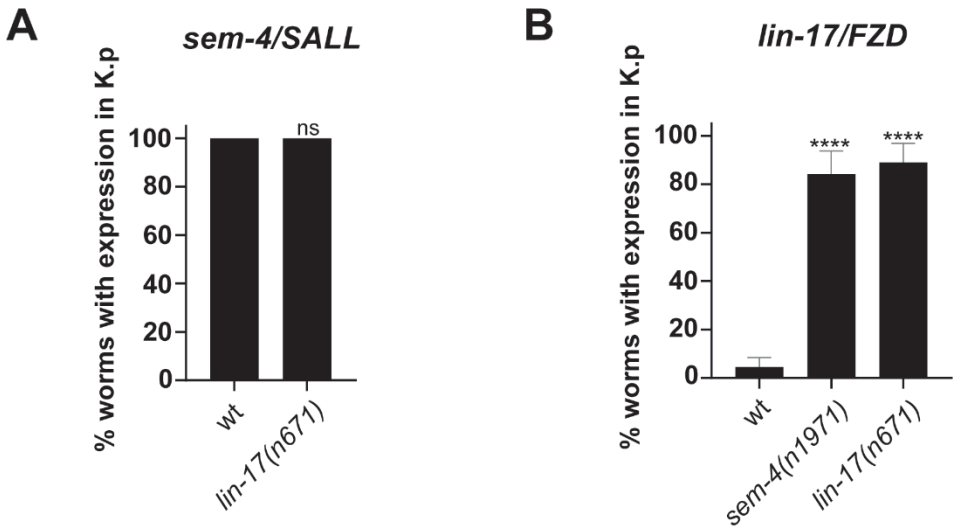

Figure 43. *sem-4/SALL* is not downstream to *lin-17/FZD*, while *lin-17/FZD* is probably indirectly downstream to *sem-4/SALLI.* A) Histograms showing the expression of *sem-4/SALL* in K.p in a control strain and in *lin-17/FZD* mutants. **B)** Histograms showing the expression of *lin-17/FZD* in K.p in a control strain and in *sem-4/SALL* and *lin-17/FZD* mutants. N=3; mean and SD shown.

17/FZD protein localises at the cell membranes and would make it difficult to discriminate at the membrane of which cell(s) it is localised. The *lin-17p(promoter)::2nls::gfp* reporter that we built allowed us to see that *lin-17/FZD* is expressed in the rectal cells F, U, K and K' at L1 stage and also in K.p after birth in both wild type and *sem-4/SALL* mutants. However, by scoring L4 larvae and adults we found that while *lin-17/FZD* remains expressed in K.p in *sem-4/SALL* mutant worms, it disappears gradually from the L2 stage to the adult in wild type worms (Figure 43B). Thus, it appears that *sem-4/SALL* is required for the repression of *lin-17/FZD* in DVB.

These data demonstrate that the Wnt signalling pathway activation is not required for *sem-4/SALL* expression (neither in K.p nor in the rectal cells). For the reciprocal relationship, we suggest that the apparent negative regulation of *lin-17/FZD* expression in K.p by *sem-4/SALL* might be indirect, as *sem-4/SALL* is expressed also in the other rectal cells where in wild type background *lin-17/FZD* is not silenced. The expression of *lin-17/FZD* appears as another molecular feature of the rectal cells (at least of F, U, K', K and later K.a) which is retained in K.p in *sem-4/SALL* mutant background as the mutant K.p remained a rectal cell. Interestingly in support to this hypothesis, we found that the expression of *lin-17/FZD* is maintained in K.p also in *lin-17(n671)* null mutant (Figure 43B).

1.5.2 *sem-4* and *wrm-1/β-catenin* mutants synergistically affect K-to-DVB

To further characterise the relationship between *sem-4/SALL* and the Wnt signalling pathway in K-to-DVB, we built a double mutant of *sem-4*/SALL and a Wnt signalling component, *wrm-1/β-catenin*, and checked the impact on DVB formation. For the Wnt signalling pathway, we could not use the available *lin-17/FZD* null mutant due to its high penetrance of DVB absence; for the same reason, we could not use the null mutant *sem-4(n1971)*, but we chose to use the hypomorphic allele *sem-4(n1378)* that showed a "No DVB" defect of around 30% at 20°C (Figure 44A). We built a double mutant with *wrm-1(n1982ts)* and scored it after shifting the embryos at the restrictive temperature: the combination of *sem-4/SALL* and *wrm-1/β-catenin* mutations leads to a synergistic effect on DVB formation, going from a "No DVB" defect of 8% and 2.5% in *sem-4/SALL* and *wrm-1/β-catenin* single mutants respectively to a defect of 46.7% in the double mutants (Figure 44B).

Altogether our results support the hypothesis that *sem-4/SALL* and Wnt signalling act through two synergistic parallel pathways in order to trigger K-to-DVB transdifferentiation, and in particular to erase K.p rectal identity.

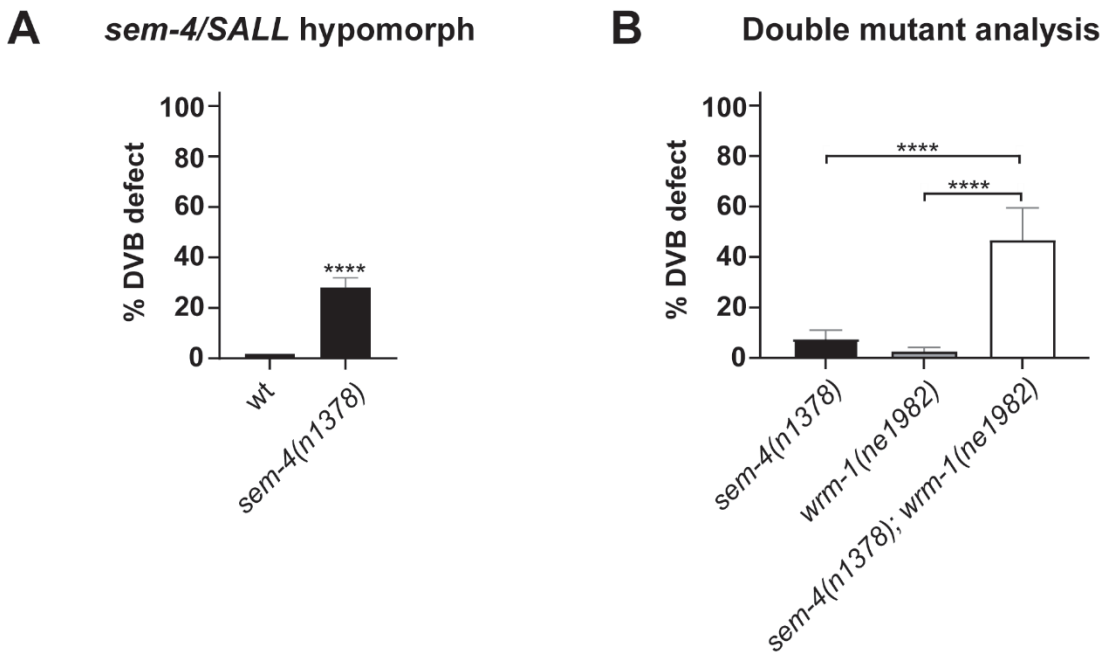

Figure 44. *sem-4/SALL* and Wnt signalling act in parallel pathway to drive K-to-DVB. A) Histograms showing the percentage of worms without DVB in a *sem-4/SALL* hypomorph mutant background grown at 20°C. **B)** Histograms summarising the absence of DVB in *sem-4/SALL* hypomorph mutants, *wrm-1/β-catenin* mutants and in double mutants at the restrictive temperature of 25°C. DVB presence based on *unc-47* expression and visible neurite. N=3; mean and SD shown.

1.6 Wnt signalling and *sox-2/ceh-6* might also act in parallel pathways during K-to-DVB

We next focused on the other conserved reprogramming factors, required like *sem-4/SALL* for Y-to-PDA and K-to-DVB but also belonging to evolutionary conserved families of reprogramming transcription factors (Julian et al., 2017; Szemes et al., 2018; Tsubooka et al., 2009): *sox-2/SOX* and *ceh-6/POU*. We aimed to dissect what their relationship with the Wnt signalling pathway could be.

By using the same approach adopted for the Wnt signalling pathway and *sem-4/SALL,* we built a strain with *wrm-1(ne1982ts)* mutation combined with nanobodyGFP-mediated *sox-2* KD (see MATERIALS AND METHODS). By shifting the worms at the restrictive temperature, we found a synergistic effect of the double perturbation of *sox-2/SOX* and *wrm-1/β-catenin* expression: the “No DVB” defects observed at 25°C affect from 6.5% and 5.5% of the worms in *sox-2/SOX* KD and *wrm-1(ne1982ts)* respectively, to 17% in the *sox-2 KD/wrm-1(ne1982ts)* strain (Figure 45).

This result suggests that *sox-2/SOX* (and probably its partner *ceh-6/*POU) and the Wnt signalling pathway act in parallel to regulate K-to-DVB.

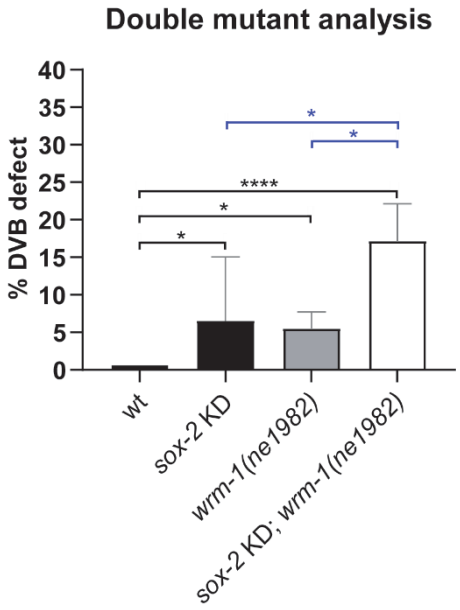

Figure 45. *sox-2/SOX* and the Wnt signalling act in parallel pathway to drive K-to-DVB. Histograms summarising the absence of DVB in *sox-2/SOX* KD strain, *wrm-1/β-catenin* mutants and in double mutants at the restrictive temperature of 25°C. DVB presence based on *unc-47* expression and visible neurite. N=3; mean and SD shown. In blue, statistical analyses of the double mutant vs the single mutants. In black, statistical analyses of mutant vs wild type worms.

1.7 Conclusions

Overall, the results showed in this chapter demonstrate that K-to-DVB is another transdifferentiation event occurring in the rectum of *C. elegans* during larval development and that it shares some molecular requirements with Y-to-PDA Td, while the cellular steps involved are different (Figure 46). DVB formation requires the K rectal cell's division and the Wnt signalling pathway which regulates the asymmetry of the division and allows the reprogramming of only one daughter cell. Interestingly, K division and the Wnt signalling pathway are not sufficient for K-to-DVB, as Y-to-PDA reprogramming factors are also needed in parallel. Nevertheless, while in Y the reprogramming factors *sox-2/SOX*, *ceh-6/POU* and *egl-5/HOX* are required for its dedifferentiation, in K they are involved at two successive steps: for the K rectal cell to divide and after division to reprogram K.p into DVB. On the other hand, the transcription factor *sem-4/SALL* is required in K.p like in Y to erase the rectal epithelial identity. Finally, *egl-27/MTA* is not very significantly required for K-to-DVB maybe because of the presence of cell division which is not perturbed in the mutants.

The impact of cell division on transdifferentiation might also be supported by the duration of K-to-DVB which is faster than Y-to-PDA, with DVB visible from the L2 larval stage while PDA

differentiates in L3 larval stage. As a probable consequence of the relatively fast K-to-DVB reprogramming, a dedifferentiate intermediate like Y.0 is not observed in K.p where for a few hours some epithelial and neuronal markers are present simultaneously (for instance *let-413* and *lim-6/LMX*).

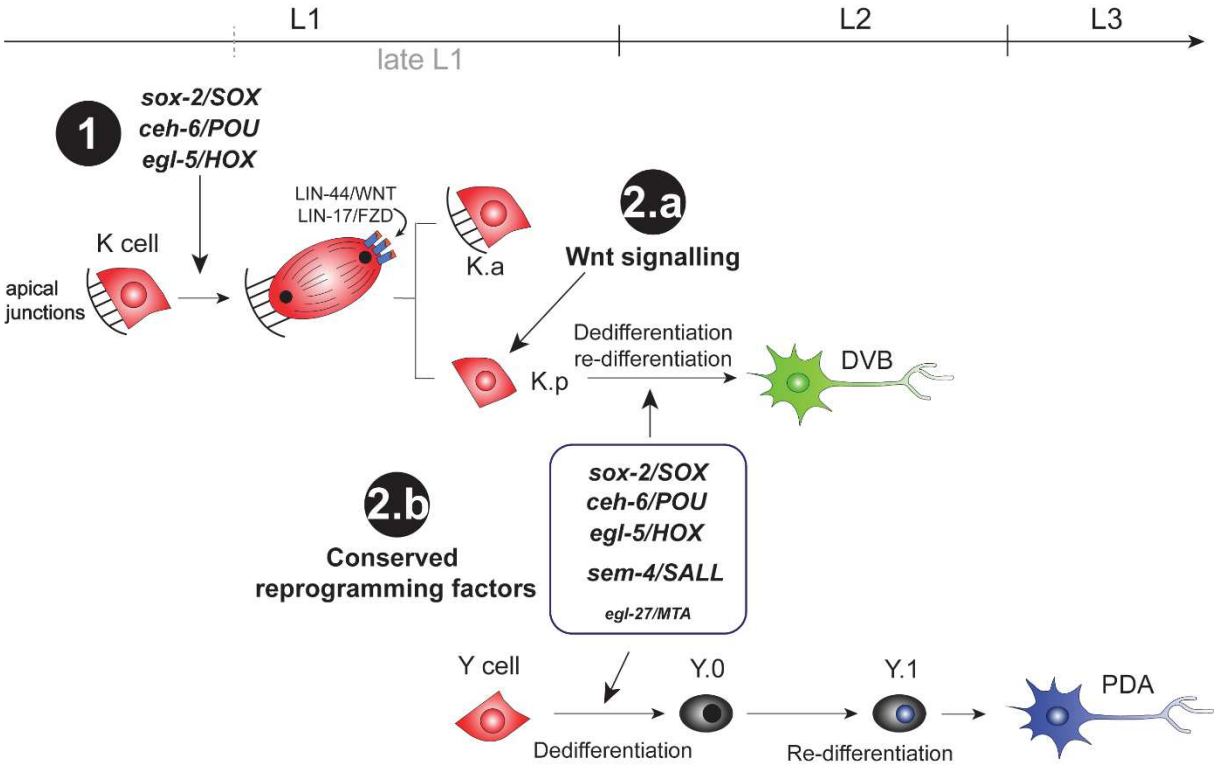

Figure 46. Model of K-to-DVB transdifferentiation compared to Y-to-PDA, summarising the cellular steps and the molecular factors involved in the two events.

2 Comparison of the molecular requirements across transdifferentiation event in C. elegans

One of the aims of this Thesis was to determine whether conserved reprogramming factors exist in *C. elegans* (see AIM OF THE THESIS). The results obtained for K-to-DVB suggest that the transcription factors *sem-4/SALL*, *sox-2/SOX, ceh-6/POU* and *egl-5/HOX* could be part of a "plasticity cassette" which allows cell plasticity in *C. elegans* differentiated cells. To further test di hypothesis, we characterised the involvement of those genes in other putative transdifferentiation events (see Introduction 4.3), after having proved that they are cellular reprogramming instances.

We focused on Y-to-PDA in males, occurring in the rectum of the worm like Y-to-PDA in hermaphrodites and K-to-DVB, and G1-to-RMH, an event which instead occurs in another organ, in the head of the worm.

2.1 Y-to-PDA in males is a transdifferentiation event in the rectum of the worm involving cell division

As anticipated in the Introduction (4.3.2), Y-to-PDA occurs also in males, but it is preceded by Y cell division (Figure 47). We aimed to compare the identity of Y and PDA in hermaphrodites and males to clarify whether this could be another transdifferentiation event and afterwards we

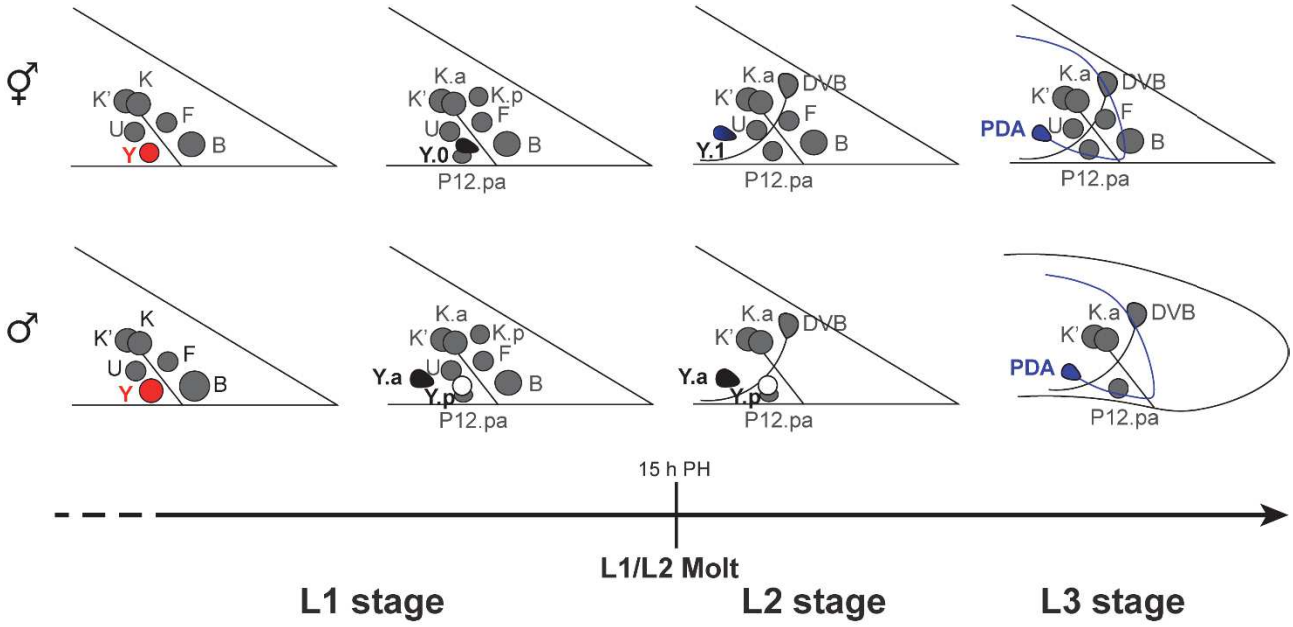

Figure 47. Y-to-PDA in males is preceded by Y cell division. Timeline of Y-to-PDA in hermaphrodites and in males compared. Y cell is represented in red; Y.0, Y.1 and Y.a are represented in black (identity not defined); Y.p is in white as it has a different fate than Y.a; PDA appears in blue.

tested whether the genes required for Y-to-PDA in hermaphrodites are necessary also in males.

2.1.1 Y-to-PDA in males is a transdifferentiation event

As for K-to-DVB, we had to prove that Y-to-PDA in males is a transdifferentiation event, that would be completed after cell division of the male Y cell. The analysis of expression of marker genes found in Y and PDA in hermaphrodites confirms that these cells hold the same identity: Y in males expresses *col-34* (collagen), *lin-26* and *hlh-16/OLIG* like Y in hermaphrodites (Figure 48Figure 47) and it forms the rectum of the worm with the other rectal cells until the L1 larval stage (Sulston et al., 1980); PDA in males, like in hermaphrodites, expresses *cog-1/NKX6-2*, *exp-1* (GABA receptor) and *unc-17* (acetylcholine vesicular transporter) (Figure 49, Table 10). *hlh-16/OLIG* is expressed also in Y cell daughters in males including Y.a, the cell that becomes PDA (Figure 48), but it disappears later in L3/L4 larval stages.

Thus, Y-to-PDA in males is a transdifferentiation event like in hermaphrodites.

***C.elegans* gene**	**Orthologues**	**Y**	**PDA**
Epithelial/rectal markers			
col-34	Cuticle collagen	+	-
lin-26	Zinc-finger transcription factor	+	-
Neuronal markers			
cog-1	NKX6-2 transcription factor	-	+
exp-1	GABA receptor	-	+
unc-17	ACh transporter	-	+

Table 10. Y is a rectal epithelial cell and PDA a motor neuron in *C. elegans* males. Summary of the epithelial, rectal and neuronal genes expressed in Y and PDA in males.

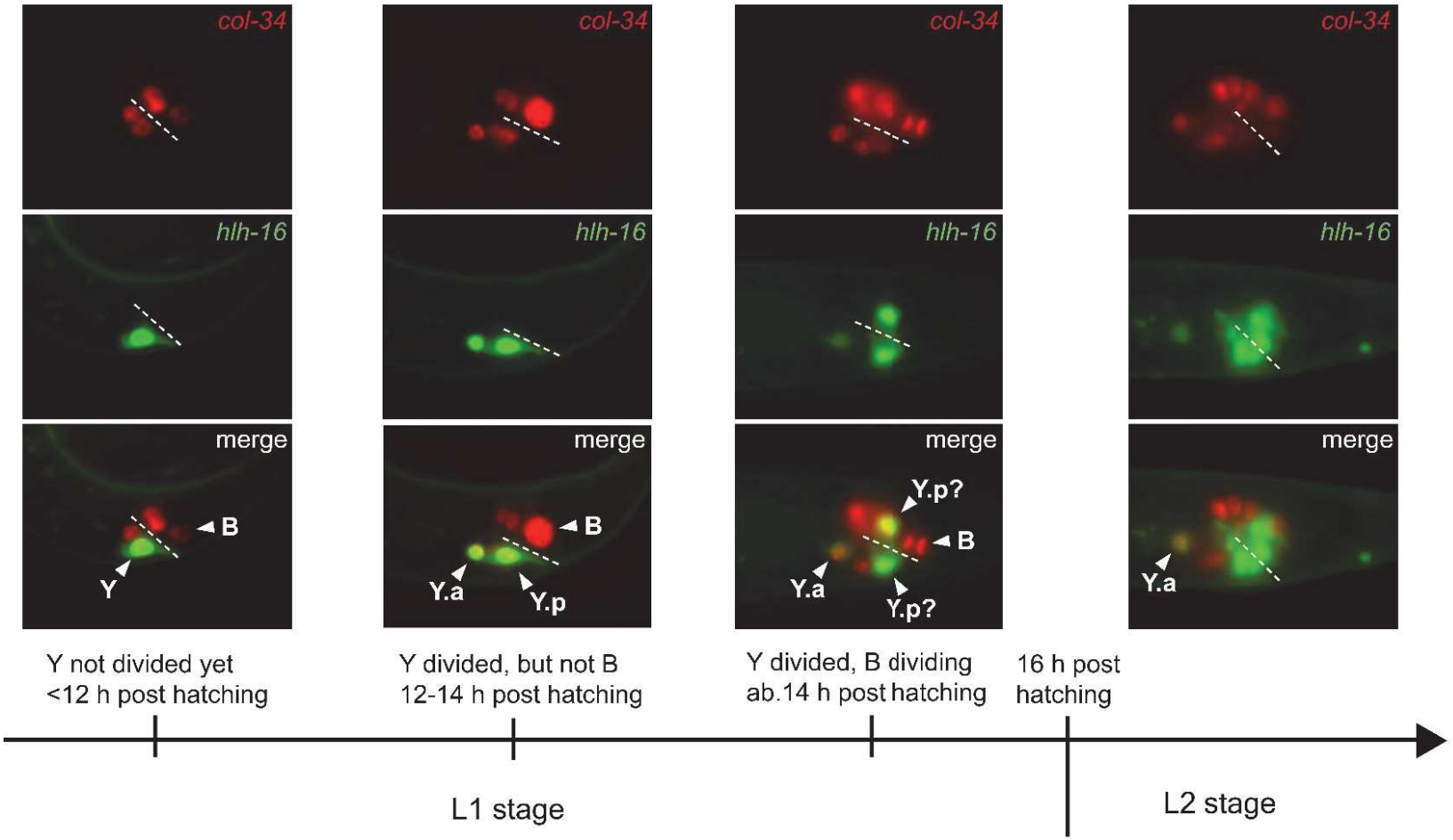

Figure 48. Y in males has the same identity as in hermaphrodites. Fluorescence microscopy pictures of Y in males from early L1, when Y has not divided yet, to L2 larval stage. Y in males like in hermaphrodites expresses collagen gene *col-34*, and *hlh-16/OLIG*. After Y division, about 12 h PH, both Y.a and Y.p express *hlh-16/OLIG* and *col-34*. However, they both start to disappear from Y.a, the future PDA while it migrates anteriorly like in the hermaphrodite.

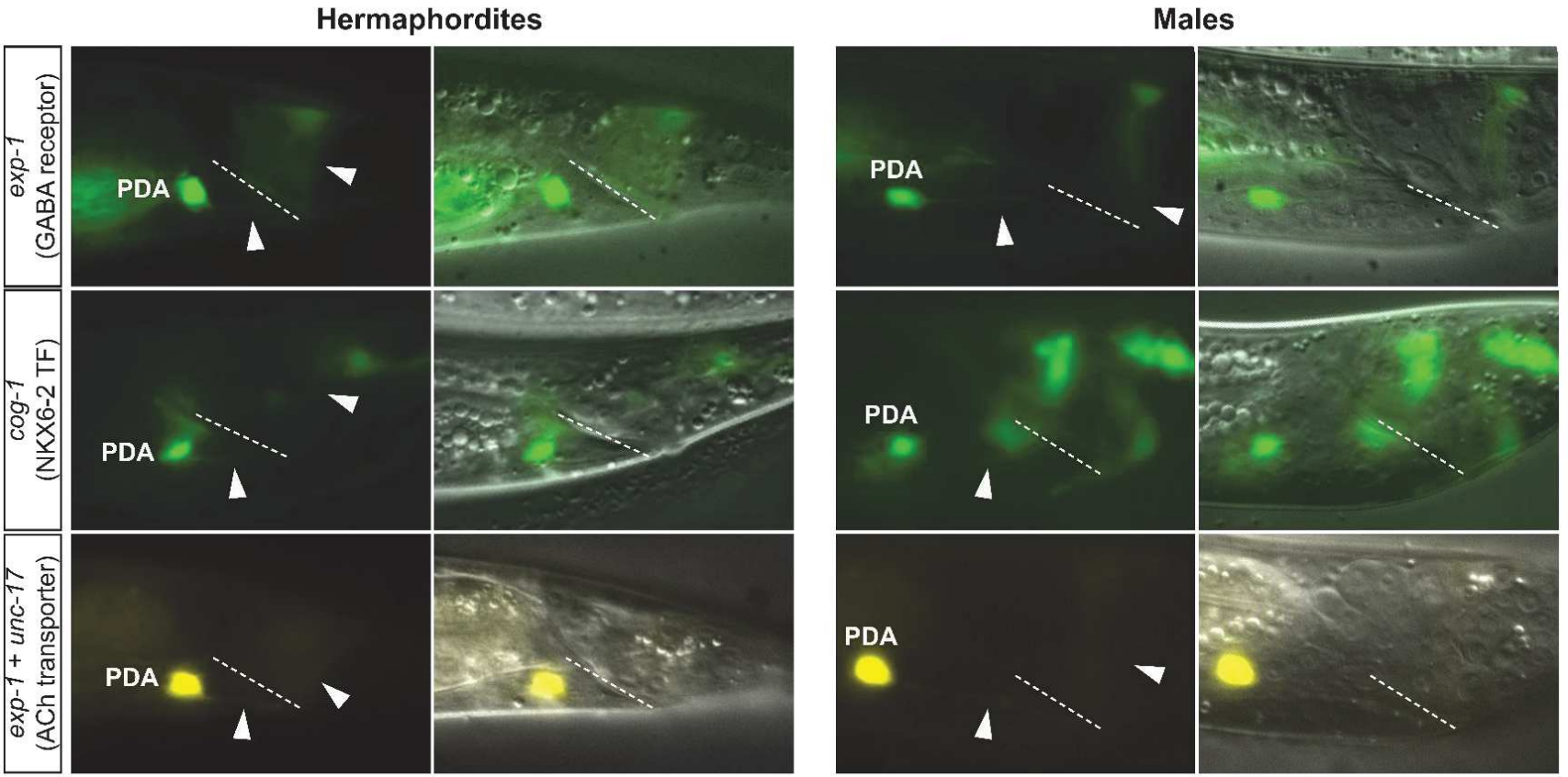

Figure 49. PDA in males is a motor neuron like in hermaphrodites. PDA in males, like in hermaphrodites, expresses *exp-1, cog-1 and unc-17* genes. It has a neurite that goes posteriorly and then turns dorso-anteriorly, posteriorly to the rectum. The neurite segment of PDA in males that goes from the cell body to the rectum is longer than in hermaphrodites as it appears that the male PDA migrates anteriorly during L3 and L4 larval development.

2.2 G1-to-RMH is a transdifferentiation event in the excretory system of the worm involving cell division

We considered a putative transdifferentiation event in the head of the worm, G1-to-RMH, which occurs between L1 and L2 larval stages (Figure 50, and Introduction 4.3.3).

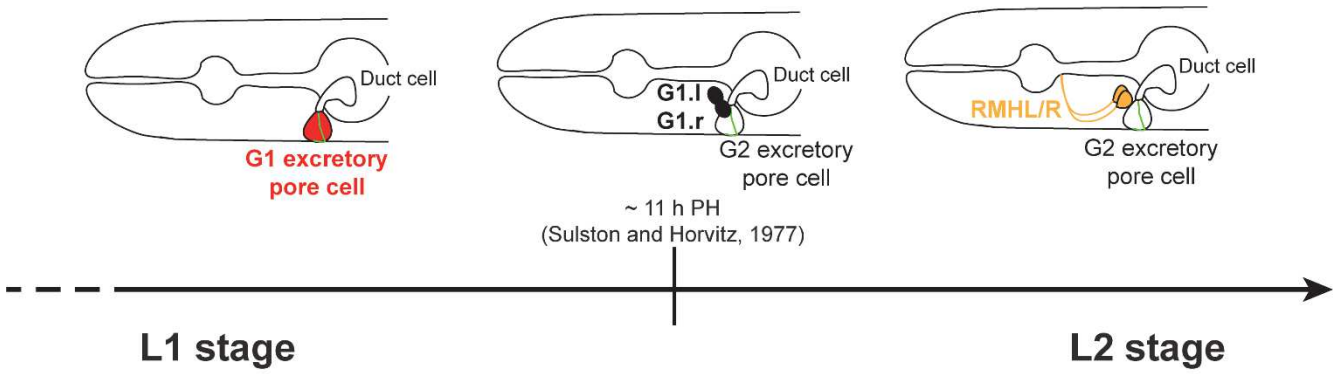

Figure 50. Timeline of G1-to-RMH. G1-to-RMH involves cell division. G1 divides 11 h PH and gives rise to RMHL and RMHR neurons by the L2 larval stage. G1 cell is represented in red; G1.l and G1.r immediately after G1 division are represented in black (identity not defined); RMH neurons appear in yellow; in green, autocellular apical junctions formed by G1 and G2 when G1 delaminates.

2.2.1 G1-to-RMH is a transdifferentiation event

The existent literature on G1 cell supports the different identity of this cell compared to its two daughter cells, the RMH neurons. In the lab we confirmed the identity of G1 excretory pore cell as an epithelial cell by using markers described in literature, useful for both identification and characterisation of its identity. A *dct-5* gene (unknown function) transcriptional reporter was used to highlight G1 cell in the L1 larvae (Abdus-Saboor et al., 2011). G1, like other epithelial cells such as the rectal cells, expresses apical junction markers *dlg-1/Discs-large* (Abdus-Saboor et al., 2011), *ajm-1* (Abdus-Saboor et al., 2011; Stone et al., 2009) and *hmr-1/E-*

Figure 51. G1 is the epithelial pore cell until L1 larval stage. DIC and fluorescence microscopy pictures of G1 pore cell in a L1 larva. In red, *dct-5* reporter allows to visualise G1 and the excretory duct cell which G1 is connected to. In green, *ajm-1* reporter allows to see the autocellular apical junctions of G1 and the apical junctions between G1 and the duct cell.

cadherin, together with *lin-26* transcription factor (Labouesse et al., 1996). It forms a tube by self-wrapping, a specialised structure which opens to the environment to execute its excretory pore cell function (Figure 51, Table 11).

On the contrary, in L2 larvae RMH neurons still express *dct-5*, but together with neuronal markers such as *cho-1* (CHOline transporter) and *unc-17* (acetylcholine vesicular transporter) as previously shown (Hobert et al., 2016). Epithelial markers are instead lost. Moreover, the SOX C family transcription factor *sem-2/SOX* is uniquely expressed in this pair of neurons in the nervous system (Vidal et al., 2015) (Figure 52, Table 11). Thus, *unc-17*, *cho-1* and also *sem-2* could be used as marker genes to identify RMH neurons in the head of the worms in combination with *dct-5* transcriptional reporter which may allow to also see their neurites. Being *dct-5* a putative zinc-finger transcription factor downstream to *daf-16/FOXO* (insulin-like growth factor signalling, Pinkston-Gosse and Kenyon, 2007), we consider it as a useful cell marker gene, but not a cell identity marker gene. Moreover, in support to this, we observed that it is expressed very broadly in the worm body. Concerning *sem-2/SOX* we considered it as a good marker for RMH identity because even though it should not be a terminal differentiation gene (being a transcription factor, differently from *exp-1* and *unc-47* used for PDA and DVB respectively), it seems not required for the acquisition of terminal RMH differentiation (Vidal et al., 2015).

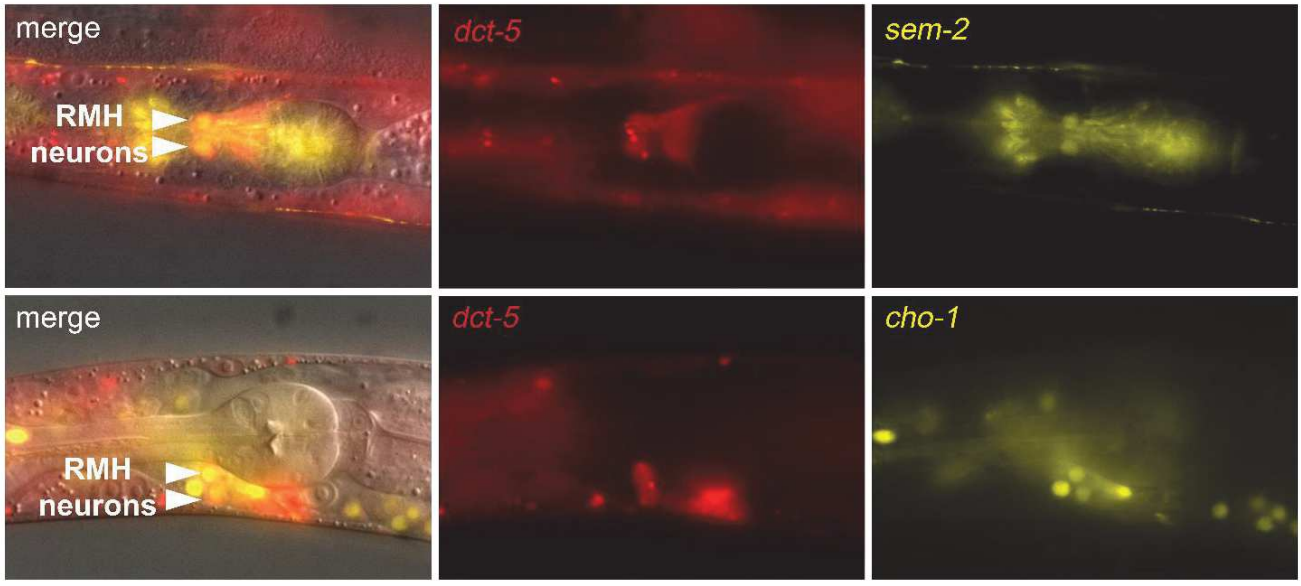

Figure 52. RMHL and RMHR are cholinergic neurons. DIC and fluorescence microscopy pictures of RMH neurons in L1 larvae. RMHL/R retain the expression of *dct-5* gene and start to express also SOX family gene *sem-2* and the choline transporter *cho-1*.

Overall, available literature and our observations demonstrate that G1 and RMHL/R have different cell identities and functions and thus support G1-to-RMH as a transdifferentiation event. Indeed the occurrence of a reprogramming event in G1 was recently suggested by experts in the worm's excretory system (Sundaram and Buechner, 2016).

C.elegans gene	Orthologues	G1	RMH	References
Epithelial markers				
ajm-1	Apical junction molecule	+	-	Abdus-Saboor et al., 2011
dlg-1	Discs-large	+	-	Abdus-Saboor et al., 2011
hmr-1	E-cadherin	+	-	This study
lin-26	Zinc-finger transcription factor	+	-	Labouesse et al., 1996
Neuronal markers				
sem-2	SOX transcription factor	-	+	Vidal et al., 2015
cho-1	Choline transporter	-	+	Vidal et al., 2015
unc-17	ACh transporter	-	+	Pereira et al., 2015

Table 11. G1 is an epithelial cell and RMHL/R are motor neurons. Summary of the epithelial and neuronal genes expressed in G1 excretory pore cell and RMHL/R neurons.

As pointed out in the Introduction, G2-to-RMF most likely also represents another transdifferentiation event. However, I did not get to characterise it because I could not find specific markers for G2 and especially for RMF neurons.

2.3 *sox-2/SOX, ceh-6/POU*, *egl-5/HOX* and *sem-4/SALL* required for Y-to-PDA in hermaphrodites and K-to-DVB are also required in Y-to-PDA in males, but not in G1-to-RMH

To reinforce the hypothesis of the existence of conserved core reprogramming factors based on the evidence obtained for Y-to-PDA and K-to-DVB, we analysed the role of Y-to-PDA and K-to-DVB factors in Y-to-PDA in males and G1-to-RMH transdifferentiation events.

2.3.1 *egl-5/HOX, sem-4/SALL*, *sox-2/SOX* and *ceh-6/POU* are required for Y-to-PDA in males

To score the impact of mutations in those genes in males, we built strains having simultaneously a mutation in *him-5* (High Incidence of Males) gene which leads to spontaneous generation of males in self-fertilising hermaphrodites (almost 40% of the progeny) that generate only hermaphrodites in wild type conditions (Meneely et al., 2012). We

observed that *him-5(e1490)* mutation alone does not lead to any PDA defect, in agreement with its function in meiotic crossover. We scored PDA presence in parallel in hermaphrodite and male siblings using *exp-1* (GABA receptor) gene as a marker of PDA terminal differentiation or in some cases *cog-1/NKX6-2* (*egl-5(n945)* and *ceh-6(gk665)*) as done previously in the lab.

We found that *egl-5/HOX*, *sem-4/SALL* and *sox-2/SOX* are required also in Y-to-PDA in males (Figure 53): the penetrance of "No PDA" defect is complete in the null mutants of *egl-5/HOX*, while it is less in *sem-4/SALL* null mutants. In males, Y-to-PDA transdifferentiation defect due to lack of *sem-4/SALL* is observed in 90% of the worms, the same percentage as observed for K-to-DVB, while around 97% defect is observed in the hermaphrodite siblings. For *sox-2/SOX* we also found an involvement in Y-to-PDA in males, but the penetrance of the defect is less than in hermaphrodites (9% vs 18%), maybe due to cell division and a possible loss of *egl-5* promoter activity in the Y.a cell, which in turn could lead to absence of sustained *sox-2* antisense expression (the antisense RNA used to KD *sox-2/SOX* in the rectal cells. See MATERIALS AND METHODS). Concerning *ceh-6/POU,* we have very preliminary results indicating that it is also involved in Y-to-PDA in males (scored using *cog-1/NKX* reporter); scoring PDA in males in these mutant background with *exp-1* reporter will allow to draw more precise conclusions about the penetrance of this defect. Moreover, if ongoing studies in the lab confirm CEH-6 association with SOX-2 in Y *in vivo*, it will be likely that *ceh-6/POU* is involved also in the male Y.

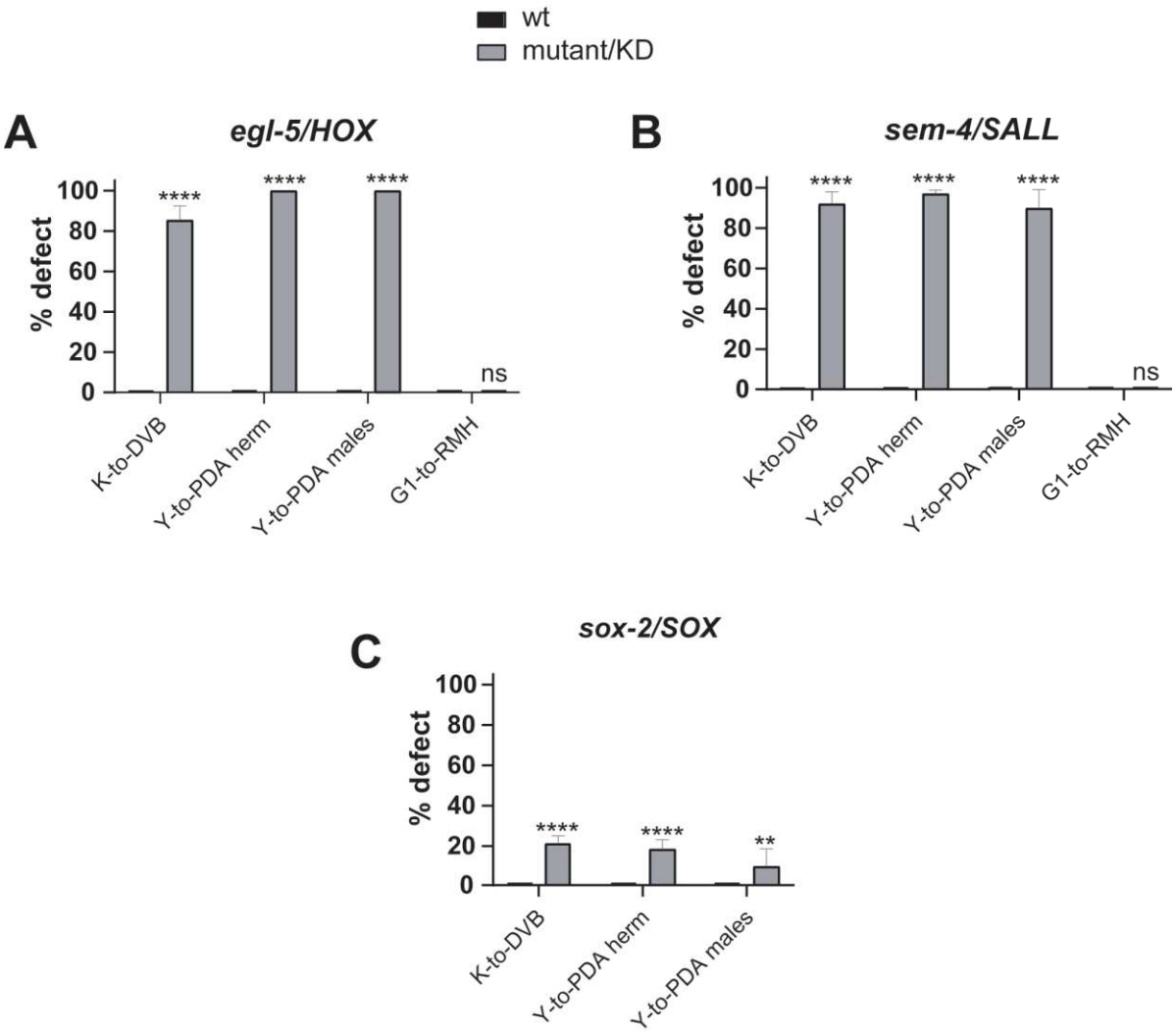

Figure 53. *egl-5/HOX, sem-4/SALL, sox-2/SOX* and *ceh-6/POU* are conserved reprogramming factors in *C. elegans* rectal cells. Histograms showing the percentage of worms with transdifferentiation defects for K-to-DVB, Y-to-PDA in hermaphrodites, Y-to-PDA in males and G1-to-RMH Tds in **A)** *egl-5/HOX,* **B)** *sem-4/SALL* and **C)** *sox-2/SOX* mutants or KDs. The role of *sox-2/SOX* and *ceh-6/POU* could not be assessed in G1-to-RMH for technical reasons. DVB presence based on *unc-47* expression; PDA presence based on *exp-1* or *cog-1* expression; RMH presence based on *cho-1* expression. N≥2; mean and SD shown.

2.3.2 *sem-4/SALL* and *egl-5/HOX* are not required for G1-to-RMH, *sox-2/SOX* and *ceh-6/POU* should not be involved as they are not expressed in G1

Next, we checked the involvement of Y-to-PDA and K-to-DVB genes in G1-to-RMH, starting from the genes with a viable null mutant available: *sem-4/SALL* and *egl-5/HOX* (Basson and Horvitz, 1996; Chisholm, 1991).

In *sem-4/SALL* mutants, we could not observe RMH neurons in 57% of the worms scored according to *sem-2/SOX* expression (Figure 58). However, when we used the terminal

differentiation gene *cho-1*, we could not detect the same defect: instead, RMH neurons were always present (Figure 53). I suggest that these contrasting results might be explained by *sem-2/SOX* being directly regulated by *sem-4/SALL* in RMH neurons as described previously in the sex myoblast (Shen et al., 2017).

In the head of the worm the *HOX* gene *egl-5* is not expressed. The *C. elegans* anterior *HOX* gene is *ceh-13* (even though it was also found in other parts of the body), while *lin-39* is expressed in the middle part and the other *HOX* genes are more posterior (Brunschwig et al., 1999; Tihanyi et al., 2010; Wang et al., 1993). Aiming to test those genes, we first excluded an involvement of the posterior *HOX* gene *egl-5* which is required for Y-to-PDA and K-to-DVB. As expected, we found that in *egl-5/HOX* null mutants RMH neurons are not affected as seen by expression of *cho-1* (Figure 53). Thus, we tested the involvement of *lin-39/HOX,* by scoring worms with a Ts allele *(n709)* grown at 25°C: however, not even *lin-39/HOX* is involved in RMH neuron formation (Figure 54), according to its posterior expression with respect to *ceh-13/HOX* (Brunschwig et al., 1999; Wang et al., 1993). I plan to test *ceh-13/HOX* in the coming weeks, as the expression pattern along the anteroposterior axis suggests that this is the *HOX* gene that could have a role in the G1 pore cell. We think it is likely that a *HOX* gene is required even in this context as supported by findings that this family of transcription factors act as transcriptional guarantors, for instance to drive terminal differentiation of neurons together with other families of transcription factors (for instance *unc-86/POU,* Zheng et al., 2015). Indeed, *ceh-13/HOX* is already known to be expressed in several neurons during development (Brunschwig et al., 1999; Tihanyi et al., 2010) and in particular in most of the pharyngeal nerve ring neurons (Regos et al., 2013).

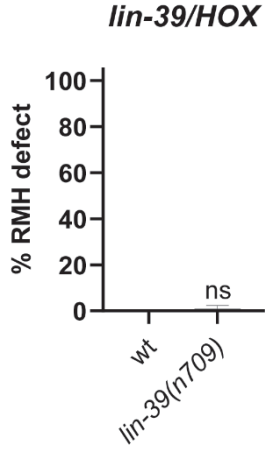

Figure 54. *lin-39/HOX*, like *egl-5/HOX*, is not required for G1-to-RMH. Histograms summarising the percentage of absence of RMH neurons based on *cho-1* expression in *lin-39(n709Ts)* mutants scored at 25°C. N=3; mean and SD shown.

Finally, concerning *sox-2/SOX* and *ceh-6/POU,* due to technical limitations we have not assessed yet their involvement in G1-to-RMH. However, we checked their expression in G1 cell in wild type early L1s, before G1 divides at around 10-11 h post hatching (Sulston and Horvitz, 1977). We could not detect neither the expression of *sox-2/SOX* (n=18) nor of *ceh-6/POU* (n=24) in G1 (Figure 55), even though *ceh-6/POU* was previously found in G1 in the embryo (Murray et al., 2012). We observed that these genes are expressed in other cells of the worm excretory system in L1 larval stage (Sundaram and Buechner, 2016): *ceh-6/POU* is expressed in the excretory canal cell (as already known, Sundaram and Buechner, 2016) and probably in the excretory gland L and R cells, while *sox-2/SOX* is present in the duct cell and two other cells at each side of G1 (that could be G2 and W cells). Given the absence of *ceh-6/POU* and *sox-2/SOX* in G1, I think that those genes should not be involved in G1-to-RMH transdifferentiation, at least cell-autonomously. However, to be sure of this conclusion, we are currently looking for a red marker for RMH neurons which could be used in parallel to *sox-2/SOX*, *ceh-6/POU* or other genes' *gfp* KIs in *grl-2p::nanobodyGFP::zif-1* strains (*grl-2* is a gene expressed in few cells in the worms, including the excretory pore cell, Hao et al., 2006).

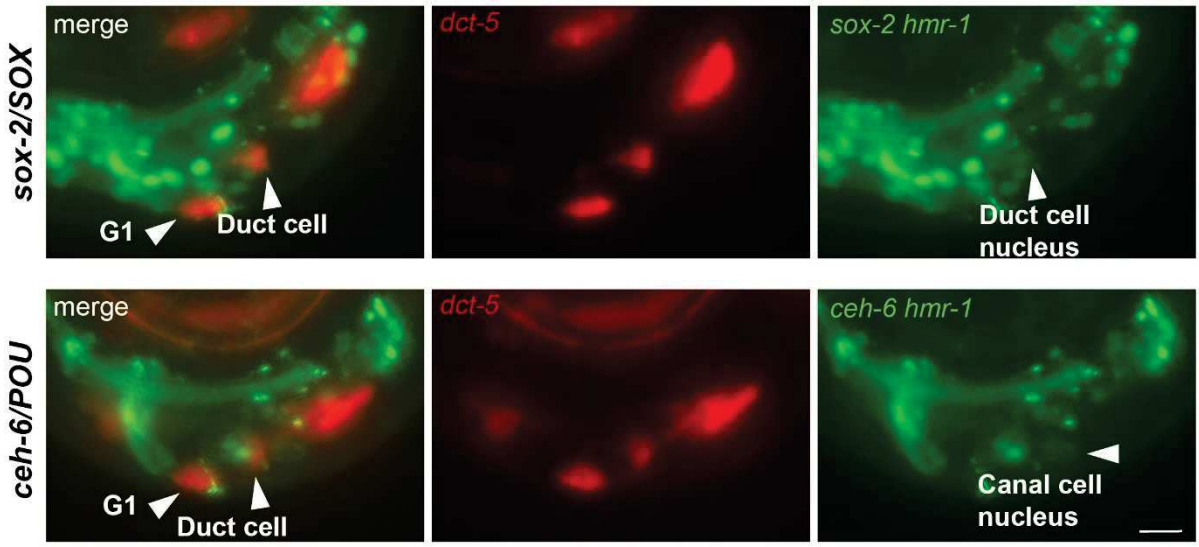

Figure 55. *sox-2/SOX* and *ceh-6/POU* are not expressed in G1 pore cell in early L1 larval stage. G1 cell and the duct cell are visible thanks to *dct-5p::mCh* reporter. Apical junctions are visible in green thanks to *hmr-1/E-cadherin* reporter. Endogenous s*ox-2/SOX* and *ceh-6/POU* expression is also visible thanks to (KI) *gfp* reporters. *sox-2/SOX* is visible in the nucleus of the duct cell but not of G1 (top); *ceh-6/POU* is visible in the nucleus of the canal cell but not in G1 (bottom).

Overall, these results reinforce the hypothesis that a conserved "plasticity cassette" exists consisting at least in *sem-4/SALL*, *egl-5/HOX*, *sox-2/SOX* and *ceh-6/POU* required for all the cell plasticity events in the rectum of *C. elegans*. Other factors, maybe belonging to the same families, might be instead required for transdifferentiation events in the head of the worm (and specifically in the excretory system where G1-to-RMH and G2-to-RMF could be compared).

2.4 *egl-27/MTA* is required with a variable lower penetrance in transdifferentiation events involving cell division

We next tested the involvement of *egl-27/MTA* in Y-to-PDA in males and G1-to-RMH. As described above, this putative component of chromatin modifier complexes is not very significantly required for K-to-DVB while it is key for Y-to-PDA initiation.

2.4.1 *egl-27/MTA* is required for Y-to-PDA in males, but with a lower penetrance compared to Y-to-PDA in hermaphrodites

Using the same approach as for the other genes and thus scoring in parallel hermaphrodite and male siblings, we observed that the long isoform of *egl-27/MTA* is also required for Y-to-PDA in males but the penetrance of "No PDA" defect is lower than the percentage found in hermaphrodites, going from 72% to 48% of animals lacking PDA (Figure 56).

Thus, *egl-27/MTA*, even though required for Y-to-PDA in males, seems to play a less - even if still - important function in presence of cell division.

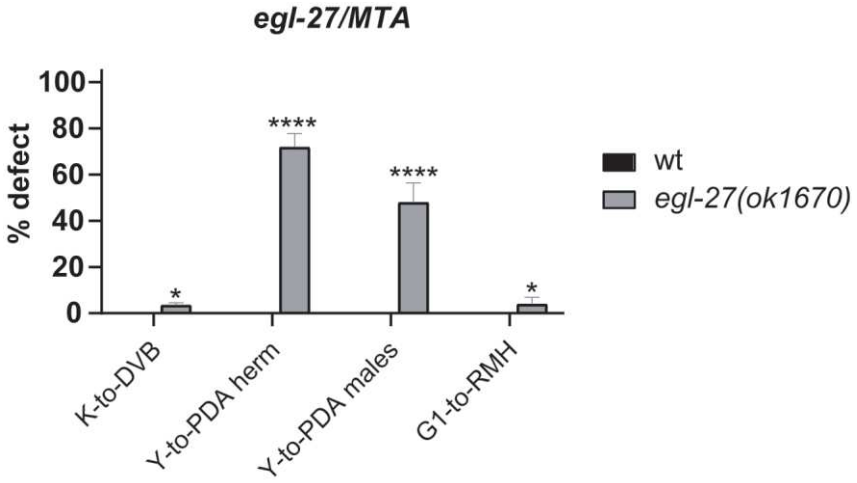

Figure 56. *egl-27/MTA* is required with different penetrance in Y-to-PDA in males and hermaphrodite, but it is not very significantly required for K-to-DVB and G1-to-RMH. Histograms showing the percentage of transdifferentiation defect for K-to-DVB (based on *unc-47*), Y-to-PDA in hermaphrodites, Y-to-PDA in males (based on *exp-1*) and G1-to-RMH (based on *cho-1*) in *egl-27/MTA* mutant worms. N=3; mean and SD shown.

2.4.2 *egl-27/MTA* is not key for G1-to-RMH transdifferentiation, as it is not for K-to-DVB

We next assessed the role of *egl-27/MTA* in RMH neurons formation by using *cho-1* marker and found a low, but statistically significant "No RMH" defect, as observed for K-to-DVB (Figure 56). We speculate that the reasons might be the same as in K-to-DVB with the cell division

contributing to chromatin remodelling or other chromatin remodelling factors at play in these contexts.

In conclusion, *egl-27/MTA* does not appear as a key factor in several reprogramming events. Differently from the Y-to-PDA initiation transcription factors, *egl-27/MTA* seems more specific to the Y-to-PDA process, both in males and hermaphrodites with a lower penetrance in males consistent with an impact of DNA replication and cell division on chromatin remodelling (Demeret et al., 2001), required for cell fate conversions (Ladewig et al., 2013).

2.5 Other reprogramming factors required for Y-to-PDA are not involved in either K-to-DVB or G1-to-RMH

Aiming to identify more conserved factors involved in transdifferentiation, other transcription factors required for Y-to-PDA discovered more recently by our lab, namely *hlh-16/OLIG, ref-2/ZIC*, were tested in all the other reprogramming events considered in this Thesis.

2.5.1 *hlh-16/OLIG* is required for Y-to-PDA in males, perhaps for G1-to-RMH, but not for K-to-DVB

We built strains to score the presence of DVB based on *unc-47/VAGT* expression and presence of the DVB neurite in mutants for *hlh-16/OLIG*. We used a hypomorphic allele obtained in the lab through mutagenesis screenings for “No PDA” defects. We found that *hlh-16/OLIG* hypomorphic allele does not affect DVB formation: in all the animals DVB is present in this mutant background even though defects in the neurites were observed (Figure 57A). Thus, *hlh-16/OLIG* which is required for Y-to-PDA transdifferentiation is not involved in K-to-DVB. Giving the neurite defects in *hlh-16/OLIG* mutants, it is possible that it is required for DVB neurite formation (cell autonomously or more probably non-cell autonomously). Indeed, the expression of those genes in Y and not in K supports these results (Bertrand and Hobert, 2009 and our observations).

Next, we tested whether *hlh-16/OLIG* is required in Y-to-PDA in males, by scoring PDA presence using *exp-1* marker. In agreement with its expression in Y and its daughter cells, we found that *hlh-16/OLIG* is required for Y-to-PDA in males and a highly penetrant “No PDA” defect is observed using the hypomorphic allele (Figure 57A).

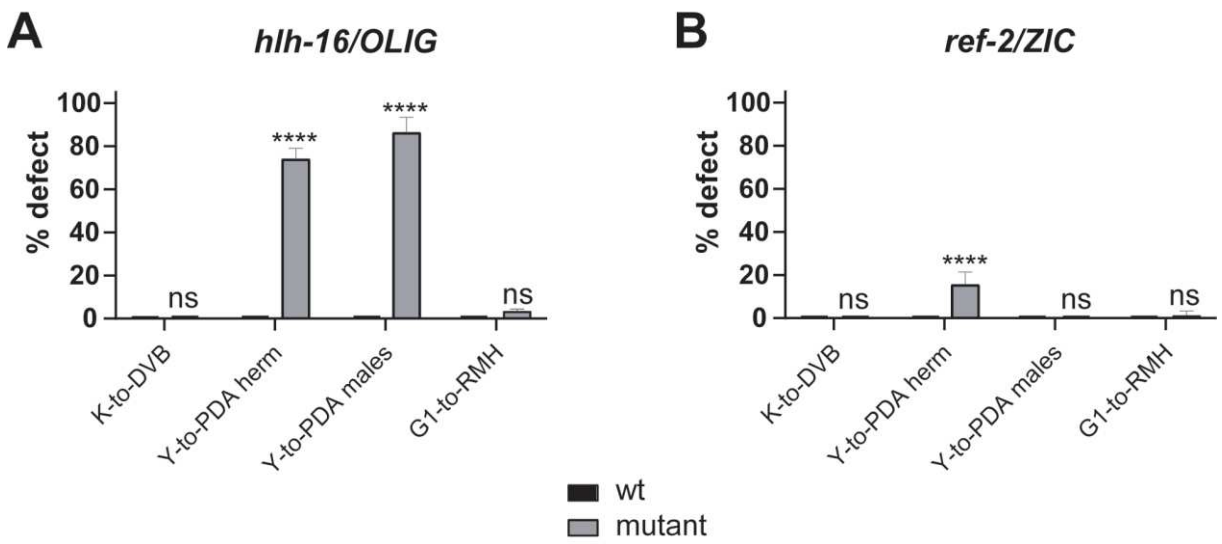

Figure 57. *hlh-16/OLIG* and *ref-2/ZIC* are event-specific reprogramming factors. A) Histograms showing the percentage of worms with transdifferentiation defects for K-to-DVB, Y-to-PDA in hermaphrodites, Y-to-PDA in males and G1-to-RMH Tds in *hlh-16/OLIG* hypomorphic mutants. **B)** Histograms showing the percentage of worms with transdifferentiation defects for K-to-DVB, Y-to-PDA in hermaphrodites, Y-to-PDA in males and G1-to-RMH Tds in *ref-2/ZIC* hypomorphic mutants. DVB presence based on *unc-47* expression; PDA presence based on *exp-1* or *cog-1* expression; RMH presence based on *cho-1* expression. N=3; mean and SD shown.

Finally, we checked the involvement of *hlh-16/OLIG* in G1-to-RMH using *dct-5* and either *cho-1* or *sem-2/SOX* marker genes for the RMH neurons. The obtained results suggest that *hlh-16/OLIG* is required for RMH formation even though low and different percentages of "No RMH" defect are observed using *cho-1* (3.4%) and *sem-2* (12.6%) markers (Figure 57A and Figure 58). This low penetrance might also be due to our *hlh-16(fp12)* allele being a hypomorphic allele. However, *hlh-16* expression has been reported at least in G1 mother cell (Murray et al., 2012), supporting this result. The difference in the result using either *cho-1* or *sem-2* as RMH markers (observed also in *sem-4/SALL* mutants) might be explained by their different nature. Being CHO-1 a choline transporter, thus a terminal differentiation gene, I preferred to always adopt this gene as a marker of RMH identity for scoring the candidate mutant genes.

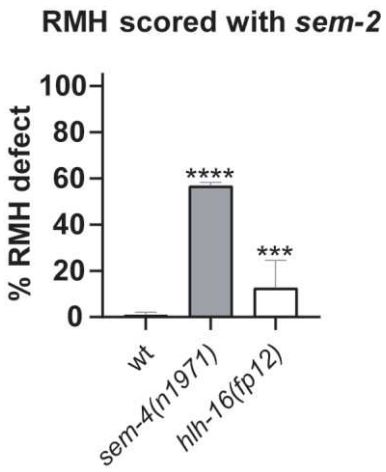

Figure 58. *sem-4/SALL* and *hlh-16/OLIG* mutants show absence of RMH neurons scored with *sem-2/SOX* marker gene. Histograms showing the percentage of RMH defect in *sem-4/SALL* and *hlh-16/OLIG* mutant worms according to *sem-2/SOX* expression. N≥2; mean and SD shown.

2.5.2 *ref-2/ZIC* might be not required for either K-to-DVB, Y-to-PDA in males and G1-to-RMH, but a null mutant is needed to confirm

The last gene that we tested for all the transdifferentiation events we are studying is *ref-2/ZIC*, involved in Y-to-PDA initiation too (unpublished).

As for *hlh-16(fp12),* we used a hypomorphic allele isolated in the lab (*ref-2(fp9)*) which showed Y-to-PDA initiation defects. However, by scoring DVB presence in this background, we could not find any defect (Figure 57B). The same observations were made for G1-to-RMH: even though *ref-2/ZIC* was shown to be expressed in G1 pore cell (Bertrand and Hobert, 2009), our hypomorphic allele does not impact on RMH formation as showed by *cho-1* expression (Figure 57B).

Concerning Y-to-PDA, surprisingly we could not find any PDA defect in males as opposite to the significant 15% “No PDA” defect observed in hermaphrodites (Figure 57B). This result might suggest that factors which do not play a key role (thus showing low penetrance of transdifferentiation defects) may become dispensable in presence of cell division (i.e. the mechanisms regulating it and its consequences). Alternatively, since *ref-2(fp9)* allele used is a hypomorphic allele (Ala→Thr at the beginning of exon 4/5 depending on the isoform), it is possible that the real impact of this gene on Y-to-PDA is not revealed with this missense allele.

2.6 Conclusions

The results presented in this chapter demonstrate that *sem-4/SALL, egl-5/HOX*, *sox-2/SOX* and *ceh-6/POU* are conserved reprogramming factors, at least in *C. elegans* rectal cells, while

other factors are more event-specific (Figure 59). Thus, the transcription factors *hlh-16/OLIG* and *ref-2/ZIC* might be specifically required for reprogramming when cholinergic neurons such as PDA, AIY (Bertrand and Hobert, 2009) (and RMH?) are generated. In support to this hypothesis, *Olig2* and *Ngn2* (orthologues of *hlh-16*) were shown to drive transdifferentiation of fibroblasts into spinal motor neurons in mice (Son et al., 2011). Finally, the impact of *egl-27/MTA* (chromatin remodelling complex component) is also event-specific and its requirement might depend on the absence of cell division.

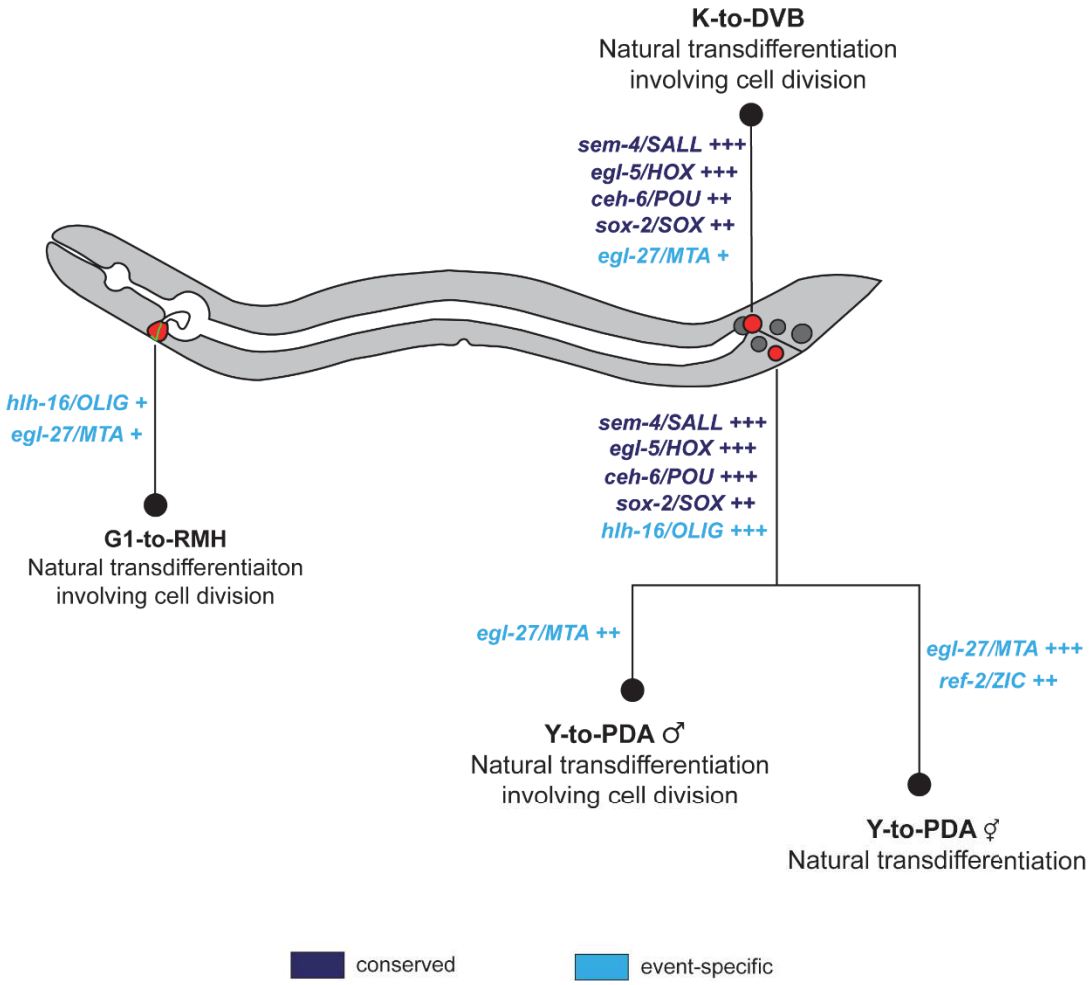

Figure 59. Schematic representation of the transdifferentiation events that we described during *C. elegans* larval development and of the factors involved in them. Factors are divided in conserved (very significantly required in more than one event) and event-specific (very significantly required only in one event). *hlh-16* is considered here as event-specific because involved only in Y-to-PDA in hermaphrodites and males according to our hypomorphic allele.

CONTRIBUTIONS OF MARTINA HAJDUSKOVA AND CHRISTELLE GALLY

Dr. Martina Hajduskova started the K-to-DVB project. She contributed to the discovery of the requirement of the Wnt signalling pathway in K-to-DVB and to the description of the orientation of K division and of K.a and K.p nuclear volumes in wild type worms vs *lin-17* and *sem-4* mutants.

Dr. Christelle Gally has been working on the K-to-DVB project with me, contributing to the results described in this Thesis. I would like to acknowledge her work by indicating which experiments she helped to perform:

- Characterisation of K and DVB markers in wildtype.
- Orientation of K division and K.a/K.p nuclear volumes.
- Score-recover-score experiments of *goa-1* and *lin-5* mutants.
- Timeline of K division and DVB differentiation.
- *lim-6*, *sox-2, ceh-6* and *unc-47* expression timing in K.p in wild type and expression in *sem-4* and *lin-17* mutants.
- Identification of TCF and SOX2 binding sites in *lim-6 intron 4*.
- Expression of *lim-6 intron 4* reporter, wild type and mutated.
- Scoring of *sem-4*, *sox-2*, *ceh-6*, *egl-5*, *egl-27*, *lin-40* and *lin-17* mutants.
- RNAi on *sox-2* paralogues (not shown).
- Cloning of pSJ721, pSJ739 and pSJ759.
- Design of the *gfp* CRISPR KI strains for *sem-4*, *lim-6*, *sox-2* and *ceh-6* genes.

Dr. Christelle Gally and Dr. Juliette Godin have been invited members of this PhD Thesis's committee.

V. DISCUSSION

1 Results summary

The work presented in this Thesis provides evidence of the existence of evolutionary conserved transcription factors which can convert cell identities *in vivo*, in natural like in induced reprogramming contexts. Moreover, for the first time we attempted to dissect the respective contribution and relationship between reprogramming transcription factors and extracellular signalling cues in driving reprogramming. The mechanism through which the Wnt/β-catenin signalling pathway works in *C. elegans*, with the occurrence of cell division, also provides an example of a reprogramming event in which transcription factors, a signalling pathway and cell division regulated by it are coordinated.

We have demonstrated that cell division is required for the transdifferentiation of the K rectal epithelial cell into the DVB GABAergic neuron, while its orientation is not. The expression and the activity of the conserved reprogramming factors *sox-2/SOX* and *ceh-6/POU,* of *sem-4/SALL* and of the *HOX* gene *egl-5* together with the activation of the Wnt/β-catenin asymmetry pathway allows the reprogramming only in the WNT-signalled daughter cell of K. We have shown that both the transcription factors, in particular *sem-4/SALL,* and the Wnt signalling pathway are necessary but not sufficient to erase the remaining epithelial identity of K.p at birth and to establish the neuronal one. In absence of these genes or of functional Wnt pathway, K.p displays a hypodermal nucleus and it fails to silence epithelial and rectal genes and later to activate pan-neuronal, terminal selector and terminal differentiation genes.

It appears that while the reprogramming factors, expressed in all the rectal cells, provide a plastic cellular context, the activation of the Wnt signalling pathway in parallel acts as a driver of transdifferentiation in only one daughter cell. The existence of a conserved “plasticity cassette” functional in the rectal cells of the worm is supported by the importance of those genes in Y-to-PDA in males too. This plasticity might also contribute (and have evolved) to confer the sex-specific capacity of the rectal cells in the male to form the male tale mating structures, as in the male B, F and U, which do not divide in hermaphrodite, undergo multiple rounds of cell divisions to give rise to different cell types including neurons. Indeed, we cannot exclude that those could be other instances of transdifferentiation.

2 K-to-DVB: a bona fide transdifferentiation event requiring the Wnt signalling pathway

The occurrence of cell division and especially the requirement of the Wnt/β-catenin asymmetry pathway with the consequent ACD could raise doubts about K-to-DVB being an actual transdifferentiation event.

As highlighted in the Introduction of this Thesis, the definition of transdifferentiation clearly states the need of unambiguously determining the differentiated identity of the initial and final cells and their lineage relationship. Both in the original definition (Okada, 1986) and in the following ones (Rothman and Jarriault, 2019), the occurrence of cell division is never indicated as a discriminating factor to identify transdifferentiation events from those which are not. In our specific case, previous evidence and our work showed that K is a fully differentiated cell, through morphological, marker gene expression and functional analyses. At the same time, DVB is also a fully differentiated cell, but its identity, as assessed by morphology, marker gene expression and function too, is completely different form the K's one. Moreover, the lineage relationship between K and DVB has been known since several years thanks to the seminal work by J. Sulston and H. R. Horvitz (Sulston and Horvitz, 1977), as already pointed out in the Introduction. Thus, K-to-DVB fulfils all the requirements to be considered a transdifferentiation event. In addition, the occurrence of K-to-DVB transdifferentiation in an ACD setting (two different daughter cells, transdifferentiation only in one, thanks to the Wnt signalling pathway) does not collide with the definition of transdifferentiation either.

Because of the requirement of the Wnt/β-catenin asymmetry pathway, a question was raised by a member of the *C. elegans* scientific community which I had the chance to present my PhD work to (*European Worm Meeting* 2018). However, the involvement of a signalling pathway required in many other different contexts (of reprogramming and not) is not an argument against the occurrence of transdifferentiation in K-to-DVB. First, it is well known that few, conserved signalling pathways are re-used at different times during animal development and that they are integrated with the specific cellular context (i.e. different gene expression) leading to different cell-specific outputs (Pires-daSilva and Sommer, 2003). Second, the study of other natural reprogramming events in several animals (both invertebrates and vertebrates) has demonstrated that signalling pathways are required in every context, together with specific transcription factors and chromatin modifiers. The involvement of a signalling pathway does not preclude the definition of those cellular events as transdifferentiation (see Table 1). In some cases, the signalling pathway was also shown to be upstream to the expression of the transcription factor(s) which drive reprogramming (see Introduction 2.1.2), something that is not observed for K-to-DVB. In K-to-DVB, the Wnt signalling pathway acts in parallel to the

reprogramming factors, at least not regulating the expression of *sem-4/SALL*. Thus, even though Wnt-driven ACD was described as a defined cellular process itself, the fact that the Wnt signalling pathway alone is not sufficient to form DVB reinforces the idea that K-to-DVB cannot be only denoted as a Wnt pathway-dependent ACD. Linked to this last point, the Wnt/β-catenin asymmetry pathway has been shown to be involved in several different cellular contexts: the EMS is an early blastomere which is pluripotent (it contributes to pharynx, muscle, neurons and other cell types); the seam cells, including the T cells, are post-embryonic blast cells; the AIY and SMDD's mother is an embryonic neuroblast (Bertrand and Hobert, 2009; Goldstein et al., 2006; Yamamoto et al., 2011). Thus, as already highlighted in the Introduction with the available literature, the Wnt/β-catenin asymmetry pathway leads to different outputs in *C. elegans* presumably because the cellular context of a pluripotent blastomere, for instance, is not the same of a post-embryonic cell. Activation of the Wnt signalling pathway is a mechanism that regulates DVB formation from K, rather than defining the cellular process itself.

To sum up, in support to K-to-DVB being a transdifferentiation event, we could highlight several points:

1) it occurs in larvae (no pluripotency anymore);
2) the initial cell is a terminal differentiated cell with hypodermal features like the other cells in the same organ;
3) the final cell is a terminal differentiated cell of a different type, neuronal;
4) there is a confirmed lineage relationship between the initial and final cells.

These observations can be also extended to Y-to-PDA in males and G1-to-RMH.

Moreover, for K-to-DVB we could add that:

5) the contribution of a signalling pathway does not preclude the definition of transdifferentiation, as inferred from Table 1;
6) the same transcription factors required in absence of cell division are required also in presence of cell division, to erase the epithelial identity.

3 Reprogramming TFs are evolutionary conserved

The transcription factors *sem-4/SALL*, *sox-2/SOX* and *ceh-6/POU* belong to families of transcription factors which are known to be master regulators of cell fate and have reprogramming activities both in reprogramming to pluripotency and transdifferentiation in mammals (and other vertebrates). It is interesting to see how the reprogramming capacity of these nuclear factors is conserved through evolution: while in *C. elegans* a few orthologues are involved in cell plasticity in the form of natural transdifferentiation, in vertebrates the

expanded families retain different reprogramming capacities which were mostly shown through their forced overexpression (Julian et al., 2017; Malik et al., 2018). The output of reprogramming of differentiated mammalian cells depends on the specific factor used to reprogram: if the POUV family transcription factor *Oct4*, absent in *C. elegans*, is used in combination with *Sox2* and other pluripotency-associated factors the result is reprogramming to pluripotency; if instead of *Oct4*, other POU factors such as the pro-neural POUIII family *Brn2* (suggested to be, based on sequence, the orthologue of *ceh-6,* Bürglin and Ruvkun, 2001) is used combined with other factors the result is transdifferentiation, while *Brn2* and the other *POU* genes are usually not able to confer pluripotency (Malik et al., 2018). The same observations were made for *Sox2* as summarised by Julian et al., 2017. The role of *C. elegans sox-2/SOX* was discussed by Vidal et al., 2015: they found that *sox-2/SOX* is not required for embryonic neural development like in vertebrates, but rather for terminal neural differentiation of post-embryonic blast cells. These blast cells are “differentiated, polarised epithelial cells” and include K, other rectal cells and also the seam cells (see Introduction 4.3). In absence of *sox-2/SOX* these epithelial blast cells fail to form the neurons that they always form in wild type conditions. However, while they conclude that K.p does not acquire the identity of its sister K.a based on *ceh-6/*POU expression (used as rectal cell marker), based on *col-34* (collagen) expression we think that K.p does remain rectal in rectal *sox-2/SOX*-depleted worms. I think that the lack of *ceh-6/POU* expression in K.p in *sox-2/SOX* mutants could be due to the parallel activity of the Wnt signalling pathway which is required for this downregulation. In any case, their data support our results of *sox-2/SOX* being a key plasticity factor in differentiated epithelial cells in *C. elegans*, which then have the capacity to give rise to neurons.

Thus, despite some differences, the activity of the *C. elegans sox-2* and *ceh-6* in Y and K reveals an ancient, evolutionary conserved capacity of those gene families in reprogramming of differentiated cells (maybe due to pioneer activity as showed for the mammalian counterparts). In addition, *hlh-16/OLIG* required for Y-to-PDA and probably in G1-to-RMH is also an orthologue of factors (*NeuroD, Neurog, Olig*) used in reprogramming to neurons in mammals (Xu et al., 2015).

Even though the nuclear factors allowing reprogramming appear conserved, there is a striking difference in the efficiency of natural reprogramming in *C. elegans* compared to induced reprogramming in mammalian cells. I think that this could be due to three main reasons:

1) in natural reprogramming the activity of more, not yet identified factors is probably required which makes it efficient and robust. On the contrary, to achieve induced reprogramming in mammalian cells a few factors are overexpressed, usually at the same moment, and their stoichiometry is probably not optimal;

2) natural reprogramming is developmentally programmed: some molecular events might "prepare" the cell before transdifferentiation may be observed at the cellular level. Reprogramming transcription factors *sox-2/SOX*, *ceh-6/POU*, *sem-4/SALL* and (for Y) *hlh-16/OLIG* are already expressed in the rectal cells since hours before the initiation of transdifferentiation in Y and K. This mechanism could be at least in part responsible of what Vidal et al. defined as "predetermined and lineage-specified differentiation program" (Vidal et al., 2015). Moreover, another explanation for efficient and robust natural reprogramming could be the existence of bivalent chromatin similar to the configuration found in ESCs (Zuryn et al., 2014);
3) finally, *C. elegans* genome lacks DNA methylation, an important epigenetic modification which contributes to progressively silence chromatin during differentiation, establishing and maintaining cell identity in vertebrates (Bogdanović and Lister, 2017). I think that this might also be one reason for which reprogramming of mammalian cells appears rarer (although widely occurring during regeneration) and more difficult to be achieved *in vitro*.

4 *egl-5/HOX* is required for transdifferentiation initiation and perhaps to specify the specific neuronal identity

egl-5/HOX gene acts downstream to *egl-27/MTA*, and likely to the NODE-like complex, in Y-to-PDA (Kagias et al., 2012). We have shown that *egl-5/HOX* is required for both Y-to-PDA (in both sexes) and K-to-DVB with a very high penetrance of "No Td" defect in the null mutant background. In absence of *egl-5/HOX*, Y remains rectal epithelial and K fails to divide in 2/3 of the worms which do not form DVB, while in 1/3 K.p remains epithelial. Thus, *egl-5/HOX* is necessary for the initiation of transdifferentiation in both contexts.

We cannot exclude that *egl-5/HOX* might be required even later for the differentiation of PDA and DVB neurons (even though *egl-5* reporters do not show expression in PDA and DVB, antibody staining allowed to see EGL-5 protein at least in PDA). As summarised by Hobert and Kratsios, 2019, *HOX* genes (defining the regional identity) have been shown to act synergistically with terminal selector genes to activate the expression of terminal differentiation genes in *C. elegans,* while in *Drosophila* they act upstream to terminal selectors. Thus, *HOX* genes may play a role in neuronal differentiation at different steps. More experiments are required to elucidate whether *egl-5/HOX* could play a direct role in DVB differentiation, either by contributing to initial activation of *lim-6/LMX* terminal selector (like in *Drosophila*) or by activating terminal differentiation genes.

If we demonstrate that *ceh-13/HOX* is required for G1-to-RMH, the same explanations may be applied to that event and it would also highlight a more general role of *HOX* genes in the plasticity of differentiated cells in addition to cell fate specification during development.

5 Some chromatin modifiers might be less required in presence of cell division

Another group of factors required for reprogramming are chromatin modifiers. However, differently from transcription factors, we could not identify conserved chromatin factors which are importantly required for all the transdifferentiation events we have been studying. EGL-27/MTA, which is key in Y-to-PDA and was shown to interact *in vitro* with SEM-4/SALL, SOX-2/SOX and CEH-6/POU (Kagias et al., 2012), is not very significantly required for the other transdifferentiation events all involving cell division. In K-to-DVB and G1-to-RMH the defect in *egl-27/MTA* mutants is below 5%, while in Y-to-PDA in males although still important it is lower than in hermaphrodites. Moreover, we tested other chromatin factors involved in Y-to-PDA for K-to-DVB (such as *lin-15A*, *lin-56* and *wdr-5.*1, not shown), but none of them is involved. These results suggest that the occurrence of cell division might bypass the requirement for some chromatin modifiers possibly because DNA replication facilitates chromatin remodelling with dilution of pre-existing modifications, even without active mechanisms. However, it is also likely that other chromatin factors are required in presence of cell division or in specific transdifferentiation events and a broader analysis should be performed to reinforce our conclusions.

Linked to the role of cell division in reprogramming at the nuclear level, histone chaperones and histone variants are other chromatin modifiers and components which would be worth to test: as non-canonical histone variants are deposited in a replication-independent manner (Henikoff and Smith, 2015), it would be very interesting to assess their function in Y-to-PDA in hermaphrodites, where cell division does not occur, compared to Y-to-PDA in males. This might be another mechanism, together with the existence of bivalent chromatin, to explain the drastic cell identity change occurring in Y in absence of DNA replication and cell division. It is likely that deposition of new histones independently of DNA replication might contribute to the erasure of the initial identity in Y in hermaphrodites while this mechanism might be (completely or partially) dispensable in males where DNA replication and cell division occur.

6 The role of orientation of K division in DVB formation

By measuring the orientation of K division in *lin-17/FZD* and *sem-4/SALL* mutants compared to wild type, we found that in some mutants the orientation appears out of the wild type range, with K.p born more ventrally or dorsally. In the case of *lin-17/FZD* mutants, we could attribute this phenotype to the function of *lin-17/FZD* and how LIN-17 protein works. As also shown in other cellular contexts in *C. elegans*, LIN-17/FZD becomes polarised in the cytoplasmic membrane on the side where WNT comes from, in the mother cell which will divide (Goldstein et al., 2006). The downstream cascade leads to differential transcription in the two daughter cells and at the same time, through the non-canonical pathway, regulates spindle orientation. Thus, the loss of *lin-17/FZD* impacts both on spindle orientation and cell fate at the same time, but the two processes can be uncoupled with respect to DVB formation as orientation is not required for DVB fate acquisition. In any case, the variations in the angle of division observed is not very high and we speculate that physical constrains might account for this (see below). Concerning *sem-4/SALL*, the explanation is more difficult as it is a nuclear factor; it is more likely that its effect on K spindle orientation is indirect.

There could be also a technical reason for the misorientation observed in both *sem-4/SALL* and *lin-17/FZD* mutants: the measurement of the orientation of K division was not performed by directly quantifying the orientation of the mitotic spindle (K division happens in a few minutes and therefore it is difficult to catch). As said above, the orientation was measured on tightly synchronised worms by quantifying the angle formed by the rectal slit with K.a and K.p nuclei alignment, maximum 1 h after K division. However, the synchronisation of mutant worms cannot be as tight as in wild types and some worms might have been older than expected. In that case I speculate that the K.p cell which remains epithelial, maintaining the cell-cell junctions (as it occurs in 90% of the worms in both mutants), is trapped in the rectal epithelium. This would explain why in some cases we see K.p between K.a and F. A similar mechanisms was shown in *Drosophila* epithelial cells, where lateral adhesion molecules allow reintegration of epithelial cells born outside the epithelial layer (Bergstralh et al., 2015).

7 Asymmetric partitioning of apical junctions: does it impact on K-to-DVB?

Cell division can contribute to cell fate diversification by allowing chromatin remodelling (discussed above), but also by asymmetrically partitioning of cytoplasmic components (cell fate determinants) through oriented ACD. Different examples of this mechanism have been described through phyla, as pointed out in the Introduction (3.2).

During K division, which appears stereotyped and oriented, we observed asymmetrical partitioning of apical junction components DLG-1/Discs-large, HMR-1/E-cadherin and AJM-1, thanks to the apicobasal polarisation of the K rectal epithelial cell and to the axis of division perpendicular to it. These proteins are inherited only by the anterior (apical) daughter cell K.a, while they do not appear in the posterior (basal) daughter cell K.p which becomes DVB. We could hypothesise that the exclusion of these proteins from K.p might contribute to K.p reprogramming (even though by looking at our reporters we cannot conclude that the respective genes are immediately silenced in K.p after K division). We have tried to test the role of *hmr-1/E-cadherin* in K-to-DVB by knocking it down with the nanobodyGFP strategy (to see whether K.a could become DVB) or by overexpressing it under the control of the *lim-6 intron 4* regulatory region (to see whether DVB was not formed): however, we failed to downregulate HMR-1 as the junctions were still visible, and the expression of *hmr-1::gfp* in DVB was very faint and difficult to score. In the few only worms marked with HMR-1::GFP (together with nuclear terminal selector *lim-6/LMX*) the DVB neurite was visible: these preliminary results suggest that the presence of HMR-1 in K.p does not prevent DVB formation. Technical improvements are required to properly carry out these experiments, for instance by integrating the *nanobodyGFP::zif-1* construct to knockdown GFP-tagged proteins (ongoing in the lab, by Christelle Gally and Allan Alcolei). These experiments will allow to clearly determine whether apical junctions, in particular E-cadherin, represent a barrier to K.p reprogramming into a neuron. Indeed, it was shown that downregulation of E-cadherin is required for efficient differentiation of PSCs into neural cells (Malaguti et al., 2013).

Although so far we could not directly address the role of apical junctions in K-to-DVB, some results would suggest that their asymmetrical partitioning should not play a role in K.p reprogramming:

- The ACDs regulated by the Wnt/β-catenin asymmetry pathway usually mainly depend on the asymmetrical partitioning of components of the pathway downstream to WNT such as FZD, DVL, WRM-1 and APR-1;
- No interaction is found between HMR-1/E-cadherin and the β-catenins WRM-1 (involved in K-to-DVB) and BAR-1 in *C. elegans* (Korswagen et al., 2000);
- The wild type orientation of K division is not required for K.p to become DVB;
- Finally, and most importantly, K.a inheriting apical junctions (as judged by position) is competent to become DVB if the polarised WNT ligand LIN-44 from the tail is missing.

Thus, it is very unlikely that apical junctions (or unidentified colocalised proteins) could act as cell fate determinants, such as in a cell intrinsic ACD setting. Nevertheless, we cannot exclude that like in seam cells (Wildwater et al., 2011), physical constrains due to the position of K in

the rectum, i.e. the cell junctional contacts with the other rectal cells and the cell shape, might contribute to the stereotyped anteroposterior cell division of K together with the polarised WNT signal. But as said previously, the orientation of K division does not play a role in K-to-DVB while the Wnt/β-catenin asymmetry pathway does. In conclusion with the data we have so far, the presence of apical junctions might have an influence on the orientation of K mitotic spindle, however without an impact on the identities of the daughter cells.

8 The Wnt pathway and reprogramming TF *sem-4/SALL* act in parallel in reprogramming

We have shown that the Wnt signalling pathway does not act upstream to *sem-4/SALL* expression, nor vice versa, although the K.p phenotypes of *lin-17/FZD* and *sem-4/SALL* mutants are indistinguishable. Moreover, we have demonstrated that in the *sem-4(n1378)*; *wrm-1(ne1982)* double mutants a synergistic increase of "No DVB" defect is seen. Even though for this double mutant analysis we did not use null alleles, because the penetrance of the defect is already too high in *sem-4(n1971)* and *lin-17(n671)*, overall our data suggest that *sem-4/SALL* and the Wnt signalling pathway act in parallel and non-redundantly to drive K-to-DVB. The identification of the molecular targets regulated by POP-1/TCF and SEM-4/SALL transcription factors would allow to see whether the two pathways converge on the regulation of common transcriptional targets to reprogram K.p into DVB, as the phenotypes of the mutants suggest. A similar mechanism where Wnt signalling contributes to reprogramming in parallel to reprogramming transcription factors was described for reprogramming to pluripotency, as pointed out in the Introduction (2.2.1.3).

9 *sox-2*/*ceh-6* and Wnt signalling might act in parallel and in an antagonistic manner to control the timing of re-differentiation

From the analysis of *wrm-1/β-catenin; sox-2/SOX* double mutant (with the same limitations as for *sem-4/SALL*), it appears that the Wnt signalling pathway also acts in parallel to *sox-2/SOX*, as it could be expected from members of a conserved "plasticity cassette". Moreover, some observations in the lab on *sox-2/SOX* and *ceh-6/POU* expression have shown that in wild type worms *sox-2/SOX* and *ceh-6/POU* are progressively downregulated in K.p after K division, in a mutually exclusive way to the activation of the DVB terminal selector *lim-6/LMX* expression (Christelle Gally), and that both regulations depend on Wnt signalling (Figure 32, Figure 60) (For *ceh-6/POU,* its loss in K.p was already known. Bürglin and Ruvkun, 2001). Considering that putative TCF binding sites are found in *lim-6 intron 4* regulatory region, that SOX2 binding

sites overlap with TCF binding sites, and given the anti-correlation between *lim-6/LMX* and *sox-2/SOX-ceh-6/POU* expression in K.p, we hypothesised a model in which SOX-2 (perhaps together with CEH-6) binds to *lim-6/LMX* regulatory regions and represses it until *sox-2/SOX* (and *ceh-6/POU*) are downregulated and POP-1/SYS-1 can instead bind to the same sites and activate *lim-6/LMX* expression. Indeed, this mechanism was described in NSCs where downregulation of *Sox2* is required for TCF-mediated activation of *Neurod1* gene, thus promoting neurogenesis (Kuwabara et al., 2009).

To test this hypothesis POP-1/TCF and SOX-2/SOX are produced in bacteria and purified, and an EMSA will be performed with probes containing the binding sequence of TCF and SOX2. We will be able to test whether POP-1/TCF and SOX-2/SOX can bind this sequence and whether their opposite activities are achieved through a competition between the two for this binding. With these results, we will draw a model to describe how conserved reprogramming factors, the Wnt signalling pathway and cell division are coordinated also molecularly to drive a natural transdifferentiation event. In particular, the regulation of *sox-2/SOX* expression by the Wnt signalling pathway and the opposite activities of POP-1/TCF and SOX-2 on *lim-6/LMX*

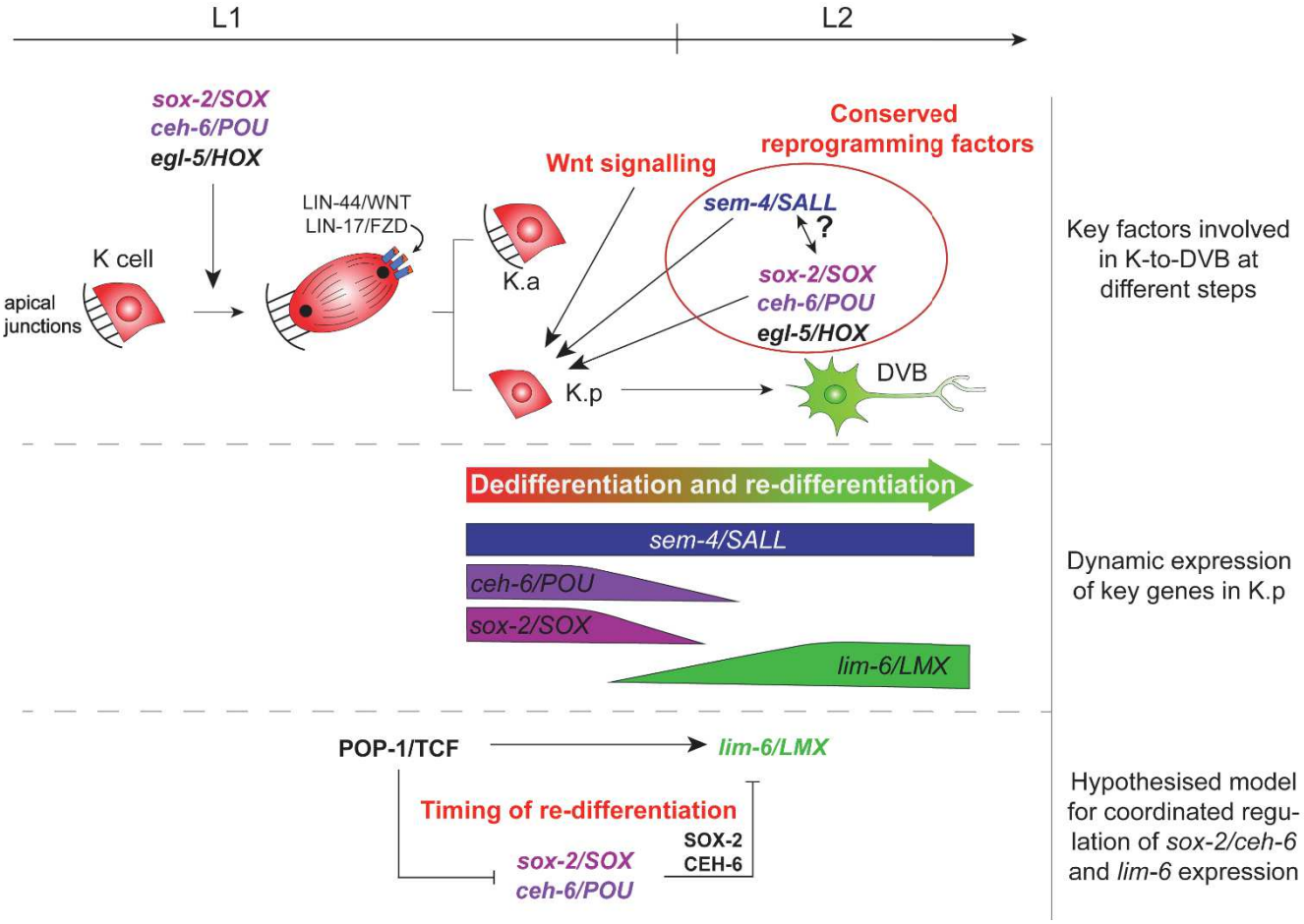

Figure 60. Model summarising the cellular steps and molecular factors required for K-to-DVB transdifferentiation, the dynamic expression of key factors in K.p and the hypothesised model for the regulation of *lim-6/LMX* expression.

regulatory regions in K.p might set a molecular timer for a tight regulation of the onset of re-differentiation (Figure 60).

In conclusion, we have shown how a signalling pathway and conserved transcription factors are required non-redundantly to ensure a reprogramming event that is 100% efficient, and how they are regulated to timely control the execution of reprogramming. This is the first study which analysed the contributions and relationships of different families of factors (i.e. signalling pathway and transcription factors) in natural reprogramming *in vivo*, by looking at the process at single cell resolution.

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ANNEX

The T blast cell putative transdifferentiation

We also aimed to demonstrate and characterise the transdifferentiation of the T blast cells which function as phasmid socket cells in early L1 and then divided several times to form hypodermal cells, neurons and the permanent PHso cells (see Introduction).

However, we decided to not focus on this event for two reasons:

1) The lack of good reporter genes to identify the cells of interest, also considering that the T blast cells undergo multiple rounds of cell divisions before forming the end-of-lineage cells. As for the other putative transdifferentiation events, I looked in the literature for good markers to identify the T blast cells and their daughters up to the PHso cells which are usually absent in mutants where the T lineage is defective (Arata et al., 2006; Yoda, 2005; Zhao et al., 2002). However, I could not find nor build enough specific and bright markers, which are not genes involved in T cell lineage regulation (see Table in the next page).
2) The absence of proof that the T blast cells function as phasmid socket cells in early L1s as seen by the DiI/DiO staining. DiI and DiO are dyes that stain *C. elegans* neurons contacting the environment with their neurites (such as the amphid and the phasmid neurons) when their support cells (socket cells) are present and functional (Hoffelen and Herman, 2009). I tried several times the staining, but I could not stain the phasmid neurons in early L1s while I could stain the amphid neurons in the head and the phasmid neurons in older worms.

Even though the staining to test the socket cell function did not work, I still cannot exclude that the T cells might be differentiated cells (perhaps like the other seam cells) which transdifferentiate during larval development. The occurrence of several rounds of cell division (importantly forming very transient cells) is not an argument against transdifferentiation as discussed in the Introduction and in the Discussion of this Thesis.

Markers for T and PHso cells		
Gene	**Array**	**Comments**
ceh-20 (Arata et al., 2006)	*mxIs28[ceh-20p::ceh-20::YFP + lin-15(+)] X*	Faint nuclear YFP seen under microscope in cells in the tail posterior (and anterior?) to the rectal slit: most probably T cells. Many nuclei observed in the VNC, P.nps and in the hypodermis. Since it's involved in cell identity acquisition, *ceh-20* is not probably good as a marker.
tlp-1 (Zhao et al., 2002)	*wgIs321[tlp-1::TY1::EGFP::3xFLAG + unc-119(+)]*	Expressed in T, T.p and T.app (the only T.a-derived cell that becomes a neuron) and hyp11, hyp10, hyp9 in the more distal part of the tail. Opposite expression pattern compared to *pop-1* and *ceh-16*. *tlp-1* mutation affects T.p and T.app cell fates.
psa-1 (Yoda, 2005)	*osEx71[psa-1::GFP + unc-76(+)]*	Not asymmetrically segregated after T cell division. Expressed in many cells. Involved in cell fate determination by chromatin remodelling. Interacts with Wnt signalling with different outputs in T.a and T.p. Epistatic to Wnt. Probably not good as markers? - ubiquitous, - same level in T.a and T.p, - involved in cell fate determination.
psa-4 (Yoda, 2005)	*osEx67[psa-4::GFP + unc-76(+)]*	Not asymmetrically segregated after T cell division. Expressed in many cells. Involved in cell fate determination by chromatin remodelling. Interacts with Wnt signalling with different outputs in T.a and T.p. Epistatic to Wnt. Probably not good as markers? - ubiquitous, - same level in T.a and T.p, - involved in cell fate determination.
alr-1 (Mains et al., 1990)	*wgIs362[alr-1::TY1::EGFP::3xFLAG + unc-119(+)]*	Not seen in larvae neither in adults. Too faint. Being a transcription factor, the translational reporter is nuclear. Need a transcriptional reporter to recognize easily the PHso cells.
ceh-16 (Cassata, 2005)	*stIs11489[ceh-16b::H1-wCherry + unc-119(+)]*	In larvae and adults, expressed only in seam cells (nuclear). Thus, it's in T in the embryo and early L1 and then along T.a lineage up to the final

		seam cell T.appa. It's lost in syncytial cells (Huang et al., 2009). Useless to follow T.p cell lineage.
ahr-1 (Qin and Powell-Coffman, 2004)	*wgIs562 [ahr-1::TY1::EGFP::3xFLAG + unc-119(+)]*	Expressed in T.p cell lineage including PHso and also in G2 and W according to literature (Qin and Powell-Coffman, 2004). Important for T.p lineage development. Observed 2 T.p cells? + other cells: may be PLM (ventral) and ALN (dorsal) neurons born before hatching. Too faint and nuclear.
lin-26 (Labouesse et al., 1996)	*fpIs108[lin-26peABCD+i::GFP + pRF4]*	Nuclear expression in T cells and PHso but also in many other cells.
lin-48	*saIs14 [lin-48::GFP; unc-119(+)]*	In PHso (or PHsh?) cells but no T cell.
dct-5	*qnEx59[dct-5p::mCherry; unc-119(+)]*	Expressed in T cells.
ajm-1	*jcIs1[ajm-1::GFP; rol-6(su1006)]IV*	Expressed in T cells and PHso cells.

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Claudia RIVA

Analysis of the core mechanisms underlying transdifferentiation in *C. elegans*

Résumé

L'objectif de cette étude est de définir quels facteurs centraux et spécifiques à l'événement affectent différents événements de transdifférenciation naturelle (Td) chez *C. elegans*. Nous nous sommes concentrés sur K-to-DVB (principalement), Y-to-PDA chez les mâles et G1-to-RMH (tous épithélial-à-neurone), des *bona fide* Tds.

Nous avons évalué le rôle des FTs de reprogrammation Y-à-PDA dans ces Tds et nous avons constaté que *sem-4*, *egl-5*, *sox-2* et *ceh-6* sont impliqués dans K-to-DVB et Y-to-PDA chez les mâles, mais pas dans G1-to-RMH. Nous avons constaté que la division de K est asymétrique et est nécessaire pour la Td; la voie Wnt est requise pour la division cellulaire asymétrique de K, pour l'effacement de l'identité épithéliale de K.p et pour la re-différenciation en DVB. L'analyse des doubles mutants suggère que les FTs de reprogrammation et Wnt agissent en parallèle pour conduire K-to-DVB.

Ces résultats démontrent la présence d'une division cellulaire asymétrique n'est pas suffisante pour permettre un changement d'identité cellulaire et des FTs de reprogrammation conservés sont nécessaires en parallèle pour conférer la plasticité cellulaire.

Reprogrammation cellulaire, plasticité cellulaire, transdifferentiation, facteurs transcriptionnel, voie Wnt, *C. elegans*

Résumé en anglais

The goal of this study is to define which core and event-specific factors are affecting different natural transdifferentiation events (Td) in *C. elegans*. We focused on K-to-DVB (mostly), Y-to-PDA in males and G1-to-RMH (all epithelial-to-neuron), all *bona fide* Tds.

We assessed the role of Y-to-PDA reprogramming TFs in those Tds and found that *sem-4*, *egl-5*, *sox-2* and *ceh-6* are also involved in K-to-DVB and Y-to-PDA in males, but not in G1-to-RMH. We found that K division is asymmetric and is necessary for K-to-DVB; Wnt pathway is required for K ACD, for the erasure of the epithelial identity of K.p and for re-differentiation into DVB. Double mutant analysis suggests that conserved reprogramming TFs and Wnt act in parallel to drive K-to-DVB.

These results demonstrate that during natural Td the presence of an ACD is not enough to allow cell identity change, and conserved reprogramming TFs are required in parallel to confer cell plasticity.

Cell reprogramming, cell plasticity, transdifferentiation, transcription factors, Wnt signalling, *C. elegans*