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Modeling HD and human striatal development using 3D cultures

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Table of contents

Abstract

Huntington's disease (HD) is an inherited disease that causes degeneration of adult neurons in the striatum and later in the cortex. Although most studies have focused on adulthood, some evidence suggests that this disease might have a neurodevelopmental component. To try to address this question, we generated a protocol to derive 3D ventral organoids from isogenic control and pathologic hES cell line to mimic *in-vitro* the development of the ventral telencephalon. Immunocytochemistry analyses show correct identity acquisition, particularly if compared with dorsal protocols. These data are supported by single cell RNA-seq analyses that highlight the presence of ventral progenitors and GABAergic neurons with no dorsal subpopulations. Compared to controls, HD organoids show a 50% reduction in the number of regions that resemble the ventricular zone of the developing brain together with abnormalities in their polarity and compromised radial distribution of $GSX2^+$ and $MASH1^+$ progenitors. These early defects are combined with aberrant cell cycle regulation as shown by a 45% reduction in the number of $KI67^+$ cells, a 95% increase in asymmetric horizontal divisions and a 15% increase in cell cycle exit ratio. Moreover, scanning electron microscopy (SEM) analyses reveal irregular neuronal architecture in HD organoids. Together our data suggest that the presence of mutant Huntingtin (mHTT) causes a developmental defect in the cellular organization of ventral organoids. This 3D model has therefore paved the way to a better understanding of the potential neurodevelopment aspects of this disease and may provide a platform to further screen and comprehend this complex disease. In line, we are now analyzing single cell data from HD organoids to unveil the transcriptional pathways affected by mHTT and in parallel we are setting up a co-culture system of control and HD cells to study cellular interactions between the two lines and to discriminate cell-autonomous versus non-autonomous defects.

Abstract (Italian)

La patologia di Huntington (HD) è una malattia ereditaria dominante che provoca la degenerazione dei neuroni adulti nello striato e successivamente nella corteccia. La maggior parte degli studi si è concentrata sul cervello adulto, tuttavia alcune evidenze suggeriscono una possibile componente già durante lo sviluppo neuronale fetale. Per indagare la questione più a fondo, abbiamo sviluppato un protocollo per organoidi ventrali a partire da una linea isogenica di cellule hES controllo e patologiche al fine di mimare lo sviluppo del telencefalo ventrale *in-vitro* in 3D. Le analisi di immunocitochimica sui nostri organoidi mostrano l'acquisizione di una corretta identità, in particolare se confrontati con organoidi di protocolli dorsali. A supporto di queste osservazioni, i dati ricavati da analisi di RNAseq su singola cellula, confermano la presenza di progenitori ventrali e neuroni GABAerigici in assenza di contaminanti sottopopolazioni dorsali. Gli organoidi delle linee HD mostrano una riduzione del 50% nel numero di strutture proliferanti simili alle zone ventricolari (VZ) del cervello durante il neurosviluppo, un'anormale polarità di queste strutture e un'alterata distribuzione radiale dei progenitori $GSX2^+$ e $MASH1^+$. Questi difetti precoci si combinano con una regolazione aberrata del ciclo cellulare, mostrata dalla riduzione del 45% di cellule $KI67^+$, dall'aumento del 95% delle divisioni di tipo orizzontale asimmetrico e dall'incremento del 15% nel rapporto di uscita dal ciclo cellulare. Inoltre, analisi tramite scanning electron microscopy (SEM) rivelano come gli organoidi HD rispetto ai controlli presentino un'irregolare architettura neuronale. I nostri dati suggeriscono che la presenza della mutazione provochi un difetto di sviluppo nell'organizzazione cellulare degli organoidi ventrali. Questo modello 3D ha quindi aperto la strada a una migliore conoscenza dei potenziali aspetti di sviluppo neurologico implicati nell'HD e può fornire da piattaforma per esplorarne e approfondirne di nuovi. Stiamo infatti analizzando i dati derivati da RNAseq su singole cellule di organoidi HD per svelare i percorsi trascrizionali influenzati dalla mutazione e in parallelo stiamo istituendo un sistema di co-coltura di cellule controllo e HD per studiare le interazioni tra le due linee e discriminare i difetti di tipo cell-autonomous da quelli non-autonomous.

Aim

It is known that normal HTT has crucial roles during fetal development; while the effect of mHTT during this period and the possible neurodevelopmental defects underlying HD are still unclear. An increasing number of studies are focusing on this quest and how altered developmental programs may lead to neuronal populations to be more susceptible to mHTT during adulthood causing cell-specific neurodegeneration.

The purpose of this project is to assess whether mHTT affects the neurodevelopmental profile, differentiation and cytoarchitecture organization of neural progenitors in human ventral organoids. In line, we would like to use control and HD organoids as a platform to discover the underlying molecular mechanisms that operate during neurodevelopment and that then may lead to a more unstable and delicate brain during adulthood.

This thesis's work is organized into three sub-goals:

- Define a protocol to generate 3D ventral organoids that mimic the development of the human ventral telencephalon and compare it with dorsal organoids of a published protocol
- Investigate mHTT activity during neural development in a 3D culture by focusing on molecular and morphological aspects such as self-organization, polarity and distribution.
- Understand cell autonomous or non-autonomous defects in HD during development by developing strategies to investigate cellular interactions between WT and HD cells.

1. Introduction

1.1 Telencephalon development

1.1.1 Relevance for this thesis

In Huntington's disease (HD), medium spiny neurons (MSNs) are the main striatal neuronal population that are lost and that cause the atrophy of this region. Later this degeneration affects also the cortex and other brain regions.

Understanding how human neuro-epithelial progenitors differentiate into MSNs is critical to develop specific differentiation protocols both for regenerative medicine and to correctly model HD during development. HD mouse models gives useful but narrow view of the disease since obvious species-specific differences exist between mice and humans. Thus, additional human model systems are needed to uncover specific human gene signalling pathways that are affected in HD. To generate these models it is possible to take advantage of human pluripotent stem (hPS) cells, both embryonic stem (hES) and induced pluripotent stem (hiPS) cells, by differentiating them towards the desired lineage. In line, with the aim of modelling the human HD counterpart *in-vitro* and to closely mimic what happens during the generation of MSNs, we differentiated control and pathologic hES cells towards 3D organoids with a specific ventral identity.

In these next sections I will describe some fundamental aspects involved in telencephalic development, in particular the striatum and cortex, that have guided us through the generation of our ventral protocol. I will then focus on the three-dimensional cultures and how we exploited known protocols to generate our ventral 3D system. Finally, I will review HD *in-vitro* models and the neurodevelopmental aspects of HD revealed by these systems.

1.1.2 LGE formation and sub-organization in VZ, SVZ and MZ

Development of the nervous system starts with neural induction, followed by neurulation that gives rise to the neural tube, and patterning along the anterior-posterior (AP) axis. The neural tube folds and is subdivided into the prosencephalon (forebrain), the most anterior or rostral part sub-organized in telencephalon and diencephalon, the mesencephalon (midbrain), and the rhombencephalon (hindbrain). The telencephalon is divided into the dorsal telencephalon (pallium), which gives rise to

the neocortex, and the ventral telencephalon (subpallium), which gives origin to the striatum, the globus pallidus and the GABAergic interneurons that migrate into the cortex.
Due to the rapid migration of neurons, three intraventricular bulges form the septum, the medial, and lateral ganglionic eminences (MGE/LGE), collectively referred to as the whole ganglionic eminence (WGE). The MGE, the most ventral eminence, gives rise to the amygdala and the globus pallidus while the LGE, that is situated more dorsally, gives rise to the striatum (Evans et al. 2012; Hébert and Fishell 2008). Within the neural epithelium of the telencephalon there are two proliferative zones that contribute to the generation of neuronal precursors, the ventricular zone (VZ), close to the lateral ventricles, and the subventricular zone (SVZ), which extends from the basal region of the VZ. After exit from the cell cycle in the VZ or the in the SVZ, neural progenitors migrate radially into the mantle zone (MZ) where they undergo terminal differentiation. Thus, the ventricular eminences are rich in neurons which ultimately must find their final destination by migration along the guide offered from the radial glia. The WGE gives rise to both projection neurons and interneurons. Specifically, striatal projection neurons are born in the LGE and make up nearly 90% of LGE neurons. Interneurons originating from the LGE populate the cortex, the olfactory bulb, and the striatum, whereas those from the MGE migrate to the cortex, the globus pallidus, and also the striatum (Evans et al. 2012; Jain et al. 2001).

Telencephalon development requires a variety of TFs interactions to ensure correct positional identity and regional subdivisions. The homeobox gene ***Gsx2*** is expressed in the VZ of the ventral telencephalon where it is involved in maintaining the identity of striatal progenitors (Corbin et al., 2000). *Gsx2* specifies MSNs and olfactory bulb interneuron identity at distinct time points during neurogenesis (Waclaw et al., 2009). *Gsx2* mutant show reduced LGE and striatum dimensions and lower expression of important genes for striatal neuronal maturation such as *Ascl1*, *Ebf1* and *Gad67* (Lopez-Juarez et al. 2013).

ASCL1, a bHLH transcriptional factor also known as MASH1 (Casarosa, Fode, and Guillemot 1999), is a downstream effector of GSX2. *Ascl1* mutants show modest alterations in striatal development; while, in *Gsx2;Ascl1* double mutants, striatal development are severely affected (Wang et al., 2009; Yun et al, 2001). Wang and colleagues proposed that *Ascl1* may also serve a negative feedback function to repress *Gsx2* in LGE murine progenitors, but the precise relationship between *Gsx2* and *Ascl1* is still unclear. Since in the WGE *Gsx2* is expressed in progenitors inside the VZ and at lower levels in the SVZ while Ascl1 shows strong expression in the border between SV and SVZ and lower

expression in the SVZ (Hansen et al. 2013; Onorati et al. 2014), it seems to recapitulate the murine development.

The neural progenitors, during their radial migration towards the MZ, undergo a progressive maturation expressing typical ventral genes such as *Ebf1, Islt11, Nolz* and later *Ctip2, Darpp32, Gad67, Foxp1* and *Foxp2*. ***Ctip2*** is central to MSN differentiation and striatal development: it is expressed by all MSN and is excluded from essentially all striatal interneurons. In the absence of *Ctip2*, MSN do not fully differentiate and fail to aggregate into patches, resulting in severely disrupted patch-matrix organization (Arlotta et al., 2008). **DARPP32** and ARPP-21 are two dopamine and cyclic adenosine monophosphate (AMP)-regulated phosphoproteins that are striatal-specific markers (Anderson and Reiner 1991). In the ventral telencephalon GABAergic neuronal differentiation is promoted by **GAD**, in the two isoforms with molecular weights of 67 and 65 kDa (GAD67 and GAD65), an enzyme needed for the synthesis of the neurotransmitter GABA (Schuurmans and Guillemot 2002).

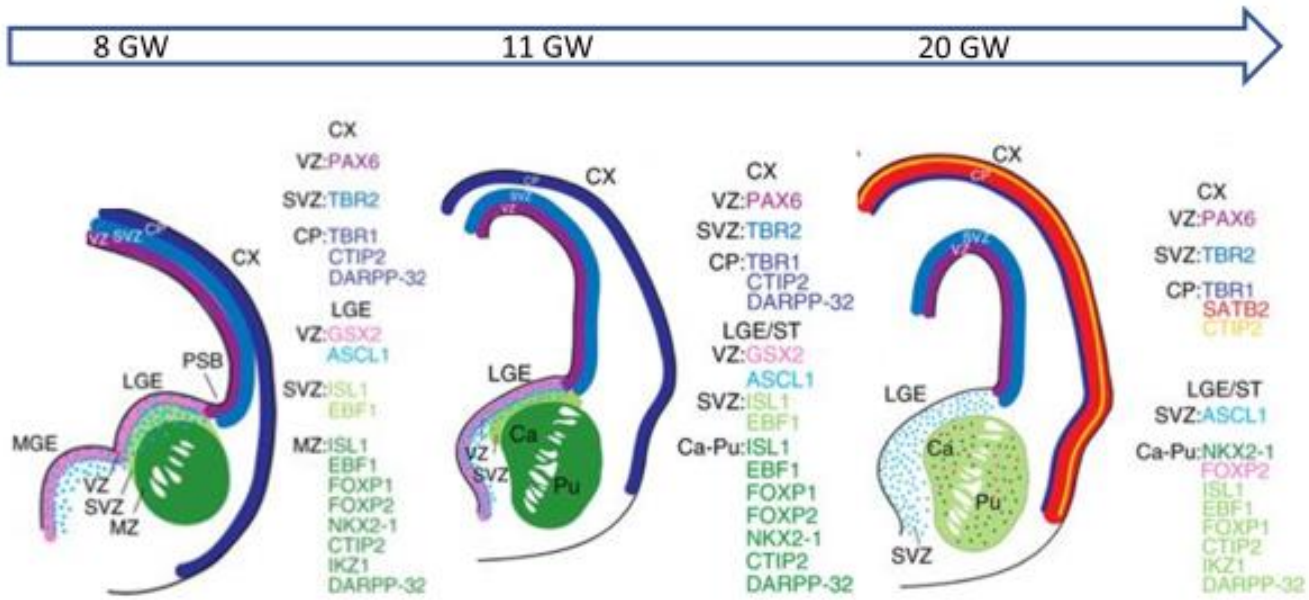

Fig 1.1: Scheme of Transcriptional Factors (TF) expression during early human fetal development, at gestational week 8, 11 and 20. *LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; CX, neocortex; CP, cortical plate; PSB, pallial-subpallial boundary; VZ, ventricular zone; SVZ subventricular zone; MZ, mantle zone; Ca, caudate; Pu, putamen. (modified from Onorati et al., 2014)*

1.1.3 Cortex formation and layering

The cerebral cortex is a six layered structure composed primarily of glutamatergic projection neurons, originated from dorsal telencephalic progenitors, and of inhibitory interneurons derived from ventral telencephalon through tangential migration.
During development the cortex contains two types of progenitor cells for glutamatergic projection neurons. The first type is the radial glia that generate both neurons and glia, express the transcription factor PAX6 and divide close to the ventricular surface. The second type, intermediate progenitor cells, are derived from radial glia, produce only neurons, express *Tbr2* and divide at more basal levels (Englund et al. 2005).

PAX6 is a paired-box transcription factor essential for cortical progenitor proliferation and for delineating the border that distinguishes the ventral telencephalon from the dorsal one (Georgala, Carr, and Price 2011). At the neural plate stage *Pax6* is expressed throughout the telencephalic anlagen. At the neural tube stage its expression is downregulated in the region that will develop the ventral telencephalon, concomitant with the upregulation of *Nkx2.1* (Corbin et al. 2003). The boundary between *Pax6* and *Nkx2.1* expression initially demarcates the dorsoventral border also called pallial-subpallial boundary (PSB) and later their expression regions become separated by the domain of *Gsx2* expression (Corbin et al. 2003). As a result, the PSB becomes defined by the intersection of *Pax6* and *Gsx2* expression (the expression domains overlap slightly). *Pax6* and *Gsx2* present a mirrored pattern of expression, with *Pax6* being expressed in a dorsal (high) to ventral (low) gradient and *Gsx2* showing the opposite gradient.

The transition from radial glia to intermediate progenitor cells (IPCs) is associated with upregulation of **TBR2**, a T-domain transcription factor, and downregulation of *Pax6*. *Tbr2* is a key molecular determinant of IPC identity, proliferation, and correct differentiation. IPC progeny contribute to the correct morphogenesis of each cortical layer (Sessa et al. 2008). Accordingly, *Tbr2* expression in progenitor compartments (the subventricular zone and ventricular zone) rises and falls with cortical plate neurogenesis.

The subsequent transition from intermediate progenitor cell to postmitotic neuron is marked by downregulation of TBR2 and upregulation of **TBR1**, another T-domain transcription factor. Cortical projection neurons express since early stages TBR1 and several bHLH genes including *Neurod2*, *Math2* and *Math3* (Fode et al. 2000). *Tbr1* is one of the few known genes with a role in the generation of a specific subpopulation of cortical neurons, it is expressed at high levels in early born neurons, in

fact *Tbr1* mutant defects are restricted to early born neurons of the marginal zone, subplate and layer 6 (Hevner et al. 2001).

Fezf2 (also known as *Fezl* or *Zfp312*), and ***Ctip2*** (*Bcl11b*) are important for postmitotic specification of deep-layer (DP) neurons. In the absence of *Fezf2* there is a significant reduction of corticospinal motor neurons (Chen, Schaevitz, and McConnell 2005; Molyneaux et al. 2005). CTIP2 is a transcription factor fundamental to establish the corticospinal tract and in *Ctip2* mutant mice, the most evident change in the neocortex is the misrouting of layer 5 axons (Arlotta et al. 2008).

In the developing cortex *Ctip2* is a direct target of **SATB2**, a DNA-binding protein important for upper-layer (UL) neuron specification. SATB2 is required to activate the UL-specific genetic program and down-regulate DL-specific genes. The lack of *Satb2* leads UL neurons to lose their identity and express genes specific of DL neurons. Moreover *Satb2* −/− mice failed to form the corpus callosum, a tract of fibers formed mostly by UL neurons (Britanova et al. 2008).

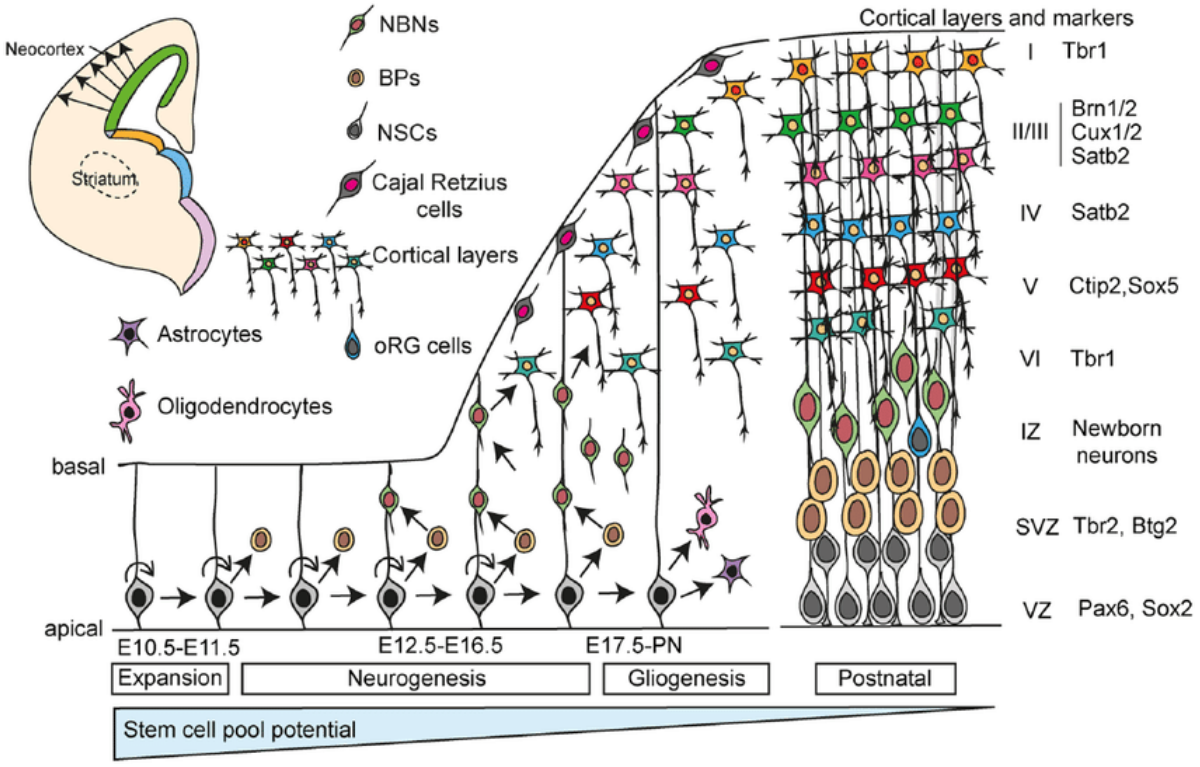

Fig 1.2: Systematic formation of the cortex layers in the dorsal telencephalon. *During early stages of cerebral cortical development (embryonic days E10.5-E11.5), NSCs predominantly undergo symmetric cells divisions to expand the NSC pool. This phase is referred to as the expansion phase. During late embryogenesis (E12-E16.5), NSCs undergo increasingly more asymmetric divisions to*

generate 1 NSC (self-renewal) and 1 BP. The BPs generate the neurons. This is the neurogenic phase. Neurons are generated in a sequential, inside-out fashion and are specified by different transcription factors. At later stages of development, NSCs generate the other cell types of the brain including astrocytes, oligodendrocytes, and ependymal cells (not shown). This is referred to as the gliogenic phase (Mukhtar and Taylor 2018).

1.1.4 Morphogens and signalling pathways

Telencephalon development requires a variety of signals secreted from surrounding signalling centres to ensure the correct positional identity of the neurons, which will populate the adult forebrain. Several gene families are involved in the coordination of telencephalon patterning and are responsible for activating downstream factors of signalling cascades allowing cells to gain a positional and molecular identity. I am going to mention here some of the most important signals which are recapitulated in the differentiation protocol (see the Materials and methods of this thesis) by molecules of agonists or inhibitors, principally: fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), WNTs (originated from the drosophila gene wingless), retinoic acid (RA), and sonic hedgehog (SHH).

BMPs belong to the TGF-β family of secreted proteins. BMPs are required for the formation of the choroid plaque (the precursor to the choroid plexus) and the cortical hem (Panchision et al. 2001). Although their inhibition is required for neuronal development, it is thought that BMPs are also needed to dorsalize the telencephalon and restrict the ventral component. BMPs are inhibited by several factors, often used in neural differentiation protocols, including chordin and noggin. TGF-β-related family of proteins comprehend also activins and Nodal. Mutants in which Nodal signalling is compromised have an enlarged telencephalon, indicating that the correct development of forebrain requires blockade of Nodal signalling (Schier and Shen 2000).

SHH signalling is crucial for ventral patterning at all levels of the nervous system (Ericson et al. 1995; Ingham and McMahon 2001). SHH operates through a concentration gradient that regulate the dorso-ventral (DV) axis at different time points to confer different neuronal identities on the developing precursors (Kohtz et al., 1998). Loss- and gain-of function analyses in several species have shown that the SHH protein is necessary and sufficient for the development of ventral neural components and the expression of related neural markers. Embryos that lack SHH fail to form normal ventral telencephalon showing markedly reduced expression of ventral markers (Rallu et al. 2002). Furthermore, ectopic expression of Shh is sufficient to induce the expression of ventral telencephalic

markers, both *in-vitro* and *in-vivo* (Gaiano et al., 1999). Throughout the development Shh presents a complementary pattern of expression from Gli3 and genetic analysis shows that their activities strongly antagonize one another. Specifically, in the absence of Shh gene, the telencephalon is strongly dorsalized, whereas in the absence of Gli3, the telencephalon is ventralized. In the absence of both Gli3 and Shh, the general aspects of DV patterning are rescued (Rallu et al.2002). SHH activates several TFs including NKX2.1 (Kimura et al., 1996; Sussel et al., 1999), GSX2 (Corbin et al., 2000; Toresson, Potter, & Campbell, 2000; Yun et al., 2001), and PAX6 (Stoykova et al., 2000).

Retinoic acid (RA) is the biologically active form of vitamin A and has been implicated in survival, specification, proliferation, and differentiation of forebrain neurons (Marklund et al., 2004; Schneider et al., 2001). It has also been reported that in vitro LGE-derived neurospheres and hES cells induce GABAergic differentiation once RA was added to the media (Chatzi, Brade, and Duester 2011). Blocking RA in chick embryos prevents the expression of *Meis2* which is expressed in progenitors of SVZ and initial MZ of the telencephalon (Toresson et al., 1999). Studies in fish and frogs have implicated WNT, RA, and fibroblast growth factor (FGF) as 'posteriorizing' (or caudalizing) factors (Altmann and Brivanlou 2001; Sasai and De Robertis 1997), indicating that inhibitors of these diffusible signals are responsible for maintaining anterior neural identity.

With respect to the antagonism of **WNT** signalling, two proteins with this activity, CERBERUS and DICKKOPF, are present in the anterior endoderm of frogs, and can induce the formation of a head (but not a trunk) when mis-expressed in frog embryos (Bouwmeester et al., 1996; Glinka et al., 1998). WNTs belong to the wingless protein family and are a class of ligands fundamental in embryogenesis and CNS development. WNTs are involved in the initial antero-posterior (AP) orientation of the neural plate and are crucial for the development of dorsal telencephalon (Houart et al. 2002). Specific concentrations of WNTs are needed to precisely define regional patterning and to induce the expression of *Pax6* (Gunhaga et al. 2003). In the absence of WNT signalling, there is an ectopic expression of ventral TFs like GSX2, DLX2, and ASCL1 in dorsal telencephalon together with downregulation of the dorsal markers EMX1, 2 and 3 (Backman et al. 2005). This ectopic expression of ventral genes facilitated the cells of the dorsal region to acquire a ventral fate and potentially to become GABAergic projection neurons. A work in mice has shown that WNT signalling is necessary for right molecular characterisation and morphology of the dorsal telencephalon before the onset of neurogenesis and that the inhibition of WNT signalling is necessary for subpallial development (Backman et al. 2005).

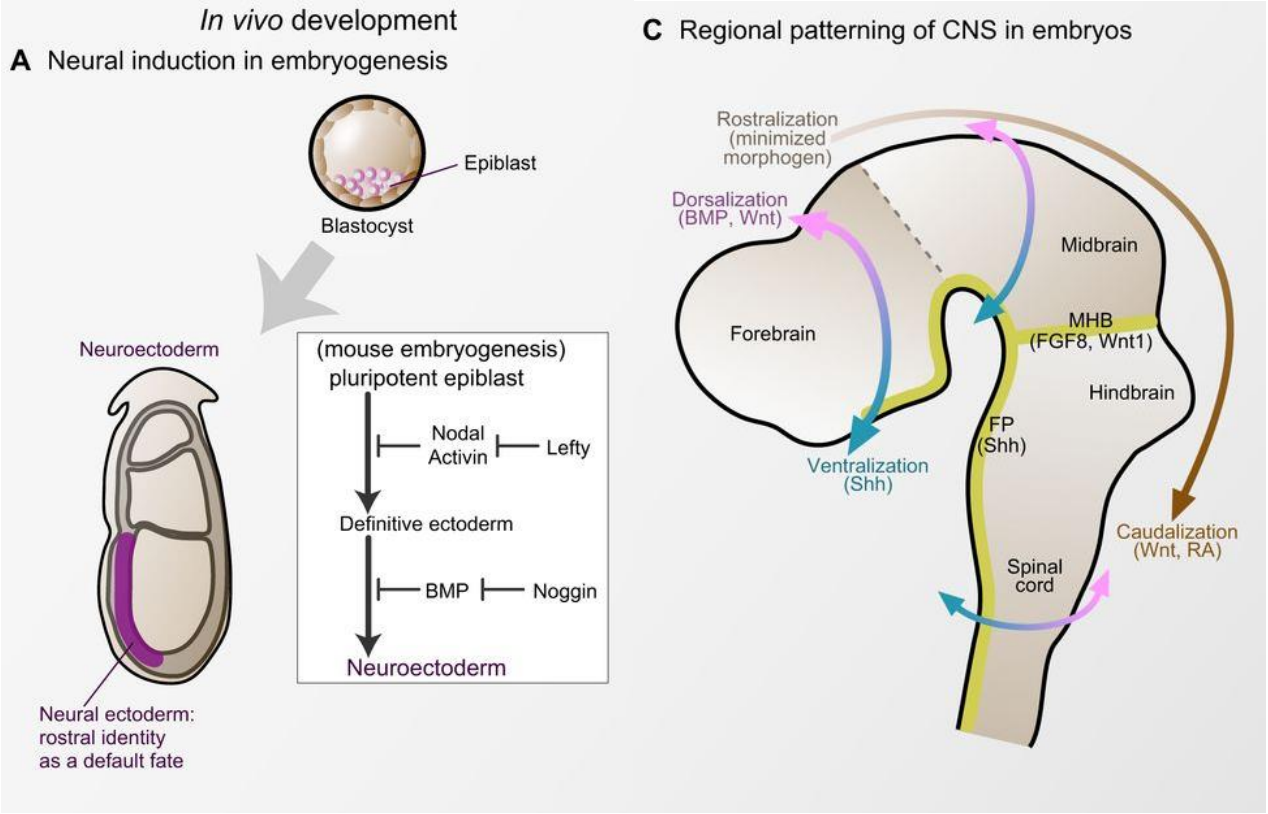

Fig 1.3: Neural induction and regional patterning in vivo*. (A) Neural induction is considered a 'default' pathway of differentiation and occurs mainly through inhibition of BMP/Nodal signaling, resulting in neural cells with rostral identity. (C) The regional identities within the central nervous system (CNS) are determined along the rostrocaudal and dorsoventral axes through the action of morphogens derived from various organizing centers (yellow). FP, floor plate; MHB, midbrain-hindbrain boundary* (Suzuki and Vanderhaeghen 2015).

1.1.5 Cell cycle regulation

The formation of the central nervous system (CNS) is governed by a tightly regulated balance between progenitor cell proliferation, cell cycle exit and differentiation to distinctive neural phenotypes. This coordination is fundamental to generate the right number of neurons and correct neuronal networks. Ontogenetic mechanisms in the developing brain are the basis for the increase in neuron's amount in specific brain regions during phylogeny. For example, the vast presence of neurons in the gyrated mammalian neocortex arise from increased progenitor numbers during development and it is considered an evolutionary distinctive tract (Mora-Bermúdez et al. 2016).

The majority of progenitor cells is within the VZ, where cells possess an epithelial polarity and comprise the stem cells of the developing nervous system, the neuroepithelial cells and ventricular (or apical) radial glia (vRGs). These cells undergo interkinetic nuclear migration (INM), with the soma migrating during the cell cycle towards the apical surface in M-phase and towards the basal surface in S-phase. Mitotic spindle orientation during M-phase is involved in the regulation of the size of progenitor cell pools in a multitude of tissues by coordinating the direction of cell division (Knoblich 2010). The cleavage plane of divisions in embryonic radial glia cells regulates the number of adult neural stem cells (aNSCs). Oblique or horizontal divisions which are considered asymmetric due to the different distribution of apico-basal components between daughter cells, are involved in asymmetric divisions leading to increased delamination of progenitor cells. The second type of divisions, the symmetric one, generates two neural progenitors that amplify their pool (Falk et al. 2017). aNSCs exhibit radial glia hallmarks, including apical end-feet with a primary cilium at the ventricle, elongated basal processes, and the expression of many radial-glial and astro-glial genes including *Glast* and *Gfap*.

During neuro-development, multipotential progenitors generate gradually more restricted precursors that will in the end produce neuronal or glial cells. A lot of key factors regulating cell cycle progression have been implicated in cell fate determination and differentiation of progenitors, while molecules important for differentiation are beginning to emerge as cell cycle regulators (Dubreuil et al. 2000; Heins et al. 2001; Nguyen et al. 2006). The neural tube, already after its formation, acquires dorso-ventral identity. The proliferation rate of neural progenitors in the dorsal part is faster than in the ventral part, whereas the opposite is true for the differentiation rate (Megason and McMahon 2002). A variety of early genes are expressed transiently at the progenitor stages and are downregulated before the exit from the proliferative zones (Ben-Arie et al. 1996; Scardigli et al. 2001). Therefore their ability to push cell cycle exit and potentiate neuronal differentiation depends on the induction of downstream genes (Bertrand, Castro, and Guillemot 2002).

Differentiated post-mitotic neurons after having exit the cell cycle, must avoid the re-entry into a proliferative state (Herrup and Yang 2007; Yang and Herrup 2007). In the adult nervous system neurons are typically found in a quiescent state, therefore they are considered as no more able to proliferate. However, some genes encoding for G1/S transition regulators can be found in different structures of the human adult brain. In some specific conditions these proteins could also lead to cell cycle re-entry. After acute stimuli such as neurotrophic factor deprivation, activity withdrawal, DNA damage, oxidative stress, and excitotoxicity, specific neurons can upregulate the expression of genes

important for reactivating the cell cycle. Under these conditions, they usually die at the G1/S checkpoint before any sign of DNA synthesis can be observed (Becker and Bonni 2004; Liu and Greene 2001).

Neurogenesis can occur also in adult brain of many species, including fish and birds, but in the mammalian brain it takes place at low levels in two specific regions: the olfactory bulb and the hippocampus. In particular in human adult hippocampus is still debated (Ernst and Frisén 2015; Kempermann et al. 2018). This process contributes to plasticity in the different regions, with regeneration not being its primary purpose and seems to be connected to higher cognitive functions such as learning and memory.

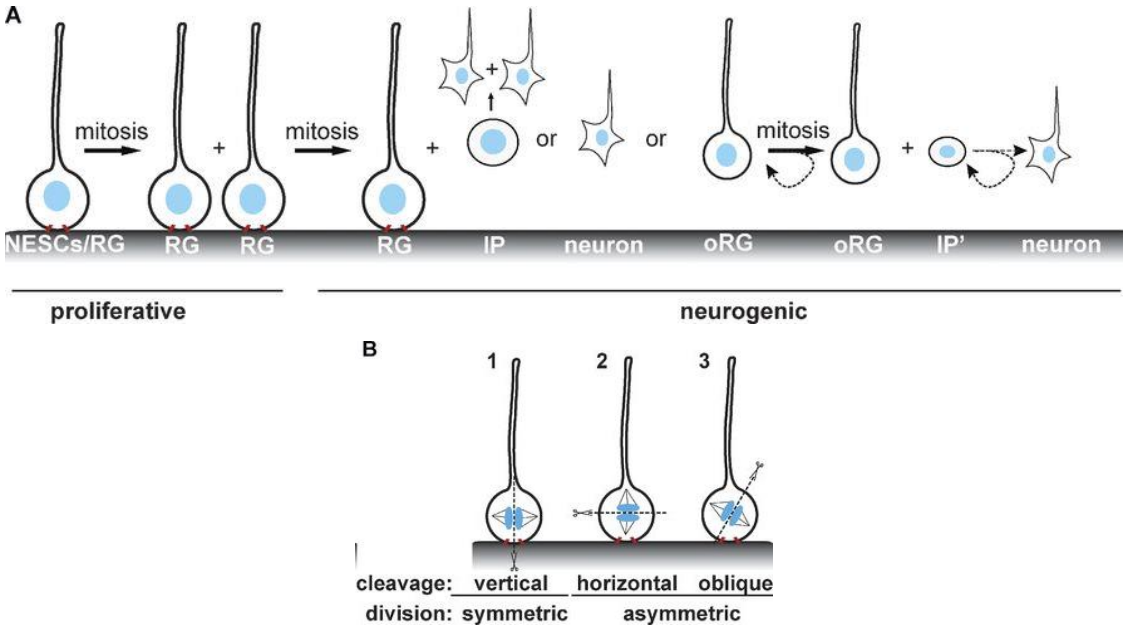

Fig 1.4: Mammalian neuronal progenitors and spindle orientation*. (A) Cell subtypes in the developing mammalian brain. NESCs, neuroepithelial stem cells. RG, radial glia. IP, intermediate progenitor. oRG, outer radial glia. IP', transit amplifying intermediate progenitors. Adherens junctions are in red. (B) A putative role of spindle orientation in the decision of symmetric vs. asymmetric division* (Noatynska, Gotta, and Meraldi 2012).

1.1.6 Primary cilium's roles in neural development

The majority of Autosomal recessive primary microcephaly (MCPH) proteins localize to the centrosomes or spindle poles (Manzini and Walsh 2011; Nigg and Raff 2009; Thornton and Woods 2009). Disruption of the centrosome or spindle -related functions are linked to the premature switch from symmetric to asymmetric cell division, mitotic entry defects, mitotic delay or arrest, and

apoptotic cell death, resulting in neural progenitors disruption and smaller brain size (Chen et al. 2014; Jayaraman et al. 2016; Sgourdou et al. 2017). MCPH associated proteins have been reported to regulate primary cilium biogenesis (assembly and disassembly) coupled with cell proliferation (Gabriel et al. 2016; Kim et al. 2011; Miyamoto et al. 2015).

In a recent paper (Zhang et al. 2019) cerebral organoids were generated to model microcephaly (WDR62 mutation) focusing on the role of primary cilia. The authors found that WDR62 deletion resulted in reduction of the size of organoids, increased percentage of ciliated progenitors, elongated cilium, reduced amount of cycling progenitors, increased asymmetric divisions, and premature cell cycle exit leading to decreased proliferation and premature differentiation (Zhang et al., 2019). Some of these phenotypes are comparable to our results in HD organoids (reported in the Results of this thesis) suggesting a possible influence of the primary cilia on the other aspects that requires further investigation. Moreover, the authors demonstrated that Cep170 (centrosome protein and marker of mature centrioles) co-localizes (IF data) and interacts (Co-IP data) with WDR62 at the basal body of the primary cilium and that Cep170 mutants (*in-vitro* data with MEFs and *in-vivo* data through in utero electroporation) presented similar ciliary phenotypes of those showed with WDR62 mutation (Zhang et al., 2019).

Interestingly Cep170 is an interactor of huntingtin (HTT) protein (HDinHD database) together with other components of primary cilium structure (Fig 1.5) such as Cep63, Cep126, IFT20 (intraflagellar transport 20, involved in the anterograde transport of proteins cargo towards to the cilium), and different members of Kinesin and Dynein motors involved respectively in anterograde and retrograde trafficking (DYNC1H1, DYNLL1, KIF2A, KIF3A, KIF5A, KIF5C). Many of these components have been demonstrated to influence the ciliary length, ciliary biogenesis and cell cycle progression (Broix et al. 2018; Kim et al. 2011; Li et al. 2011) leading to cortical and gyral pattern abnormalities associated also with microcephaly (Poirier et al. 2013). These data reinforce the link between MT-dependent processes at the level of primary cilia and neurodevelopmental pathologies. Indeed, centrioles in basal bodies influence cilia function while in centrosomes they mediate cell division. Cell cycle entry is preceded by cilium resorption, whereas exit from mitosis and differentiation are accompanied by cilium assembly. Thus, defects in cilia contribute to causing proliferative disorders and alterations in cellular differentiation (Bisgrove and Yost 2006).

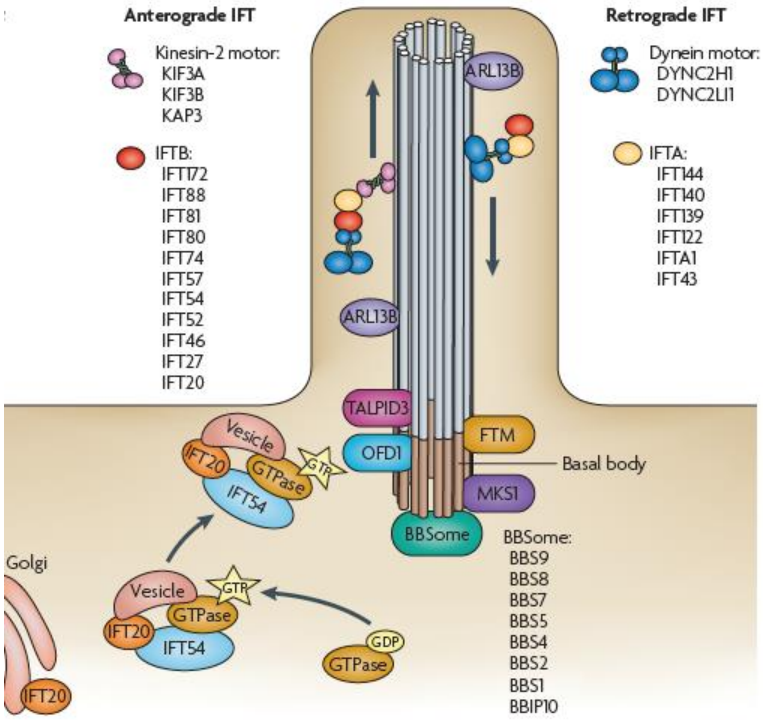

Fig 1.5: Cilia structure and intraflagellar transport *(Goetz and Anderson 2010).*

In neural tissue, the cilium is required for Shh signaling and trafficking of Gli, a transcriptional effector of this signaling pathway. As Gli transcription factors can activate the expression of cell cycle regulators, including cyclin D1, failure of cilia function could block Gli-dependent activation of cyclin D1 control for G1–S (Jackson 2011). Evidence that vertebrate Shh signaling depends on cilia came from studies of mutants for IFT proteins that showed loss of the ventral cell types in the neural tube specified by high levels of Shh. Disruption of the kinesin-2 motor in KIF3A^{-} null embryos also caused similar defects in Shh-dependent neural patterning (Zhou 2009).

All the key components of Shh pathway are enriched in cilia: IFT proteins act at the heart of the Shh pathway, downstream of the membrane proteins patched 1 (PTCH1) and smoothened (SMO) and upstream of the Gli transcription factors that implement the pathway (Goetz and Anderson 2010). In the absence of Shh ligand, PTCH1 is localized to the base of the cilium and SMO is not associated with cilia (Corbit et al. 2005) while upon exposure to ligand, PTCH1 exits and SMO moves into the cilium (Fig 1.6).

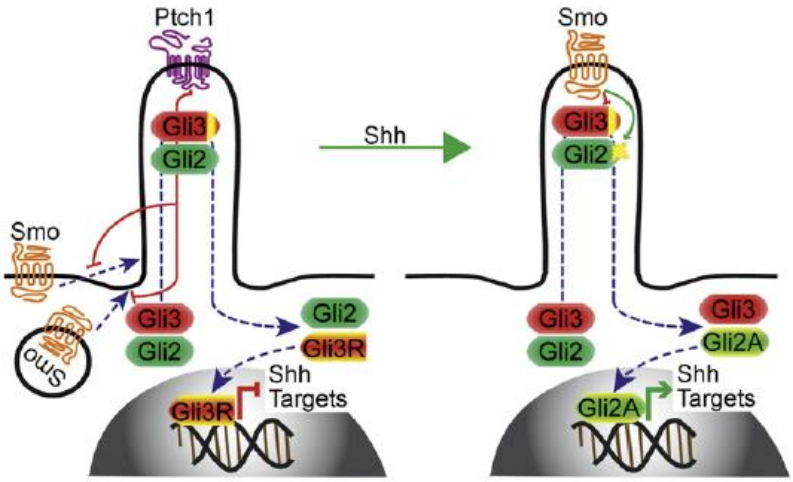

Fig 1.6: Role of primary cilia in Shh signaling, in absence and in presence of the ligand *(Han and Alvarez-Buylla 2010).*

Shh expression in the notochord and limb buds of IFT mutants is normal, but the expression of downstream targets such as PTCH1 and Gli1 in responding tissues is reduced, consistent with a loss of Shh signal transduction. Interestingly, IFT mutants display a loss-of-function Shh phenotype in the neural tube, where Gli activators (Gli1, Gli2) have a major role in pattern formation, and a gain-of-function Shh phenotype in the limb, where the Gli3 repressor (Gli3R) plays the main role (Bisgrove and Yost 2006).

Moreover, additional indications of the primary cilia's role in telencephalic patterning and morphogenesis can be found in mutants for the ciliopathy gene Ftm (Besse et al. 2011). Telencephalic neuroepithelial cells of $Ftm^{-/-}$ mice lack primary cilia and their subpallium is expanded at the expense of the pallium, in parallel with a decreased production of the short form of the Gli3 protein. Indeed, primary cilia produce short repressor form of Gli3, essential for neurodevelopmental patterning. These mutants showed defects in dorsoventral identity acquisition with dorsal Ngn2 and Pax6 expression markedly reduced in the anterior telencephalon whereas the Dlx2 and Gsx2 expression domains were expanded anteriorly and dorsally (Besse et al. 2011).

1.2 Brain Organoids

1.2.1 Why Organoids?

Since organs and tissues are three-dimensional (3D), important aspects influencing organogenesis are missing in conventional two-dimensional (2D) cultures. This problem has recently led to the development of protocols for *in-vitro* 3D cultures, called *organoids*, capable of developing organo-typically and exerting organ-specific functions. Concerning the HD modelling, we thought that the 3D strategy could highlight phenotypic differences between control and HD cells not only in terms of specific gene expressions but also for morphological and spatial organization, providing new methods to study brain development and to model Huntington's disease in-vitro.

1.2.2 Organoids definition and characteristics

Organoids exhibit some level of self-organization, due to the ability of stem and progenitor cells to autonomously generate the architectural complexity. The basic definition implies several important features that are characteristics of organs. First, it must contain some of the cell types of the modelled organ; second, it should present some function specific to that organ; and third, the cells should be spatially organized similarly to the organ itself. This similarity is also in terms of organization during development, through two major processes that regulate morphogenesis: "cell sorting out" and "spatially restricted lineage commitment" (Lancaster and Knoblich 2014). The first process is the capacity of cells to segregate and reorganize based on similar adhesive molecules and surface protein while the second is the correct progenitors' differentiation in a temporally and spatially restricted manner. Following this second principle, progenitors develop into more mature populations which because of spatial constraints and division orientation, are forced into a more distal localization that promotes their differentiation. These cells can sometimes further divide to give rise to more differentiated progeny, which are further displaced.

3D differentiation protocols require specialized tissue-culture conditions and in order to maintain their 3D growth: low-attachment plates and methods to increase oxygen exchange. This can be achieved by culturing in a high oxygen environment (40%), which requires a special incubator and access to O2 lines (Eiraku et al. 2008; Kadoshima et al. 2013; Sakaguchi et al. 2015) or by agitation, either in a spinning bioreactor or orbital shaker (Lancaster et al. 2013; Lancaster and Knoblich 2014; Qian et al. 2016). Finally, some 3D methods also need manual mechanical steps such as the isolation

and aggregation of rosettes (Mariani et al. 2015) or the embedding of cerebral organoids in droplets of coating gel, usually Matrigel, to mimic extracellular-matrix components and give growth support (Lancaster et al. 2013; Iefremova et al. 2017). Organoids usually present large brain lobes containing fluid-filled cavities that resembled ventricular zones (VZ) of developing fetal brain, rather than the small neural tube-like lumens seen in rosettes of 2D cultures. These proliferative VZ like regions contain neural stem cells that, over time, produce a multilayered radial distribution of maturing cells around a central empty lumen (Di Lullo and Kriegstein 2017). In the case of cortical organoids, these structures included a marginal-like zone containing Reelin-positive Cajal–Retzius cells, a subplate-like region, and a cortical plate-like zone with cells positive for markers of deep and superficial layer neurons. These regions could reveal many aspects about cell polarity and migration and therefore we decided to focus on their morphology in our control and HD ventral forebrain organoids (see the Results' section of this thesis).

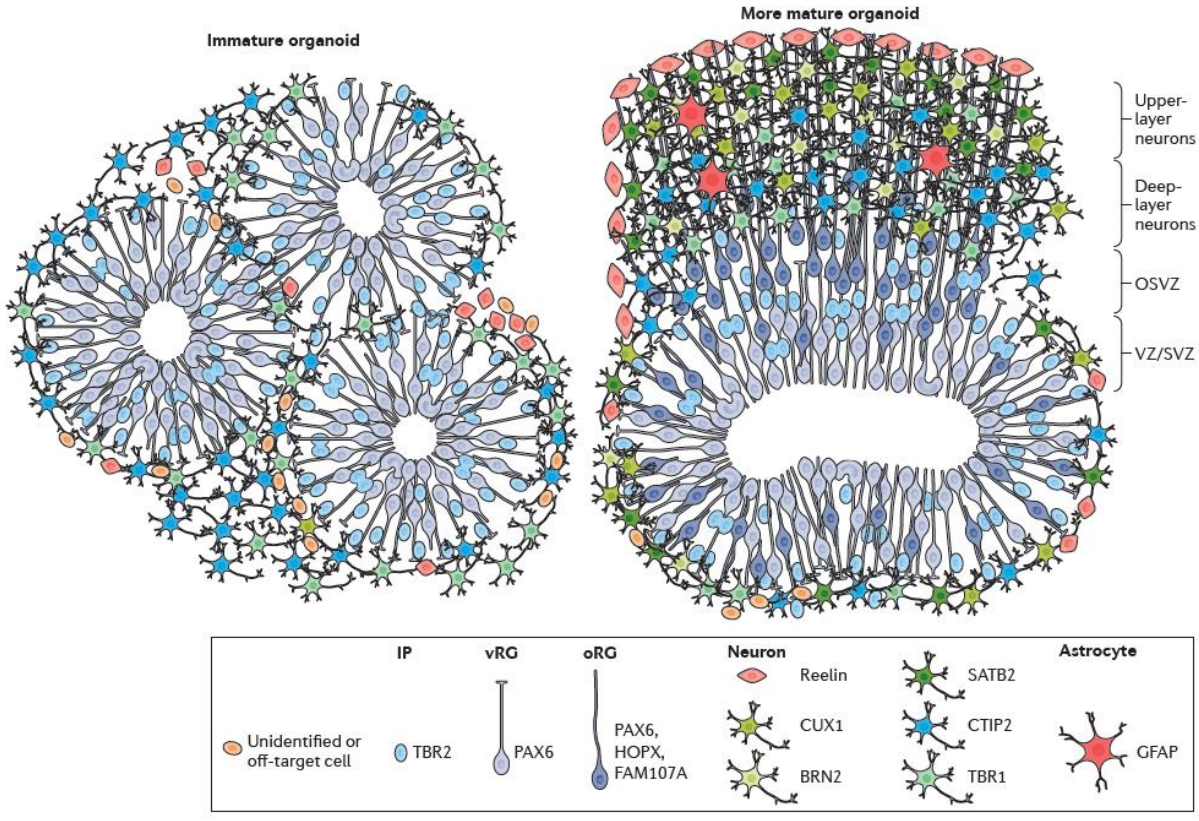

Fig 1.7: Schematic representation of cortical organoids generated with current protocols. *Immunohistochemical analyses reveal rosette-like structures in immature and mature organoids. These contain neuroepithelial stem cells and ventricular radial glial cells (vRGs) that divide at the apical surface and form a ventricular-like zone (VZ) and a subventricular-like zone (SVZ). Intermediate progenitors (IPs) and outer radial glial cells (oRGs) surround the VZ forming the outer subventricular zone (OSVZ) while neurons and astrocytes are localized more external* (Di Lullo and Kriegstein 2017).

1.2.3 History

The origin of organoid methods can be traced back to studies of developmental biology through experiments of cell dissociation and reaggregation from tissues (for example embryonic chick tissues) to investigate morphogenetic movements of cells *in-vitro* and their ability to segregate and re-organize. A major advancement in the history of brain organoids was the development of embryoid bodies (EBs) technique. EBs are multicellular 3D aggregates of PSCs that are often produced as an early step in stem cell differentiation protocols especially for neurons, to follow later modification resembling pre-gastrulation embryo. These aggregates were usually plated on coated dishes to generate clusters of neuroepithelial cells that self-organized in 2D culture to form rosettes (Shi et al. 2012; Elkabetz et al. 2008; Koch et al. 2009).

As 3D aggregation was not essential for efficient neural differentiation many neuronal protocols were then developed to skip the EBs steps and keep cells in monolayer adherent cultures for the entire protocol lifespan (Chambers et al. 2009; Espuny-Camacho et al. 2013; Tornero et al. 2013). Another important achievement was the study from Ying et al. in 2003 with the details to differentiate ESCs into neural precursors without serum or growth factors (Ying et al. 2003), highlighting the hPSCs' ability to spontaneously acquire neural identities due to autocrine signalling. The combination of the EB-derived rosette approach and the absence of serum lead to establish the so-called SFEB (serum free culture of embryoid bodies) method to obtain forebrain neural precursors from EBs with specific inductive signals and later plated in 2D on coated plates (Watanabe et al. 2005). This method was subsequently improved from the same laboratory with the protocol termed SFEBq (Eiraku et al, 2008), allowing the formation of large rosettes. This protocol take advantage from self-organization with patterning factors to induce the dorsal forebrain identity, such as polarized dorsal neuroepithelium, intermediate progenitors of the cerebral cortex and distinct types of cortical excitatory neuron.

Thus, the SFEBq method developed by the group of Yoshiki Sasai can be considered as something in between of 2D and 3D cultures that led to further publications of many protocols for different brain regions. More recently was published a modification of SFEBq method based on reaggregation after plating, showing large rosettes and mimicking the forebrain development (Mariani et al, 2015). The first entirely 3D neural culture was published in 2011 with the generation of optic cups from human PSCs (Eiraku et al. 2011), providing the proof of principle of maintaining neural tissue in 3D floating culture, which will self-organize to replicate physiological tissue architecture. A following method employing simple culture conditions and scaffold-free growth was developed to generate cortical

spheroids containing dorsal forebrain excitatory neurons (Paşca et al. 2015). This entirely 3D culture method includes a step of neural-progenitors expansion to promote the production of nonreactive astrocytes, cells that usually develop when the peak of neurogenesis is complete, suggesting high tissue maturation.

In parallel, the studies of Clevers, Sato and others on 3D cultures to mimic the intestinal crypts (Sato et al. 2009) revealed that embedding cells in a supportive extracellular matrix gel rich in laminin, like Matrigel, improve the self-organization of these cells into spatially defined epithelia with typical apicobasal polarity (Lancaster et al., 2013). The key discovery of Matrigel as a supportive matrix and the addition of later agitation in the floating cultures promoted formation of much larger tissues (up to 4 mm in diameter) than previously described and widened the field. In the last few years many protocols have been published to model different brain regions, including cortex (Lancaster et al. 2013; Kadoshima et al. 2013; Qian et al. 2016; Quadrato et al. 2017) hippocampus (Sakaguchi et al. 2015), midbrain (Jo et al. 2016; Monzel et al. 2017) cerebellum (Qian et al. 2016; Muguruma et al. 2015) retina (Quadrato et al. 2017; Eiraku et al. 2008) and adenohypophysis (Suga et al. 2011). Currently in the scientific community there are debates as to at which level of complexity an embryoid body becomes an organoid but also regarding the correct specific nomenclature (spheroids, brain organoids, cerebral organoids…) to better classify different type of 3D cultures. This lack of unique consensus about nomenclature may be due to the recent and short window of publications' delivery.

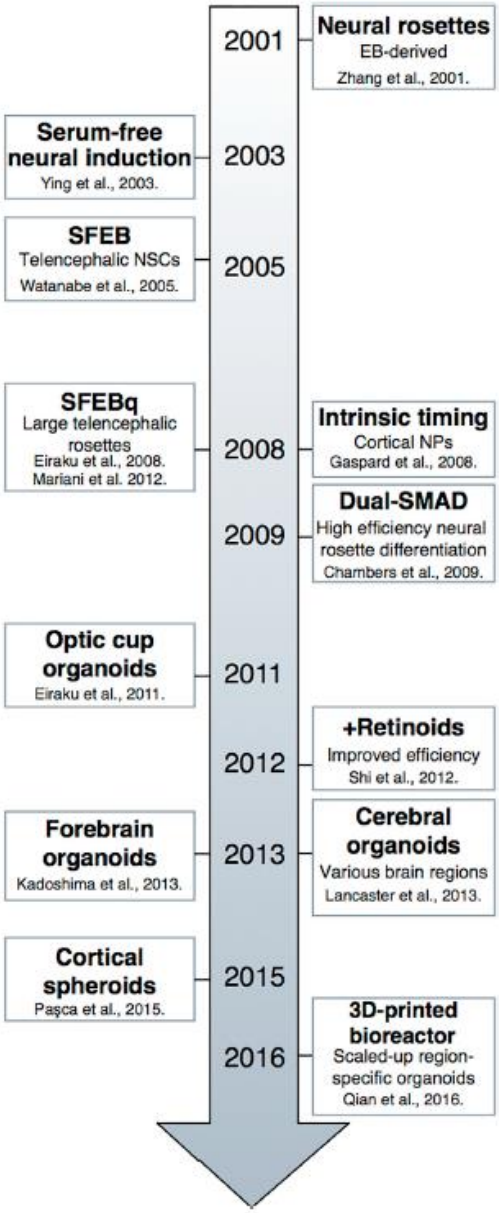

Fig 1.8: ***Timeline of recent in vitro methods of neural differentiation.*** *A simplified representation of the recently developed in vitro methods for the generation of neuronal tissue from human and mouse PSC (Kelava and Lancaster 2016, Cell Stem Cell).*

1.2.4 Homogeneity, complexity and variability

Current models of human neurodevelopment in vitro span a wide range of complexity. 2D traditional cultures of unpolarized NSCs are the least complex ones, presenting limited differentiation potential but high homogeneity and therefore suitable for high-throughput screening (Pollard et al. 2006). Although the neural rosettes are still cultured in monolayers, they are characterized by increasing complexity in fact these polarized neuroepithelial cells self-organize into radial arrangements that

mimic the neural tube (Shi et al., 2012). The protocols for SFEBq and spheroids could be considered as a modification of 2D rosettes, the tissue is plated on coated dishes but doesn't flat and maintain an intermediate level of complexity with extended apical surfaces.

Organoids are at the end of the spectrum as they show high complexity with expanded tissue organization not visible in 2D culture but also increased heterogeneity. Thus, 3D organoid approaches are currently preferable to address questions regarding cellular architecture and interactions. The balance between complexity and homogeneity is an important feature to consider when deciding which method to use.

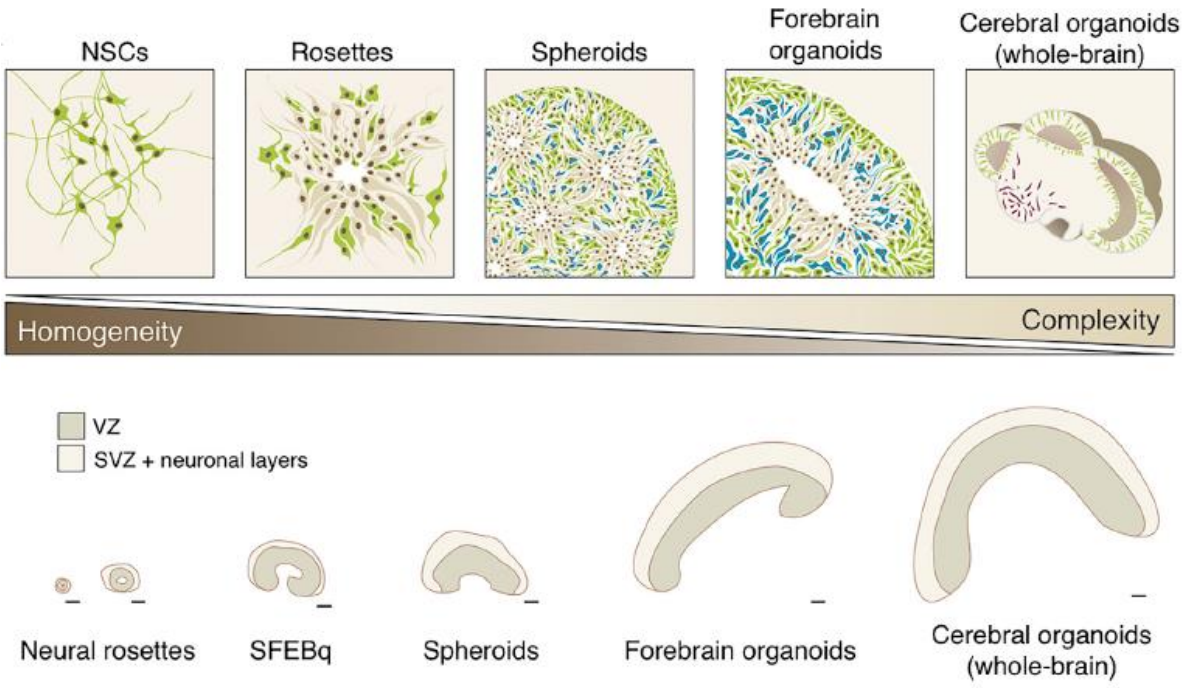

***Fig 1.9**: **The Trade-Off between Homogeneity and Complexity**. A scale showing the relationship of the complexity of the cells/tissue produced by individual protocols, the homogeneity of the cells/tissues generated and their relative size (Kelava and Lancaster 2016, Cell Stem Cell).*

This complexity of organoids, due to their spontaneous self-organization nature could lead to variability between different areas inside each sample or between different batches of samples (Lancaster et al., 2013; Quadrato et al., 2017). Concerning the common issue of variability, some organoids protocols started to use extrinsic patterning molecules to promote defined identity. It is still debated at which extent free self-organization should be favoured over guiding patterning molecules, because both protocols present advantages and disadvantages.

In the first type of protocol the spontaneous neural induction is achieved without patterning signalling molecules, promoting the generation of diverse brain regions (Lancaster et al. 2013; Li et al. 2017; Lindborg et al. 2016; Watanabe et al. 2017). Although such regional diversity is appealing it also could give a variable outcome with differentiation of some cells into non-ectodermal cell types (Camp et al. 2015; Quadrato et al. 2017). The second type of protocol optimize neural induction by mimicking endogenous patterning through the application of exogenous molecules. Commonly, neural induction includes inhibition of SMAD signalling to block mesoderm and endoderm formation (Chambers et al. 2009; Hemmati-Brivanlou and Melton 1994), followed by morphogens like sonic hedgehog or retinoic acid to specify dorso-ventral and rostro-caudal identity. The organoids generated with these protocols are generally more homogenous in cellular composition and regional identity. On the other hand, free un-patterned organoids obtained with the first type of protocol should in principle be able to mimic a largest diversity of cell types. Furthermore, differentiation of multiple cellular types of the brain (neuronal and glial) in a single organoid could favour maturation of the cells produced, implementing the formation of active neural networks.

Considering the variability of organoids, the classification of cells types is a fundamental requirement but methods generally used in neurobiology (for example, immunohistochemistry) might not fit well, mainly because the combination of few markers per cells cannot conclusively identify the specific cellular subpopulations. The recently developed high-throughput RNA sequencing analysis of thousands of single cells (scRNAseq), became extremely useful to establish fine molecular mapping and classification of the organoids' cellular composition. For example, a recent paper used droplet-based single-cell mRNA sequencing to analyse gene expression in 80,000 cells from 31 brain organoids after 3 and 6 months *in vitro* (Quadrato et al. 2017). Taking advantages from principal component analysis, 10 sub-populations of cells (better reported as "clusters") were identified in 6-month old organoids. These older organoids presented a greater diversity and a further maturation than the younger ones. Interestingly the same group has more recently published a new detailed work focusing on organoid-to-organoid variability through scRNAseq analyses of 21 single organoids and

proving their reproducibility also in the case of using different stem cell lines (Velasco et al. 2019). These studies highlighted the utility of scRNAseq approach in analysing complex samples like organoids to evaluate their level of heterogeneity.

This aspect is particularly critical because the heterogeneity can lead to issues with reproducibility, making it challenging to perform high-throughput screening with a robust and reliable readout and requiring careful morphological analyses. Technical improvement of the methods are now aiming at producing homogeneous organoids while maintaining a level of self-organizing capacity (Lancaster et al. 2017). Morphogenetic signals for example, with future engineered technics could be delivered by signal releasing beads or by growing organoids on carriers coated with signalling molecules to generate gradients and get a higher spatially or temporally controlled delivery. An example that exert more control over regional topography and tissue architecture is the recent publication from Studer's lab (Cederquist et al. 2019) in which they introduced by co-culture a sort of "signalling centre" into forebrain organoids to specify the positional identity with dorso-ventral and antero-posterior axes.

1.2.5 Modelling neurodevelopment and diseases

With the aim of understanding the level of similarity between *in vitro* methods and *in vivo* brain development several studies have employed gene expression analyses on cortical cultures by microarrays (Mariani et al.,2012; Paşca et al.,2015), RNA-seq (Mariani et al. 2015; Qian et al. 2016; van de Leemput et al. 2014) or single-cell RNA-seq (Camp et al. 2015; Velasco et al. 2019), and compared it to gene expression of the developing brain (Camp et al. 2015; Kang et al. 2011; Miller et al. 2014). All of these studies suggested that *in vitro* methods replicate remarkably well early *in vivo* brain development (middle and end of the 8–10 gestation weeks (GW)) with some of them reporting that the *in vitro* parallels the *in vivo* up to late mid-fetal period (19–24 GW) (Paşca et al. 2015; Qian et al. 2016). This means that such *in vitro* approaches are highly suitable for studies of human-specific brain normal and pathological development.

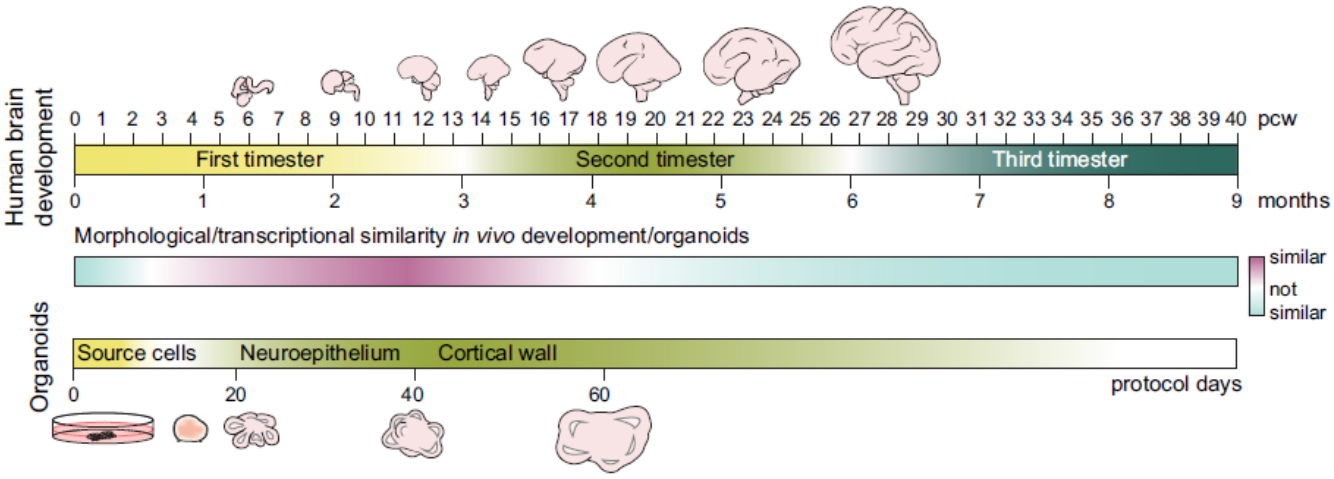

***Fig 1.10**: **Timeline of human brain development**. A timeline showing relative similarities between human in vivo brain development and the brain organoid protocol timeline. The relative similarity (cyan-purple gradient) is based on cell-biological and transcriptomics data from several studies (Kelava and Madeline A. Lancaster 2016, Dev. Biol.).*

Consequently many studies have concentrated on diseases such as Microcephaly, a condition in which the head circumference is smaller than normal because the brain has not developed properly or has stopped growing (Lancaster et al., 2013, Qian et al. 2016), Lissencephaly like Miller-Dieker syndrome characterized by nearly absent cortical folding often associated with reduced brain size (Bershteyn et al. 2017; Iefremova et al. 2017) and ZIKA virus infections, whose exposure during early pregnancy can cause severe fetal brain defects (Cugola et al. 2016; Dang et al. 2016; Garcez et al. 2016; Watanabe et al. 2017).

Organoids are incredibly used, also to overcome the lack of translation from animal studies, due to species-specific features such as differences in developmental programs, cytoarchitecture, cell composition, and genetic background. Many uniquely human cognitive and behavioral diseases like autism spectrum disorder (ASD) or schizophrenia, present polygenic etiology, making them arduous to study with existing animal models. Organoids derived from patients with genetic diseases give the possibility of focus on disease mechanisms and discover human-specific phenotypes. Moreover, organoids are also appropriate for studies that require live, functioning tissue, such as the analysis of electrophysiological features or dynamic cell behaviours. Whole-organoid transcriptome analyses can be used to identify molecular and cellular alterations that may underlie neuropsychiatric disorders.
In a publication from Vaccarino's group, forebrain organoids were generated from severe ASD patients showing dysregulated expression of *Foxg1* gene, accelerated cell cycle and increased

production of GABAergic neurons with consequent imbalance of excitatory/inhibitory networks (Mariani et al., 2015). Brain organoids have also been used from Pasca's group to model Timothy syndrome, a severe disease characterized by ASD and epilepsy (Birey et al. 2017). In this study, dorsal and ventral telencephalic organoids were fused to mimic interneuron migration, showing a cell-autonomous migration's defect in pathological samples. The assembly of organoids of different brain regions in culture was published in the same year from other two independent groups to analyse neuronal migration or networks and represents a huge advancement to overcome the limitation in studying brain cellular interactions with previous singular regional identity organoids (Bagley et al. 2017; Xiang et al. 2017).

Other studies have suggested that brain organoids may be relevant approaches even for late-onset diseases such as Alzheimer disease (AD), thanks to the presence of AD-like phenotype such as amyloid aggregation, hyperphosphorylated tau protein, and endosome abnormalities (Choi et al. 2014; Raja et al. 2016) pushing further these 3D models.

1.2.6 Limitations and challenges

Organoids replicate morphological organisation of fetal ventricular zones and some aspects of cortical layering, but additional levels of radial glial scaffold, gyrification and proper cortical layering still needs to be established. The heterogeneity in terms of diverse cell types in organoids could be an advantage for modelling complex cellular interactions but requires validation for the correlated variability. An improvement in terms of reproducibility that still maintain organoids self-organization was published by Lancaster and Knoblich groups (Lancaster et al. 2017) with the engineered use of bio-compatible material microfilaments as internal scaffold and the addition of dissolved Matrigel. The slow maturation of organoids may limit studies of later developmental events or latest stages of disease progression. Finally, the absence of body axes, the under-representation of peculiar cell types involved in brain development (such as endothelial cells, microglia and astrocytes) and the lack of proper neuronal connections are currently under consideration, for example with co-culture protocols (Giandomenico et al. 2019).

Vascularization is considered one of the major limitation of organoids approach. Early development of the neocortex progresses without it before blood vessels invade the cortical wall (Vasudevan et al. 2008) but later stages of development are strongly dependent on it. In the SVZ blood vessels work as a niche for neural progenitors (Javaherian and Kriegstein 2009) and are necessary for efficient neural

progenitor differentiation (Lange et al. 2016). Another linked issue is the difficulty of oxygen penetration that renders the centre of the organoid necrotic, interfering with its normal development and physiology. It will be necessary to focus the efforts on delivering signalling molecules deep inside the tissue, by cell culture modifications or engineering strategies. An example could be the use of microfluidic chambers (Bhatia and Ingber 2014; van Duinen et al. 2015) to have a fluid flow through the organoid with the combination of signaling molecule carriers to establish the right patterning.

Recently an important accomplishment in this direction was obtained by Gage's group, showing that intracerebral transplantation of brain organoids in mice results in growth of blood vessels with better cell viability and maturation, compared to organoids maintained *in-vitro* (Mansour et al. 2018). Interestingly the host vascular system invades the grafted organoids with blood flow and migration of some host microglia. This approach needs future improvements to limit possible damages in the host's brain but has the advantage of being comparable to the developmental angiogenesis and allows investigation of the host-organoid interaction.

The failure of many neurotherapeutic approaches to translate from animal models to clinical practice underscores the need for better predictive models, and despite the mentioned limitations, brain organoids may help bridge this divide.

1.3 HD in vitro models

1.3.1 Fundamental aspects

In comparison to most other neurodegenerative disorders, such as Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), or Alzheimer's disease (AD), HD is a monogenic disorder. This is an advantage for generation of hPSC models. Moreover, animal models have been useful to elucidate many aspects of the disease but because of species-specific differences studying human neurologic disease remains challenging. The human forebrain is significantly increased in cell number and complexity (Clowry, Molnár, and Rakic 2010), and the normal HTT CAG repeat tract is expanded from 7 in rodent to ~20 in humans. Furthermore, the human HTT locus encodes for multiple mRNA isoforms, thus generating multiple HTT proteins, some of which are present only in higher apes and humans (Ruzo et al. 2015). Therefore, a comprehensive set of *in-vitro* and *in-vivo* models ranging from mouse to human may be necessary in order to study HD.

Traditionally, HD cellular models consisted of rodent neurons expressing expanded polyQ repeats in HTT. Since these latter can be limited in their disease manifestation or proper genetic context, human HD PSCs are currently being adopted both for modelling and drug screening. It is fundamental to define which lines of HD cells should be employed, particularly in terms of polyQ length. As HD onset can occur with a broad spectrum of polyQ tracts (36–180+), researchers would ideally use multiple lines carrying a wide range of repeats to faithfully resemble disease pathogenesis and even understand whether any CAG-length dependent phenotypes are present. Therefore, as obtaining hES lines from embryos is limited, patient skin biopsies with different polyQ lengths are typically collected to derive fibroblasts and reprogram them into hiPSCs.

The next critical question relates to the choice of proper control lines (Mattis and Svendsen 2017). Controls can be gene-edited cells by the correction of the gene and the generation of isogenic cell lines (An et al. 2012) such as small interfering RNAs, short hairpin RNAs, or antisense oligonucleotides (Mattis et al. 2015) to knockdown the expression of mHTT. Recent studies in fact moved toward the "gold standard" isogenic lines as controls (An et al. 2014; Ruzo et al. 2018; Xu et al. 2017), because having the identical genetic and epigenetic backgrounds they may better replicate the differences in polyQ length and severity (J.-M. Lee et al. 2012), as well as individual genotypic variability (Boulting et al. 2011; Hu et al. 2010). It has been show that CRISPR/Cas9 can be targeted to single nucleotide differences between expanded and nonexpanded alleles (Hsu, Lander, and Zhang 2014), in order to specifically delete the mutant allele. Ideally, the best cellular system to replicate

CAG repeat phenotypes would result from a wide cohort that combines isogenic lines together with lines from individuals with different genetic backgrounds (iPSCs).

The majority of publications focused on HD phenotypes in hiPSC (Park et al. 2008), neural stem cell (NSC) (Bradley et al. 2011; HD iPSC Consortium 2012; Naphade et al. 2018; Niclis et al. 2009; Zhang et al. 2010), glia (Juopperi et al. 2012), or different neuronal cultures (Conforti et al. 2018; McQuade et al. 2014; Nguyen et al. 2013; Niclis et al. 2013).

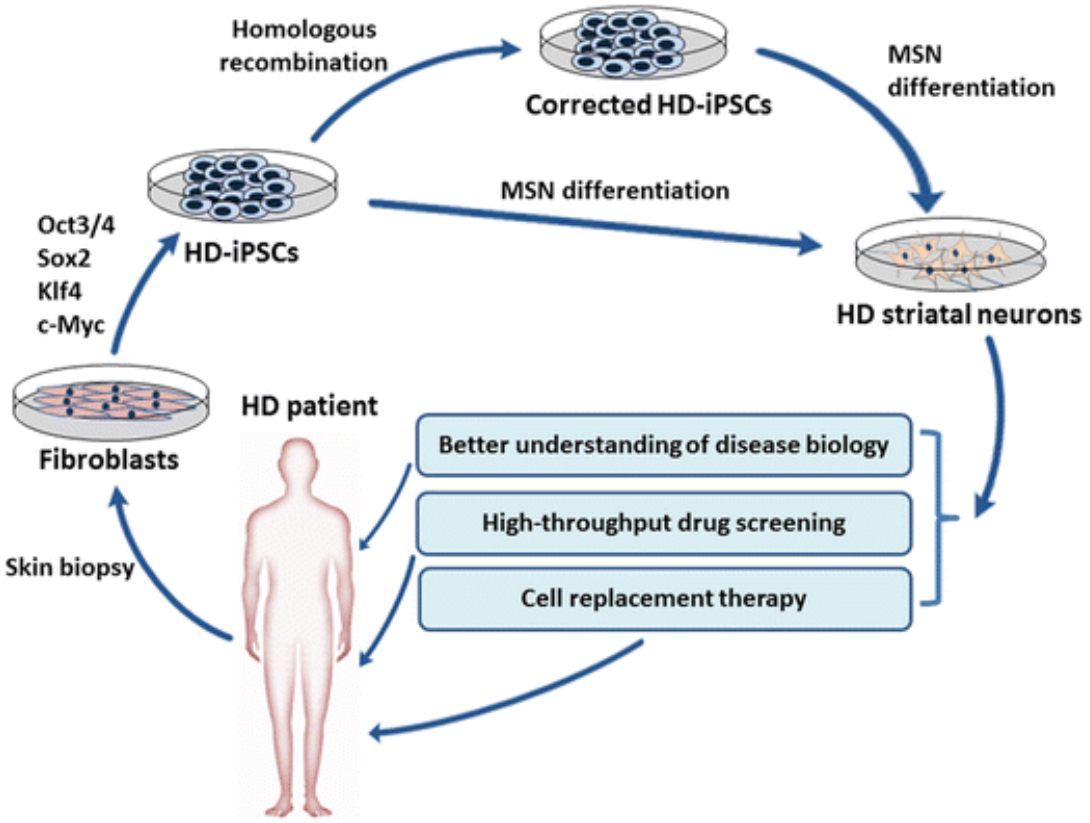

Fig 1.11: The generation and application of iPSCs in HD research. *HD patient-specific iPSCs can be obtained by reprogramming of skin fibroblasts. Established iPSCs can be used as a tool for better understanding the molecular basis of HD. Moreover, HD-iPSCs can be differentiated into specific cell types predominantly affected in the disease (striatal MSNs). Emerging gene therapies make the genetic correction of HD-iPSCs become feasible, useful for autologous transplantation strategies* (Liu et al. 2016).

1.3.2 Neurodevelopmental models and neurodegenerative disease

One of the major open question in the field is how to study an adult-onset disease with a developmental model, such as *in-vitro* differentiation of hPS cells. Several strategies have been developed in order to artificially "age" PSC-derived HD models, for example employing various cellular stressors (An et al. 2012; HD iPSC Consortium 2012). PD researchers managed to rapidly age hPSCs by overexpressing the gene mutated in the aging disease Progeria (Miller et al. 2013). This method, although quite advantageous, was artificially design and opened the question to what extent the effect observed mimics physiological or pathological aging.

It is known that normal HTT has crucial roles in neural development, in fact the lack of huntingtin in mice results in embryonic lethality soon after gastrulation, while its early embryonic knock out from pyramidal neurons yield reduced cortical and striatal size due to the lower amount of neurons. Additional suggestions can be found in the depletion of HTT in postmitotic projection neurons during embryonal development, that leads to the mis-localization of layer-specific neuronal populations in the murine neocortex (Dragatsis et al. 2018; Zeitlin et al. 1995).

On the other side the effect of mHTT during this period and the possible neurodevelopmental defects underlying HD are still unclear. In fact, despite HD is characterized by neurodegeneration in adult life, an increasing number of studies are focusing on changes occurring during fetal development, that may lead specific neuronal populations to be more susceptible to the mutations with consequent later neurodegeneration (Nguyen et al. 2013). These results are suggesting a possible neurodevelopmental component in the disease (Kerschbamer and Biagioli 2016; Mehler and Gokhan 2000). A piece of evidence supporting this hypothesis comes from pre-manifest HD (pre-HD) individuals who present brain abnormalities years before the clinical onset. Moreover, neurological changes occur 15–20 years before symptoms (Gómez-Tortosa et al. 2001; Philpott et al. 2016; Tabrizi et al. 2009), whereas neurocognitive, psychiatric and motor signs are evident <10 years prior (Stout et al. 2011). Furthermore, prodromal HD patients seem to have a smaller intracranial volume (Lee et al. 2012; Nopoulos et al. 2011), linking the HD mutation to characteristics of neural development. Indeed, the intracranial volume is due to the maximal brain growth obtained during development and not a potential shrinkage as a part of a disease. The interpretation, still debated in the community, is that these defects of altered development can compromise neuronal homeostasis and increase cellular vulnerability to later stressors during adult life (Ramocki and Zoghbi 2008). While large-scale effects may not appear until adulthood, subclinical transcriptional alterations could create a different starting

point in HD relative to non-HD brain and could contribute to incorrect structural connectivity (Novak et al. 2015).

Experiments of conditional mouse models helped in revealing this neurodevelopmental component, such as the conditional deletion of huntingtin gene in the whole brain from E14.5, presenting progressive degenerative neuronal phenotype and resembling several HD phenotypes (Dragatsis, Levine, and Zeitlin 2000). Another strong evidence comes from a recent paper (Molero et al. 2016) that revealed how conditional mice expressing mHTT only until postnatal day 21 presented a phenotype similar to mice expressing mHTT throughout their entire life. In line with these results, there are also evidences on developmental abnormalities in striatal and cortical neurogenesis in HD mice. Furthermore, a previous study from the same lab (Molero et al. 2009) proved that the striatum of Hdh-Q111 (HD) embryonal mice was structurally different compared to Hdh-Q18 (WT) ones. Particularly, the HD mice exhibited delayed acquisition of early striatal cytoarchitecture with aberrant expression of progressive markers of MSN neurogenesis (Islet1, DARPP-32, mGluR1, and NeuN).

Recent identification of genetic factors that are able to modify the age of HD onset proposes that the pathologic process is modifiable prior to clinical diagnosis (Genetic Modifiers of Huntington's Disease (GeM-HD) Consortium et al. 2015) suggesting that intervening before disease onset might be of some therapeutic value.

1.3.3 Characterization of HD in-vitro models

One of the first results with hiPS cells to model HD was published from Ellerby's group (Zhang et al. 2010). They generated $NESTIN^{+}/PAX6^{+}/SOX1^{+}/OCT4^{-}$ neural stem cells (NSCs) from HD-iPS cells taking advantages from the embryoid body method. HD-NSCs were then subjected to a differentiation protocol with morphogens and neurotrophins to induce striatal lineage identity. HD-NSCs showed enhanced caspase activity upon growth factor deprivation compared to normal NSCs. Additional results revealed that neurons generated from patient-derived hiPSC exhibited similar phenotypes to those observed in human HD patient tissue and HD animal models, such as genetic anticipation (Swami et al. 2009), dysregulated calcium and glutamate signalling (Bezprozvanny and Hayden 2004; Greenamyre et al. 1985; Laforet et al. 2001) and aberrant BDNF trafficking (Drouet et al. 2009; Gauthier et al. 2004). Transcriptomic studies highlighted categories like signalling, cell cycle, axonal guidance, and neural development as significantly altered in HD hiPSCs compared to control counterparts (An et al. 2012; HD iPSC Consortium 2012; Ring et al. 2015).

In a recent paper (Hung et al. 2018), the researchers used human telomerase reverse transcriptase (hTERT) to immortalize fibroblasts obtained from individuals with different age, sex, disease onset, and CAG repeat length. The pathological cells presented some HD phenotypes, such as altered morphology, growth rate, increased sensitivity to oxidative stress, aberrant ADP/ATP ratios, and hypo-phosphorylated huntingtin protein. They also noticed dysregulated reactive oxygen species (ROS)-dependent huntingtin localization to nuclear speckles in HD cells.

As discussed above (see chapter 1.3.2), normal HTT has essential roles in the developing brain. Few recent reports have begun to indicate that some aspects of HD pathogenesis might start during embryogenesis (Godin and Humbert 2011; HD iPSC Consortium 2017; Lopes et al. 2016; Molero et al. 2009). HD iPSCs-derived neurons display defects in their maturation, electrophysiology, metabolism, cell adhesion and, ultimately, cell death, consistent with an impairment during human neurogenesis. RNAseq analyses from the work of HD Consortium (HD iPSC Consortium, 2017) identified downregulated expression of genes involved in glutamate and GABA signaling, axonal guidance and calcium influx in HD cultures. Particularly affected were the pathways regulating neuronal development and maturation, suggesting that specific gene networks could represent potential therapeutic targets. The authors then tested a small molecule, isoxazole-9 (Isx-9), because of his connection with many of the dysregulated gene networks. Isx-9 rescued some CAG repeat-associated phenotypes in HD iPSC-derived neural cultures, as well as cognition and synaptic pathology in a mouse model for HD (R6/2), proving that these deficits are almost reversible with final improvement of synaptic homeostasis (HD iPSC Consortium, 2017).

The generation of the first isogenic hESC line with CRISPR/Cas9 to model HD has been recently published (Ruzo et al., 2018). They revealed that these HD-hESCs recapitulate some previously reported neuronal phenotypes, thus validating the model, but also a new unexpected phenotype. Chromosomal instability during *in-vitro* neurogenesis resulted in the generation of multinucleated neural progenitors and neurons, at a frequency proportionally correlated to polyQ length. Together, these data support the idea that HD is not merely an adult-onset disease but also has a neurodevelopmental component. In the same work, they observed phenotypes on rosette size and organization that is in line with a role of HTT in polarity, as reported from reduced cell adhesion in HD-iPSC neural progenitors, neural tube defects in zebrafish embryos, randomization of epithelial polarity in the skin of frog embryos, and reduced rosette formation in mESCs (Conforti et al. 2018; Haremaki et al. 2015; Lo Sardo et al. 2012).

CRISPR/Cas9 approach has also been used in a previous study to correct HD hiPSCs from Pouladi's group (Xu et al. 2017), proving that the defects observed in pathological neural cells, including impaired neural rosette formation, increased susceptibility to growth factor withdrawal, and mitochondrial respiration deficits, are rescued in isogenic controls. Importantly, by genome-wide expression analysis, they showed how some apparent gene expression alterations between HD and non-related control lines were undetectable between HD and corrected isogenic counterparts. This analysis suggests that the first variances were likely due to the different genetic background rather than HD-specific effects, highlighting the importance of isogenic controls for disease modelling with hPSCs. The same group has more recently published (Ooi et al. 2019) the use of genome editing to obtain an allelic series of isogenic HD (IsoHD) hESC lines carrying different CAG repeat lengths in the first exon of HTT. To investigate tissue-specific effects, the IsoHD panel was differentiated into neural progenitors, neurons, hepatocytes, and muscle cells. Functional analyses in differentiated neural cells revealed HD-related abnormalities in mitochondrial respiration and oxidative stress, as well as elevated markers of DNA damage and enhanced susceptibility to drug-induced DNA damage with reduced cellular proliferation. Through whole-transcriptome and whole-proteome analysis they discovered CAG repeat length-dependent phenotypes with cell-type specificity.

The lab, in which I am doing my PhD, has recently described that HD iPSC lines are defective in their ability to acquire both ventral ad dorsal telencephalic identity, in 2D cultures. In particular, the differentiation efficiency of HD iPSCs was reduced compared to CTRL lines, presenting defects in specification and maturation (Conforti et al. 2018). In the same work, cortical organoids were differentiated revealing disrupted cytoarchitecture in HD lines. The gene expression analysis revealed how control organoids overlap with mature human fetal cortical areas, while HD counterparts correlate with the immature zones (VZ/SVZ). The authors also showed that down-regulation of mHTT or inhibition of one of its effectors, ADAM10, successfully rescues neuronal differentiation, suggesting that early intervention may revert neurodegeneration later in life.

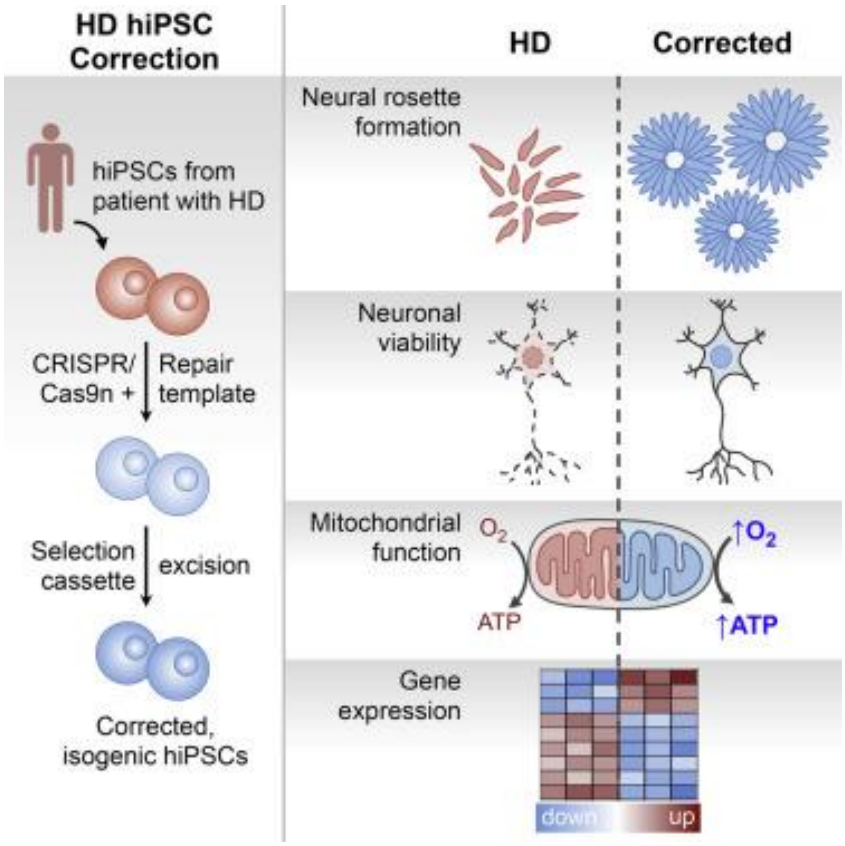

Fig 1.12: Reversal of phenotypic abnormalities by CRISPR/Cas9-mediated Gene Correction in HD hiPSCs. *Both HD and corrected isogenic hiPSCs can be differentiated into forebrain neurons, demonstrating that phenotypic abnormalities in HD hiPSC-derived neural cells, including impaired neural rosette formation, increased susceptibility to growth factor withdrawal, and deficits in mitochondrial respiration, are rescued in isogenic controls (Xu et al., 2017).*

1.3.4 Organoids' approach to study HD

The previous work performed in the lab focused on cortical organoids from iPSC (Conforti et al. 2018), while this thesis regards organoids with ventral telencephalic identity, the region that during human fetal development give rise to the striatum, using a panel of hESC isogenic lines. These cells were generated with CRISPR/Cas9 from hESC RUES2, differing only by the length of the CAG repeats to avoid noise due to different genetic background. We received them from professor Ali Brivanlou at the Rockefeller University in the context of a collaboration.

Our interest is to investigate in a simplified system like *in-vitro* culture, the possible neurodevelopmental component of HD focusing on aspects hard to appreciate in a 2D culture, such as polarity, morphogenesis and migration. We decided to take advantage of organoids because of their cellular composition and organization. The use of a patterned protocol with ventralizing signalling molecules gave us a 3D model of ventral telencephalon development with an intermediate level of complexity, appropriate for deriving information about cellular interactions.
Moreover, we want to use control and HD organoids as a platform to discover underlying pathological mechanisms, that may be already present during neural development.

2. Results and discussion

2.1 Mimicking the human telencephalon development with organoids

2.1.1 Generation of telencephalic organoids with dorsal or ventral identity

Here we first focussed on the recapitulation of human neural development verifying how organoids acquire correct dorsal or ventral identities. We first took advantage from the Lancaster protocol (Lancaster et al. 2013; Lancaster and Knoblich 2014) to produce cortical telencephalic organoids, introducing an adaptation of the original protocol to obtain quite homogeneous organoids without bioreactors. The major differences consist in putting the aggregates on rotation since day5 and maintaining them in rotation after matrigel embedding while the media are the same from the published protocols (see the materials and methods chapter of this thesis).

We kept the cortical organoids in culture until day 45 and then analysed them by immunohistochemistry. The organoids are characterized by regions of progenitors and although they differ in dimensions this internal organization is maintain between different samples (Fig 2.1).

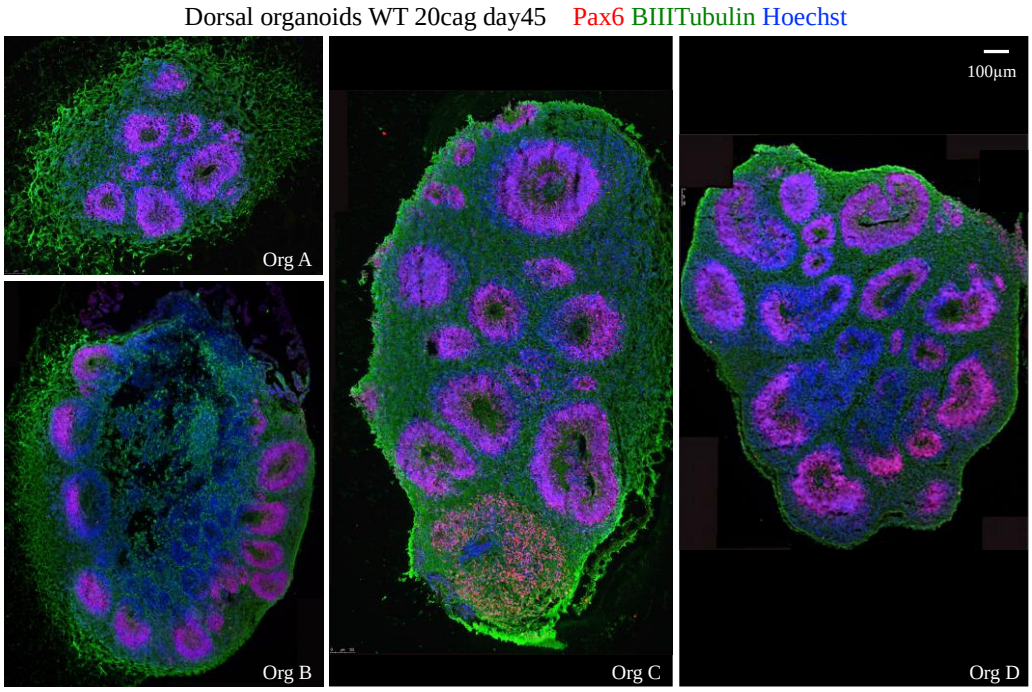

Fig 2.1: Immunohistochemistry analyses of WT dorsal organoids at day 45 of differentiation for PAX6, βIIITUBULIN and Hoechst. Different organoids are shown. Scale bar 100µm on the top right.

Investigating the dorso-ventral identity, we found cells positive for dorsal markers such as TBR2, CTIP2, TBR1 and negative for ventral markers like GSX2 (Fig 2.2). For this reason, we will refer to them as “dorsal” organoids.

In conclusion we were able to replicate the published protocol with hES Rues2 cell line, although compared to the original one it seems to be very dorsalized, maybe due to our adaptation. In the published protocol, in fact, the authors declared of having some organoids (around 34%) with few ventral regions marked by NKX2.1, while all of them (100%) presented dorsal cortical identity (Lancaster et al. 2013). This characteristic is in-line with the tendency of hPSC to acquire by “default” a dorsal lineage (Gaspard et al. 2008; Muñoz-Sanjuán and Brivanlou 2002).

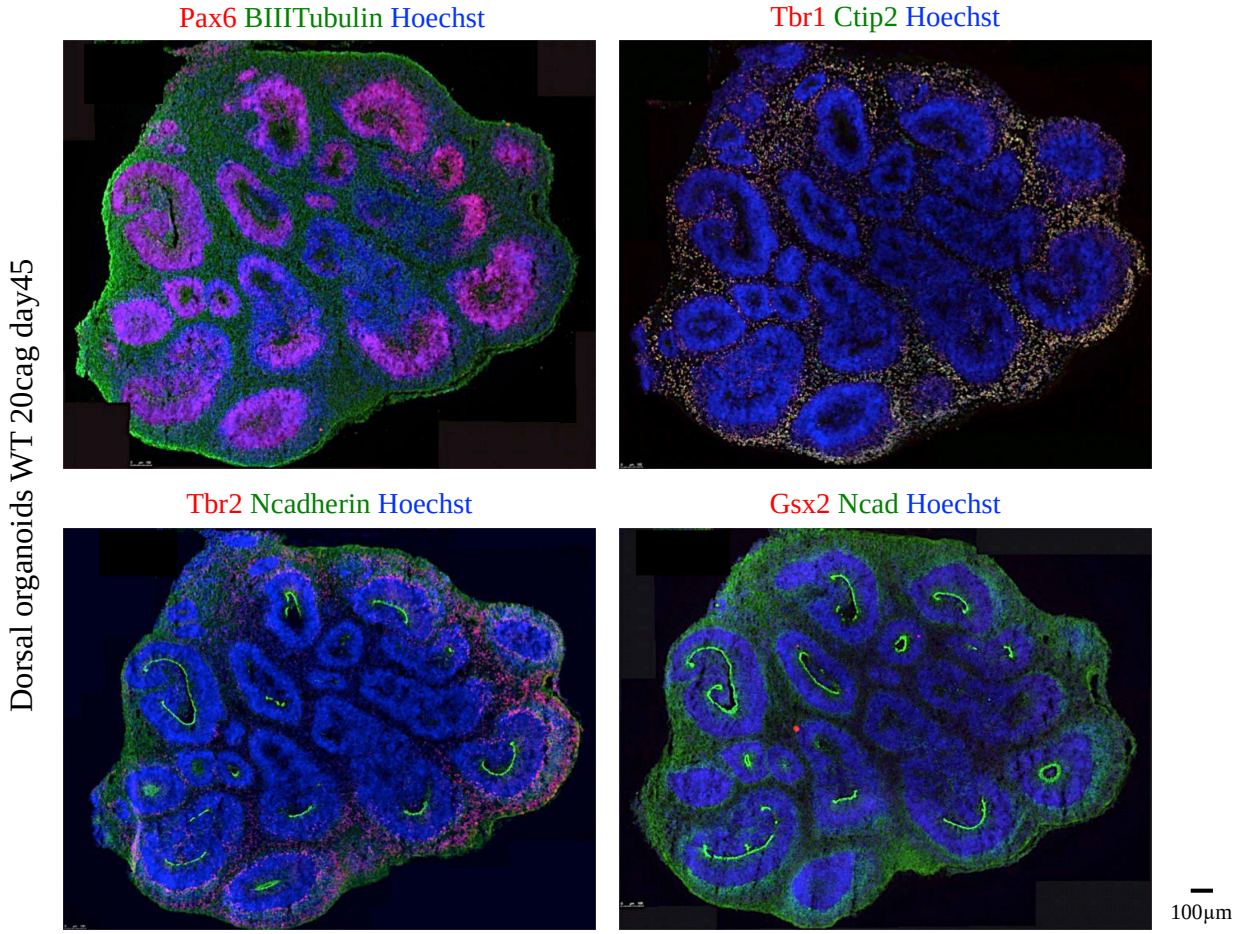

Fig 2.2: Immunohistochemistry analyses of WT dorsal organoids at day 45 of differentiation for PAX6, TBR2, TBR1, GSX2, N-CADHERIN, βIIITUBULIN and Hoechst. Different slices of the same organoid are shown. Scale bar 100µm on the bottom right.

In literature it is possible to find many protocols to derive organoids for different brain regions but methods that resemble ventral telencephalon are less developed. The major ones are three independent and quite simultaneous studies (Bagley et al. 2017; Birey et al. 2017; Xiang et al. 2017) in which ventral organoids resemble the MGE, the region that give rise to inhibitory interneurons. These organoids are then fused with dorsal ones to model interneurons' migration in the cortex during development. To achieve a strong ventral identity they used patterning factors for SHH signalling such as Purmorphamine and SHH in Xiang et al., 2017 and Smoothened agonist (SAG) in Birey et al., 2017 and Bagley et al., 2017.

To generate our ventral protocol we took inspiration from the differentiation protocol that the lab, in which I am doing my PhD, published in 2013 (Delli Carri et al. 2013) for adherent cell culture to get medium spiny neurons (MSN). The mechanical steps are similar to the dorsal organoids' protocol shown above but the media are different. In particular, neural induction in 3D is achieved with the dual SMAD inhibition strategy with SB and LDN (Chambers et al. 2009). The ventral identity is then pushed by SHH (Gaiano et al. 1999; Rallu et al. 2002) and by Dkk1 that inhibits WNT (Backman et al. 2005; Suzuki and Vanderhaeghen 2015).

In our protocol the organoids show neuroepithelial EBs characteristics around day 15-17, before the Matrigel embedding, with cells expressing SOX2, NESTIN, KI67, initial levels of NKX2.1, BIIITubulin and no pluripotency (Oct4) (Fig 2.3 upper line). At day 35 the ventral organoids present organized structures of progenitors (SOX2/NESTIN/BLBP) and neurons (βIII-TUBULIN) with telencephalic ventral identity (GSX2/MASH1/NKX2.1/ISLT1) (Fig 2.3 central line). At later time points, day 50-60, these organoids show markers specific for mature ventral telencephalon (EBF1, CTIP2, GAD67) and they maintained the presence of organized structures of progenitors (SOX2) and neurons (MAP2) with few astrocytes marked by GFAP (Fig 2.3 lower line).

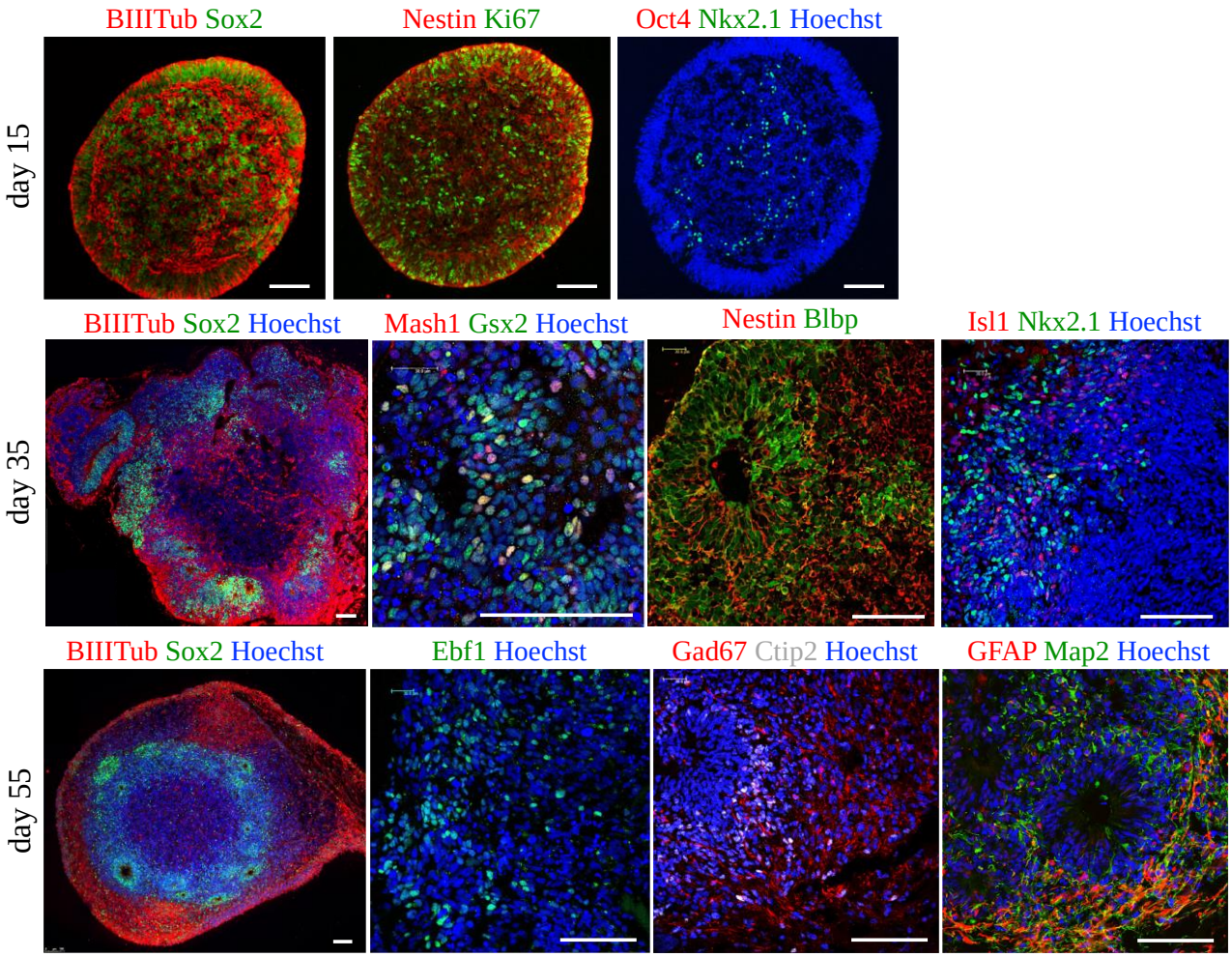

Fig 2.3: Immunohistochemistry analyses of WT ventral organoids at day 15, 35 and 55 of differentiation. Markers at day 15 in the upper line: βIII-TUBULIN, SOX2, NESTIN and KI67. Markers at day 35 in the central line: βIII-TUBULIN, SOX2, MASH1, GSX2, NESTIN, BLBP, ISLT1 and NKX2.1. Markers at day 55 in the lower line: βIII-TUBULIN, SOX2, EBF1, GAD67, CTIP2, GFAP, MAP2. Different organoids are shown. Scale bar 100µm on each image.

2.1.2 Ventral telencephalic organoids from a single cell perspective

Nowadays high-throughput RNA sequencing analysis of thousands of single cells became extremely useful to establish fine molecular mapping of the organoids' cellular composition. A recent publication from Arlotta's group focused on dissecting the organoid-to-organoid variability through scRNAseq analyses of 21 single organoids and proving their reproducibility also in the case of using different stem cell lines (Velasco et al. 2019). Other works took advantages from this recent technic for comparison with human cortical development (Camp et al. 2015; Giandomenico et al. 2019) and for evolutionary studies (Mora-Bermúdez et al. 2016; Pollen et al. 2019), highlighting the utility of scRNAseq approach with heterogeneous and complex samples like the 3D cultures.

We planned scRNAseq analyses with the final aim of understanding at which transcriptomic level WT and HD organoids are different and furthermore to visualize possible misrepresented sub-populations in HD samples. For example in HD organoids, compared to WT, we could find an unbalanced composition between progenitors and mature neurons or the presence of mis-differentiated cells towards unwanted identities. Our preliminary experiment is focused on three individual ventral organoids WT (line edited with 20CAG) and three HD (line edited with 56CAG) at day 45 of differentiation. At the moment of writing this thesis the bioinformatic analyses are still ongoing, giving the priority to WT samples to understand their variability and characterize their ventral identity from a single cell perspective.

Quality control analyses performed on the three WT organoids at day 45 reveal similar results between these samples, suggesting that there is no variability or batch effects at least in terms of quality check. There is a low expression of mitochondrial genes with only a few outlier cells, indicating that the process of dissociation in single cells suspension with following steps of emulsion (see Material and methods, paragraph 4.8.2) did not stress the cells (Fig 2.4 left graph). Another important parameter is the numbers of sequenced genes, indicator of the correct procedure, where the few outlier points could represent emulsion drops with more than one cell (Fig 2.4 right graph). The values in these analyses are comparable with others obtained from our lab with human fetal brain samples at 9 and 11 weeks of age.

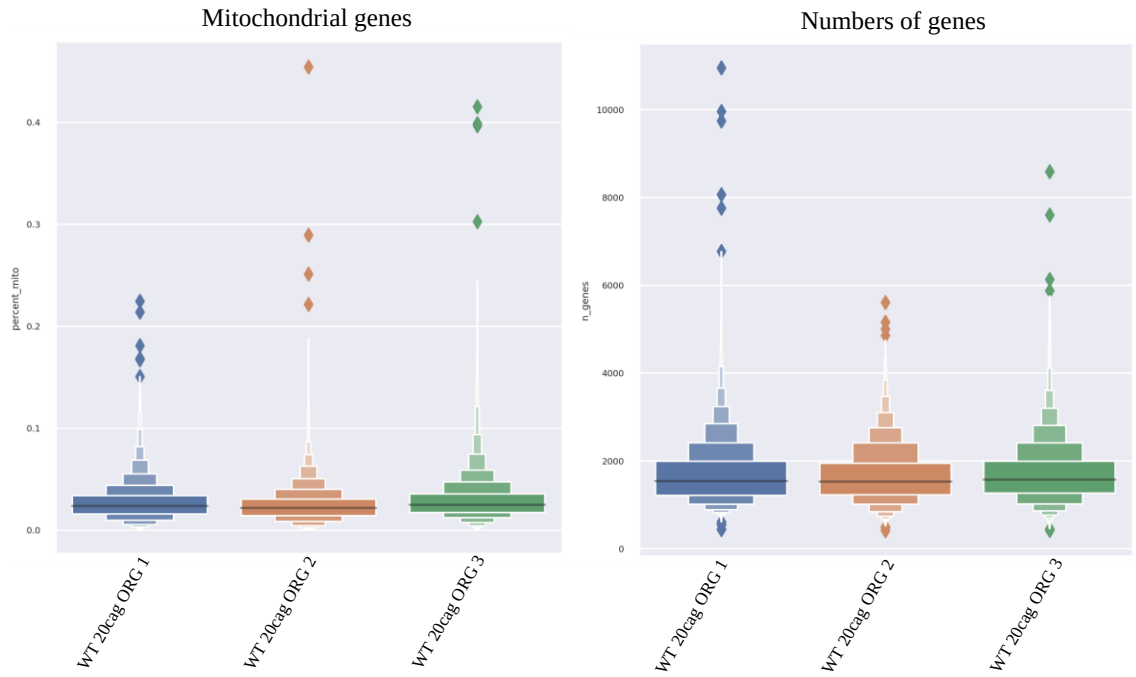

Fig 2.4: Boxplot graph of quality control analyses on mitochondrial genes expression and number of sequenced genes of three singular organoid WT 20CAG at day 45 of differentiation.

Analyzing together the WT organoids, the louvain algorithm identified ten different clusters, shown in the tSNE plot in Fig 2.5. These clusters are generated by the cells of all the samples with no batch effects, suggesting good reproducibility between the three individual WT organoids (Fig 2.5 on the right). This aspect was really important to us, because a common issue with organoids is the usually high heterogeneity that inherently leads to low reproducibility.

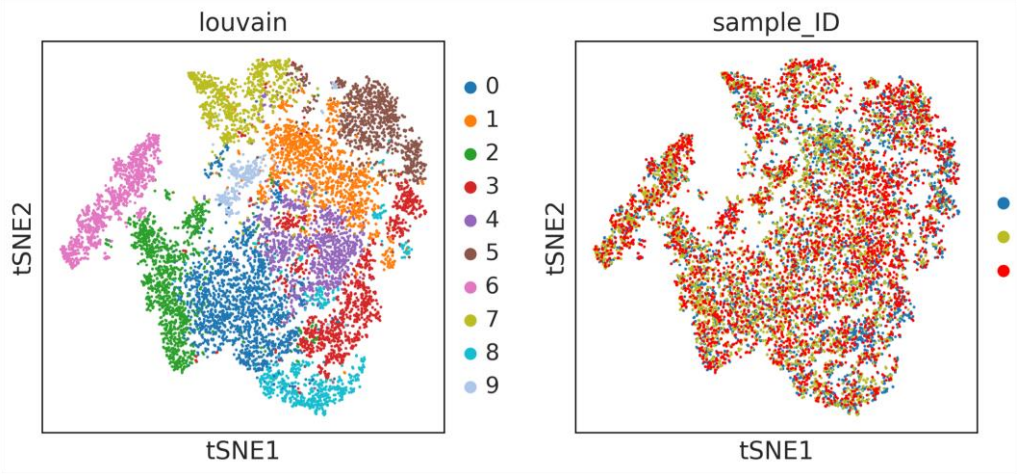

Fig 2.5: tSNE plots of single cells clustering with louvain algorithm of three singular organoid WT 20CAG at day 45 of differentiation.

We then looked for specific markers of ventral telencephalon development inside the clusters (Fig 2.6). Almost all the cells express *Foxg1* with similar expression levels between different clusters (recognizable from the scale on the right), indicating general telencephalic identity. *Sox2*, *Gsx2* and *Ascl1* are more expressed in cluster 6, 2 and 0, suggesting ventral progenitor status (Ferri et al. 2013; Pauly et al. 2014). The *Dlx* genes are shown in the order of their temporal expression during fetal subpallium development: *Dlx2* →*Dlx1* →*Dlx5* →*Dlx6* (Panganiban and Rubenstein 2002) highlighting the sub-divisions into progenitors (clusters 6, 2 and 0) and neurons (clusters 7 and 5). Doublecortin (DCX), microtubule-associated protein, is expressed by immature and mature neurons in development (Walker et al. 2007) and is fundamental for neuronal migration by regulating the stability of microtubules (Gleeson et al. 1999). DCX is expressed in all clusters except number 6 reinforcing his identification as a population of early progenitors. We then checked the expression of glutamate decarboxylase gene *Gad2*, that encodes for GAD65, which is present preferentially in presynaptic terminals for synthesis of GABA (Le et al. 2017). *Gad2* showed the same pattern of DCX, suggesting immature identity of cluster 6 and the presence of GABAergic neurons inside our organoids (Fig 2.6).

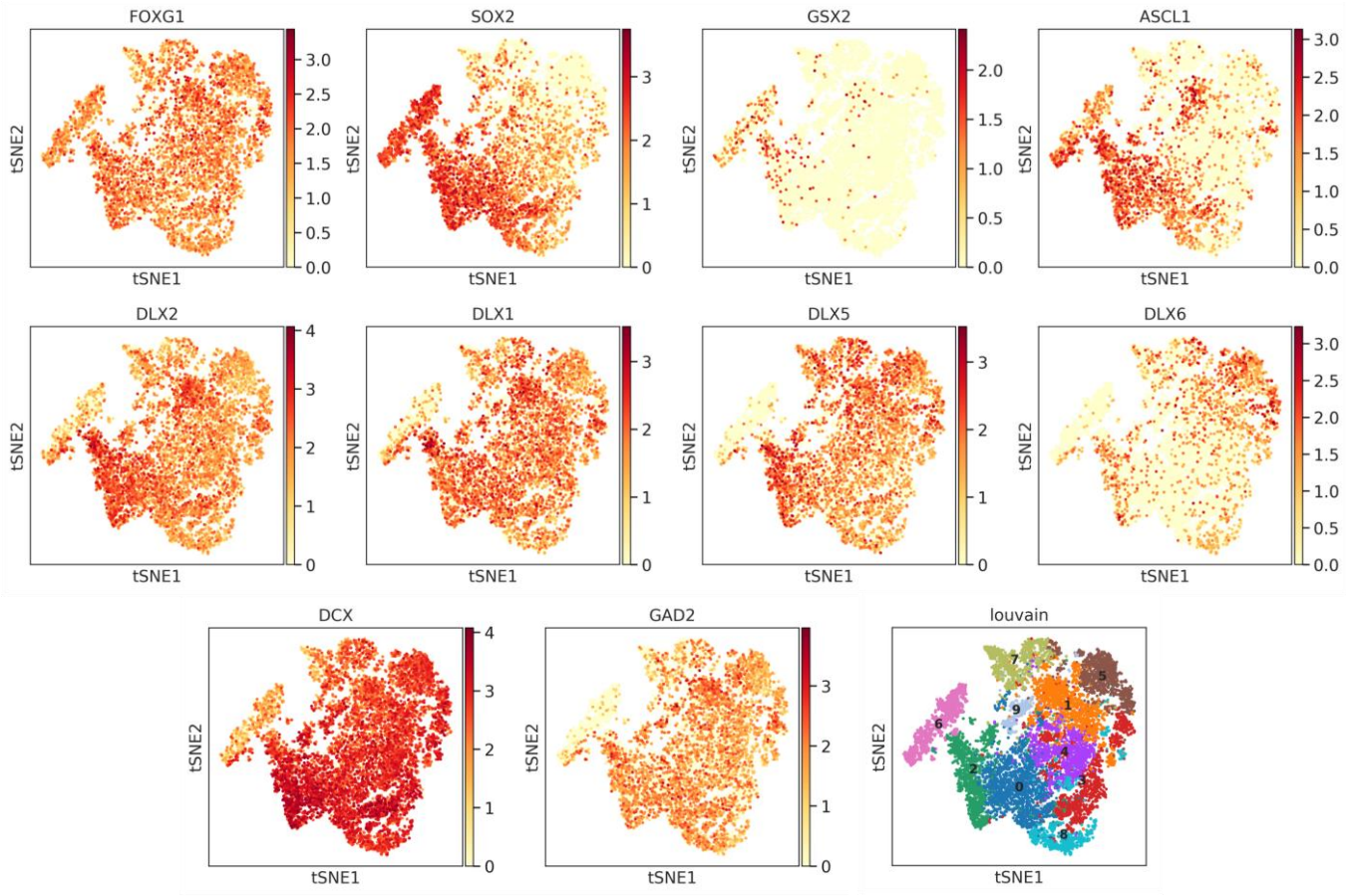

Fig 2.6: tSNE plots of single cells clustering for specific markers of ventral telencephalon development of three singular organoid WT 20CAG at day 45 of differentiation.

As negative controls, we looked for gene expression patterns of dorsal markers such as *Pax6, Tbr1* and *Slc17a6* (or vGlut2) (Englund et al. 2005; Fremeau et al. 2004; Vigneault et al. 2015). These genes are nearly absent in all the clusters of our organoids confirming their ventral identity (Fig 2.7). Only *Pax6* shows low expression in cluster 6, the putative sub-population of early progenitors, that could resemble neuroectodermal cells and the first VZ layers of the human LGE (Hansen et al. 2013).

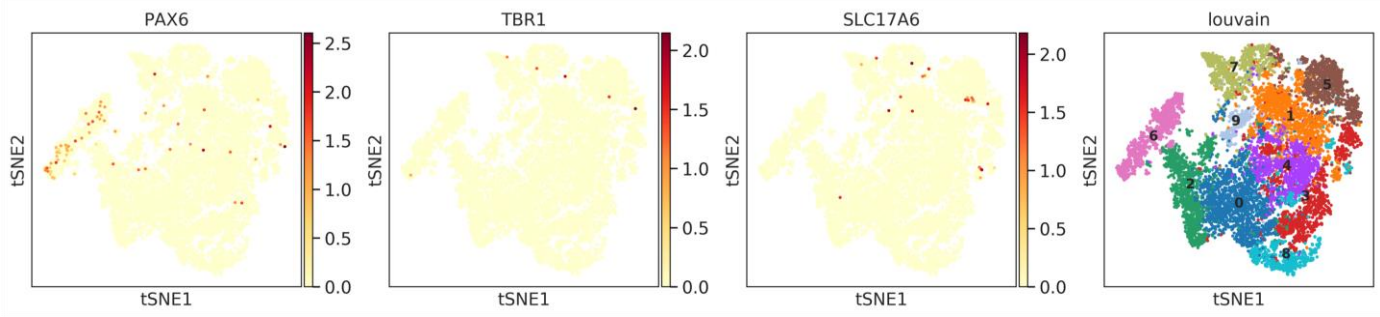

Fig 2.7: tSNE plots of single cells clustering for specific markers of dorsal telencephalon development of three singular organoid WT 20CAG at day 45 of differentiation.

These preliminary results confirm previous immunohistochemistry data, shown above, regarding the presence of both neural progenitors and more mature neurons with ventral telencephalic identity in our organoids. In particular, the cluster number 6, of putative early progenitors, results to be small compared to the total amount of cells and this is reasonable considering the maturation of WT organoids at day 45, time of the analysis. Further detailed investigations are still necessary to classify each cluster in terms of specific cellular subpopulation. Moreover, future analyses will also focus on HD counterparts to verify their internal variability and understand if there are different subpopulations compared to the WT organoids.

2.1.3 Comparison of the two protocols

The comparison between dorsal and ventral organoids generated by the two different protocols presented above, showed correct identity acquisition since the stage of progenitors cells inside the proliferative rosettes-like structures. In particular, as shown in figure 2.8 TBR2 is present only in dorsal organoids while GSX2 and MASH1 are present only in the ventral ones (Fig 2.8).

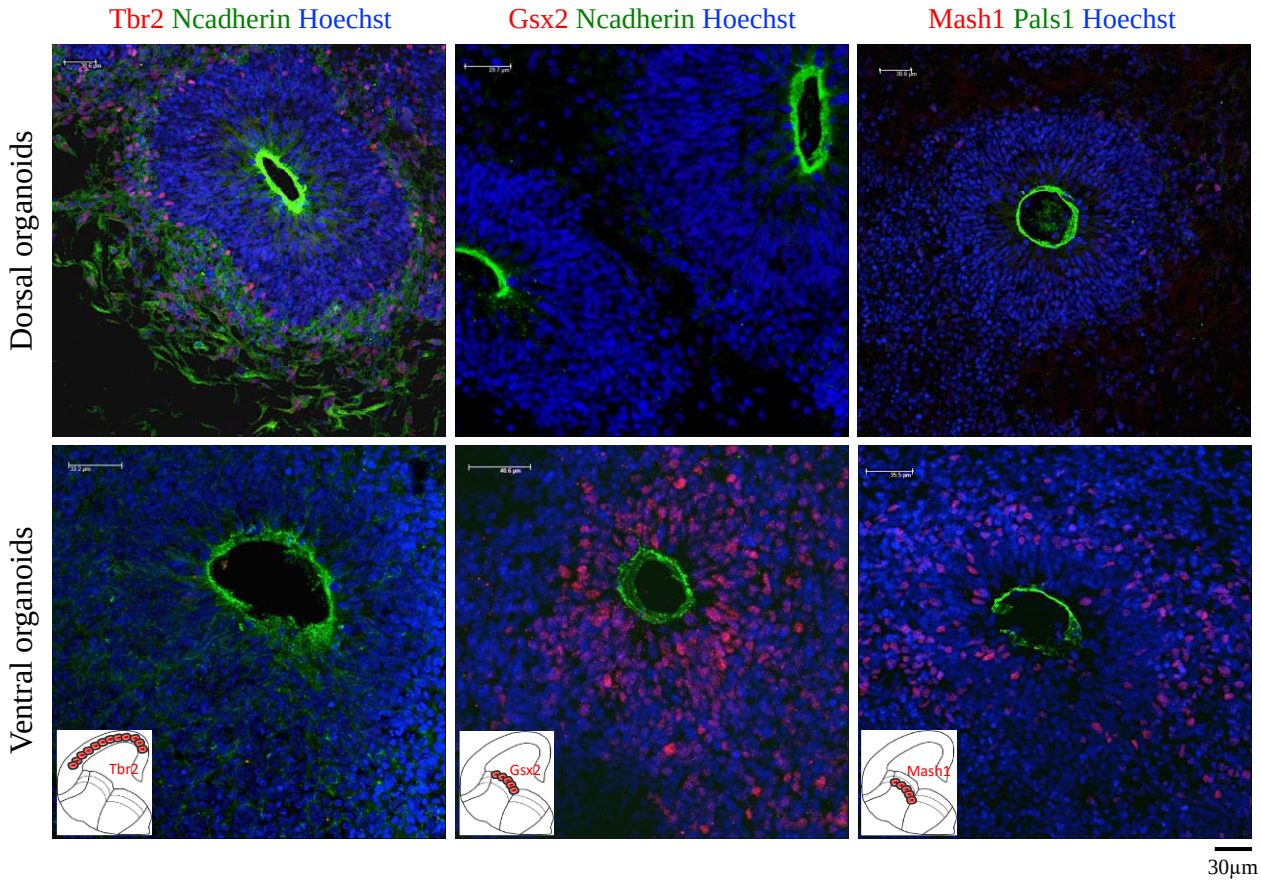

Fig 2.8: Comparison of immunohistochemistry analyses of WT dorsal and ventral organoids at day 45 of differentiation for TBR2, GSX2, and MASH1. Lumens of VZ structures marked by N-CADHERIN or PALS1. Schematic representation the different marked regions in developmental brain at the bottom. Scale bar 30µm on the bottom right.

Concerning the formation and organization of progenitors' structures, we compared the two protocols for dorsal and ventral organoids. The structures' amount inside the two types of organoids is similar. Indeed, the quantification of these VZ like structures per slice of organoids that does not present significative differences (Fig 2.9). We noticed that they present different morphology as the cortical ones are thicker compared to the ventral one. This quantification takes into consideration the internal area (derived from lumen's markers like PALS1) and the outside area (derived from the lower signal of BIIITubulin). The lumen size (PALS1 area) is similar while the thickness (differences between external and internal area) or the ratio (ratio of the two areas) are significantly different showing structure with broader expansion in dorsal organoids (Fig 2.9).

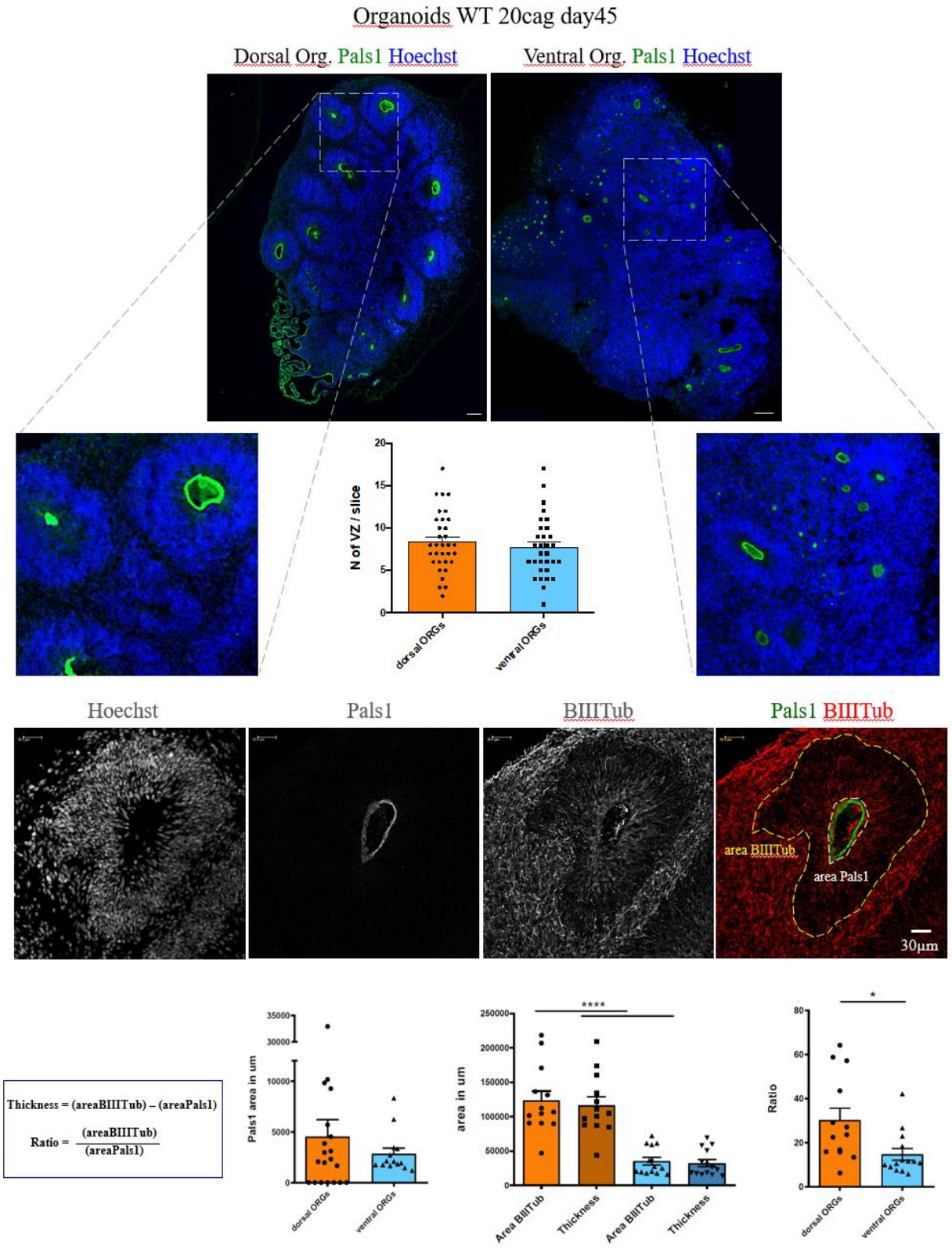

*Fig 2.9: Immunohistochemistry analyses of WT dorsal and ventral organoids at day 45 of differentiation. Quantification of numbers of VZ like structures per each slice by PALS1 staining at the top. Quantification of thickness and ratio of VZ like structures by BIIITubulin and PALS1 staining at the bottom. Scale bar 100µm for the top images and 30µm for the bottom images (Manual measurement. N= 13 VZ structures per protocol. Anova One Way, Bonferroni post test, *p< 0,05, **** p<0,0001)*

Although we don't know the precise reason of this morphological difference, there could be a physiological meaning. The cortex is the most expanded region in the brain with evolutionary trend so that the human have the biggest surface organized in folds, due to the amplification of cortical progenitors in VZ and SVZ (Lui, Hansen, and Kriegstein 2011; Otani et al. 2016). Interestingly a similar difference between dorsal and ventral organoids seems to be present also in a recent publication (Bagley et al. 2017) in which the two structures were separately generated and then fused to model inhibitory interneurons' migrations.

2.1.4 Recapitulation of LGE organization in ventral organoids

We then wondered if progenitors' structures in ventral organoids are representative of LGE development. We noticed that ventral organoids showed progenitors structures organized like VZ of LGE, with GSX2 closer to the lumens of the structures and MASH1 more far (Onorati et al. 2014). For this physiological conformation we also called them "VZ like structures". To investigate the distribution of these markers we created concentric areas around the lumens of the structures (Fig 2.10) and quantify the percentage of cells positive for each marker (GSX2 and MASH1) in each area (1, 2, 3). This assay is designed by ImageJ with manual drawing of the lumen's staining and sequential automatic drawing of the concentric areas as a progressive scale of the lumen. Then it was possible to manually count the cells in each area with the Cell Counter plugin of ImageJ. This approach highlight that WT ventral organoids are able to self organize in progenitors structures that resemble the LGE organizations. In fact, $GSX2^+$ and $MASH1^+$ populations are characterized by an opposite and significant trend. $GSX2^+$ progenitors display closer to the lumen with higher percentage of positive cells in area 1, while MASH1 positive cells are more localized in area 3 (Fig 2.10).

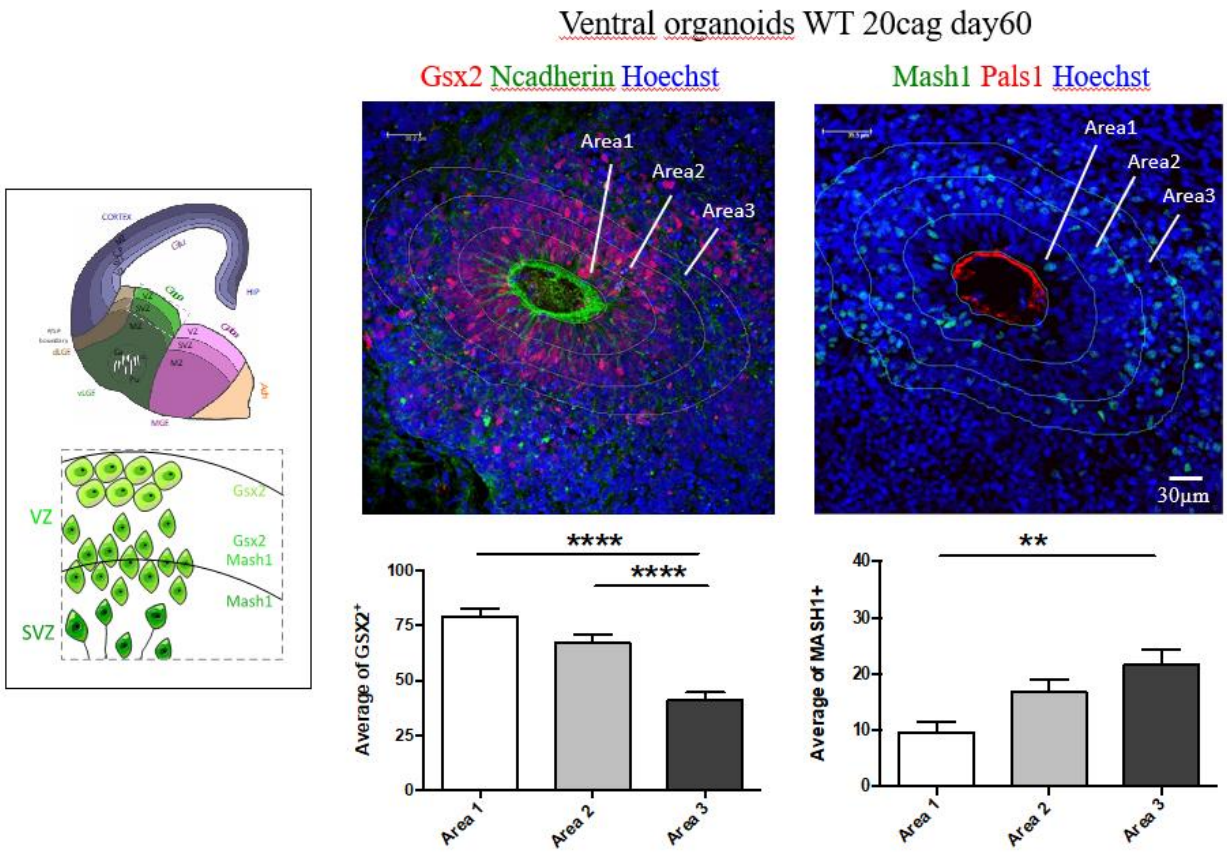

*Fig 2.10: Immunohistochemistry analyses of WT ventral organoids at day 60 of differentiation for GSX2, MASH1, N-CADHERIN and PALS1. Quantification of numbers of cells positive for GSX2 or MASH1 in each concentric area at the bottom. Schematic representation of LGE sub-regions in developmental brain on the left. Scale bar 30µm (Manual counting. Anova One Way, Bonferroni post test, **p<0,01, **** p<0,0001. N= 15 VZ structures from 12 organoids of 3 biological replicates.)*

To verify how consistent is this distribution, each singular VZ like structure quantified for GSX2 is showed in Fig 2.11. By measuring area and perimeter of the lumens (with PALS1 and N-CADHERIN staining) we demonstrate that the assay is independent from the lumens' size. This consideration is important because lumens are variable from structure to structure but their progenitors' distribution is an intrinsic characteristic that resemble the radial migration of cells in LGE from VZ to SVZ (Fig 2.11).

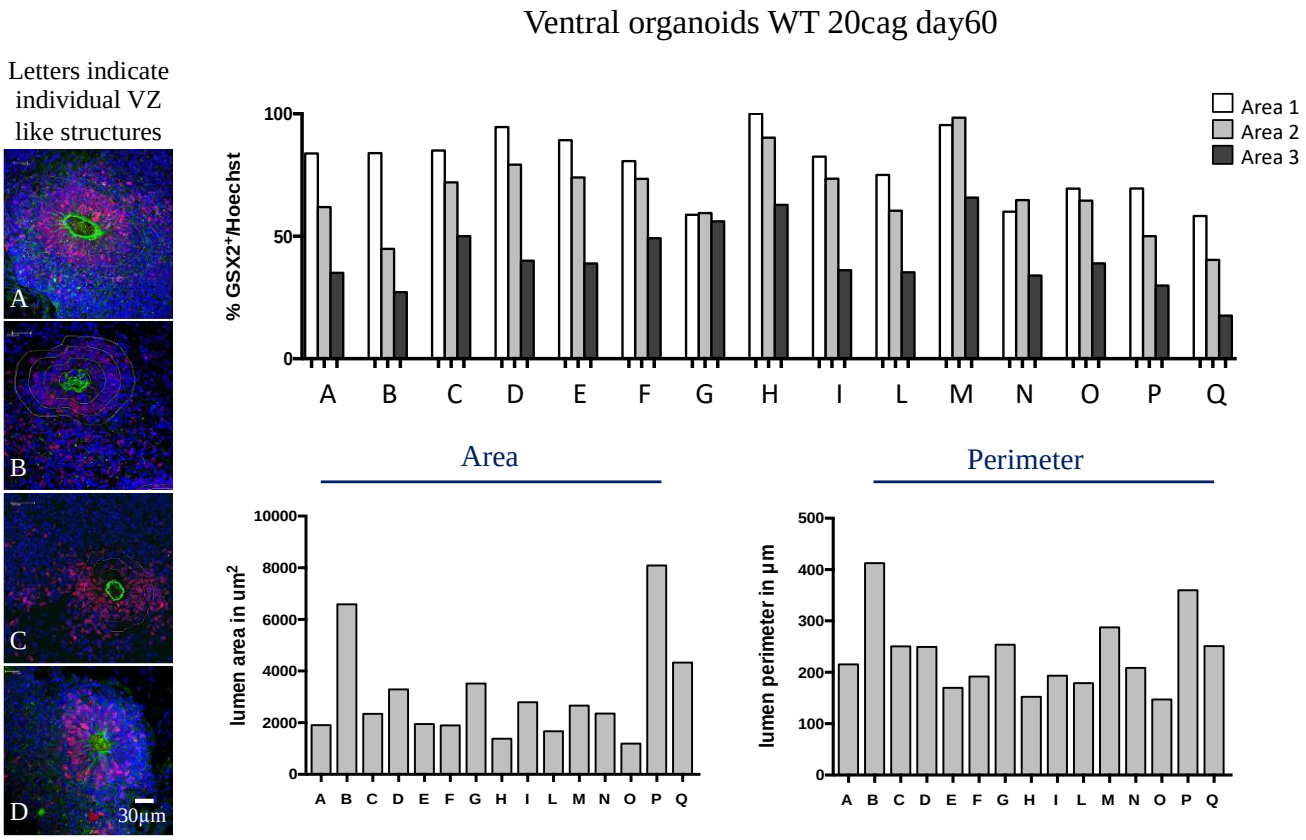

Fig 2.11: Immunohistochemistry analyses of WT ventral organoids at day 60 of differentiation for GSX2, N-CADHERIN and PALS1. Quantification of numbers of cells positive for GSX2 in each concentric area for each singular VZ structure at the top. Measurement of area and perimeter for each singular VZ structure on the bottom. Scale bar 30µm (Manual measurement. N= 15 VZ structures from 12 organoids of 3 biological replicates)

2.1.5 Reliability of the assay and recapitulation of cortex organization in dorsal organoids

Taking in consideration literature data (Di Lullo and Kriegstein 2017; Paşca et al. 2015; Qian et al. 2016) in which cellular organization in radial areas seems peculiar of 3D culture, we replicated similar analyses in dorsal telencephalic organoids (generated with the adaptation of Lancaster organoids shown above). The assay of concentric areas can be applied to these organoids (Fig 2.12) to study the correct distribution of cortical markers revealing its suitability and robustness. Also these dorsal telencephalic organoids have radial distribution of cells maturating from the center of VZ like structures that mimic the development of human cerebral cortex with PAX6 closer to the lumens, TBR2 in the external part of the structures and CTIP2 or TBR1 more distant (Fig 2.12).

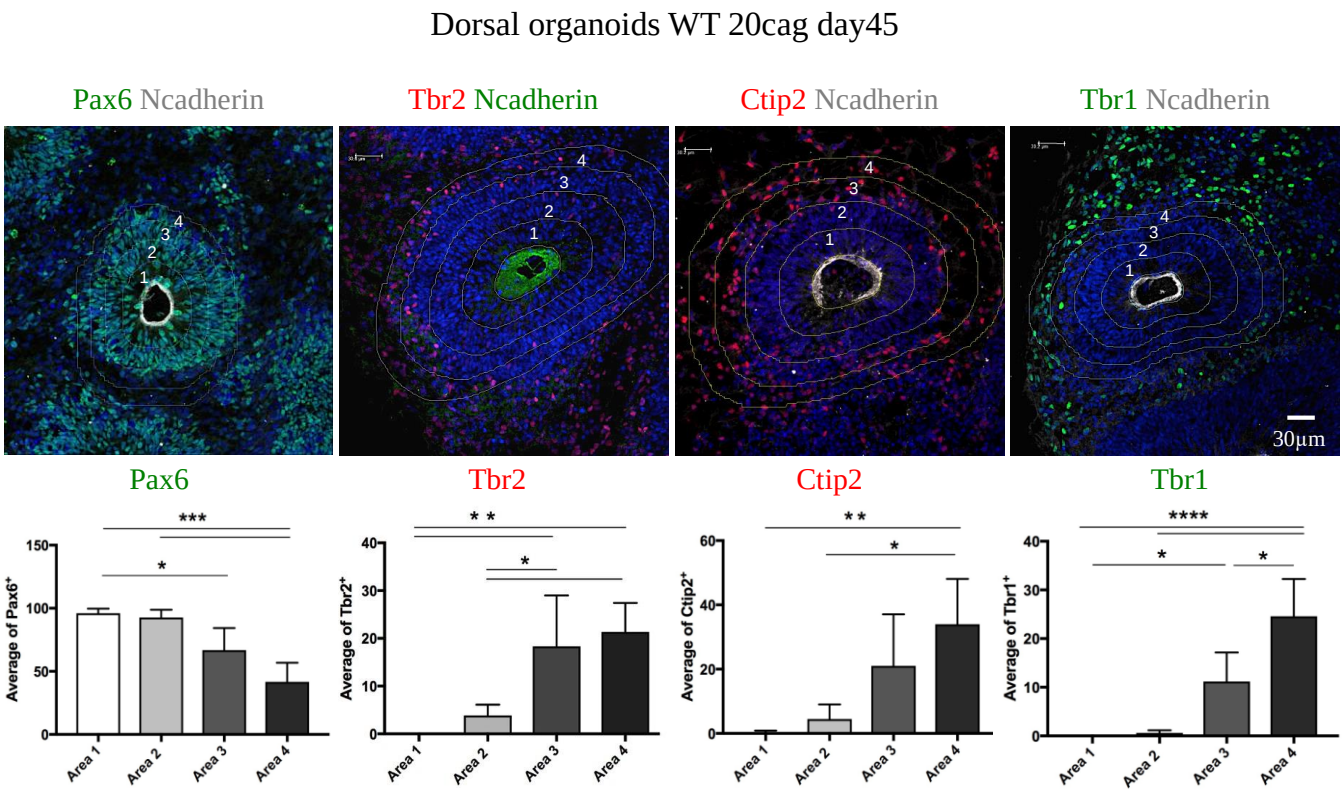

*Fig 2.12: Immunohistochemistry analyses of WT dorsal organoids at day 45 of differentiation for PAX6, TBR2, CTIP2, TBR1 and N-CADHERIN. Quantification of numbers of cells positive for each marker in each concentric area at the bottom. Scale bar 30µm (Manual measurement. Anova One Way, Bonferroni post test, * p< 0,05 ** p< 0,01 *** p<0,001 **** p<0,0001. N= 4 VZ structures from 4 organoids of 1 biological replicate.)*

Although many publications have already compared the progenitors' rosette-like regions of organoids to the VZ and SVZ or IZ regions of embryonal brain in development (Jo et al. 2016; Paşca et al. 2015) they usually define layers based on the distribution of markers for specific cell populations. A clear example can be found in the quantification of layers thickness, subdivided in VZ, SVZ and CP, based on the area occupied by SOX2 and CTIP2 positive cells (Qian et al. 2016). Instead in our analyses we tried to have a slightly different approach in which the concentric areas are based on each structures' morphology to later investigate the distribution of specific markers in these pre-defined areas. An example similar to our method is the subdivision of the entire neuroepithelium span into five equal portions (called bins) in which are then quantified SOX2 and the outer radial glia marker HOPX (Li et al. 2017). However, in this case the bins could result more arbitrarily defined, because of the lack of a precise end of the neuroepithelium span.

2.1.6 Preliminary conclusions about mimicking the human telencephalon development

On the basis of the above evidence we conclude that:

i. We have developed a new protocol for ventral telencephalic organoids that show typical ventral markers of neural induction at early time points and of GABAergic maturation at later time points.

ii. Preliminary scRNAseq results from ventral organoids confirmed the immunohistochemistry data and the absence of dorsal neurons' sub-populations

iii. The comparison of protocols for dorsal and ventral organoids showed the correct identity acquisition since the progenitor's stage. These cells presented a radial distribution in concentric areas around lumens that resemble cortex and LGE in development.

iv. Dorsal and ventral organoids present progenitors' structures in equal number but with different thickness

Despite differences in technical details, all the other published protocols for ventral organoids (Bagley et al. 2017; Birey et al. 2017; Xiang et al. 2017) resemble the MGE region of the brain with the majority of the cells being positive for NKX2.1. Our protocol have in common with them several ventral markers but seems to be more in the direction of the LGE, with only few Nkx2,1^{+} cells and some GSX2^{+} or ISLT11^{+} cells. Additional future characterizations with high-throughput rtPCR will clarify better these differences in precise region identity.

2.2 Modelling Huntington's disease in-vitro with ventral organoids

The previous work done in the lab (Conforti et al. 2018) exploit 2D culture and 3D cortical organoids from iPSC while this thesis is focussed on the use of control and HD organoids with ventral telencephalic identity, in order to mimic the development of the LGE and investigate whether the presence of mHTT can affect it.

In this project I used and isogenic allelic serie of hESC line, generated with CRISPR/Cas9 from hESC RUES2, differing for the number of CAG repeats but having the same genetic background, with the advantage of avoiding side effects not CAG related (Ruzo et al. 2018). We received the lines from Brivanlou's lab at the Rockefeller University as part of a a collaboration. We used two lines for WT (the parental not edited line and the other one edited with 20 CAG) and three lines for HD (edited respectively with 48 or 56 CAG and more recently we included in some analyses also the 72CAG).

2.2.1 VZ like structures organization in control and HD ventral organoids

We wondered if the radial distribution of progenitors in VZ like structures, described above (see paragraph 2.1.3) is affected in HD ventral organoids.

We analysed WT and HD cell lines organoids by immunohistochemistry for GSX2-MASH1 and quantification of positive cell inside each concentric area. As expected, the significant radial distribution of GSX2 and MASH1 progenitors is present in CTRL lines (mean of 2 WT lines: the un-edited parental line and the edited with 20CAG) while in contrast this is absent in HD organoids both with 48CAG and 56CAG. Moreover HD organoids are also less efficient in differentiation demonstrated by reduced percentages of $GSX2^+$ and $MASH1^+$ cells in each ventricular area as shown in Fig 2.13 (33% reduction of GSX2 in area 1 for 48CAG and 47% for 56CAG, while 25% reduction of MASH1 in area 3 for 48CAG and 60% for 56CAG).

This result suggests defects in correct differentiation and distribution of HD ventral progenitors inside organoids.

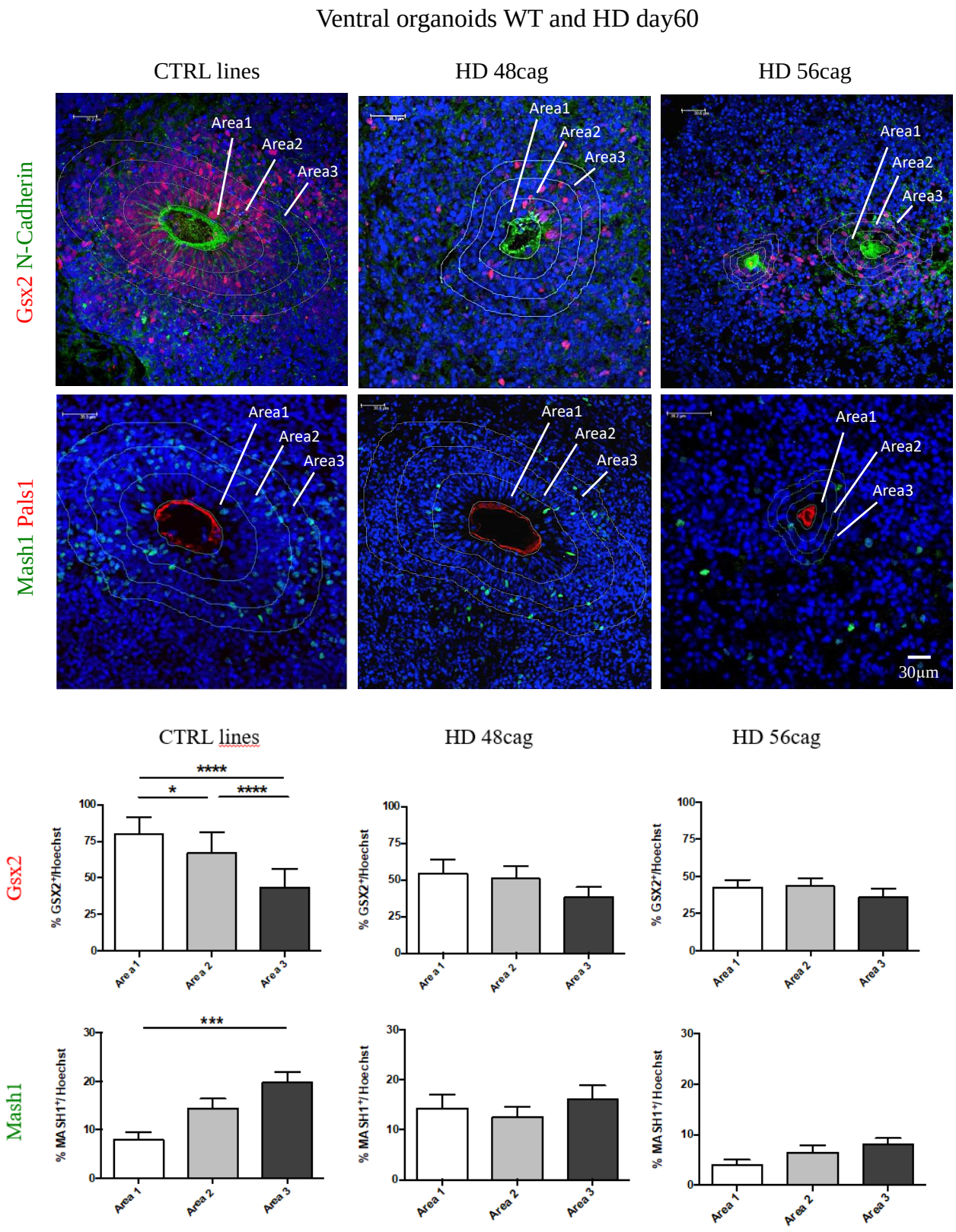

*Fig 2.13: Immunohistochemistry analyses of WT (20CAG) and HD (48 and 56 CAG) ventral organoids at day 60 of differentiation for GSX2, MASH1, N-CADHERIN and PALS1. Quantification of numbers of cells positive for GSX2 or MASH1 in each concentric area at the bottom. Scale bar 30µm (Manual counting. Anova One Way, Bonferroni post test, * p< 0,05, ***p<0,001, **** p<0,0001. N ≥ 15 rosettes from 12 organoids of 3 biological replicates for each cell line (2 for 48CAG line))*

Our interest was then to investigate if mHTT affects the formation of VZ like structures in ventral organoids. The presence of progenitors and radial glia was checked by general markers such as NESTIN, BLBP, VIMENTIN and SOX2 (Pilz et al. 2013; Pollen et al. 2015) in WT and HD ventral organoids at day 35. As shown in figure 2.14, organoids from all cell lines present markers for radial glia within the region of the VZ like structures.

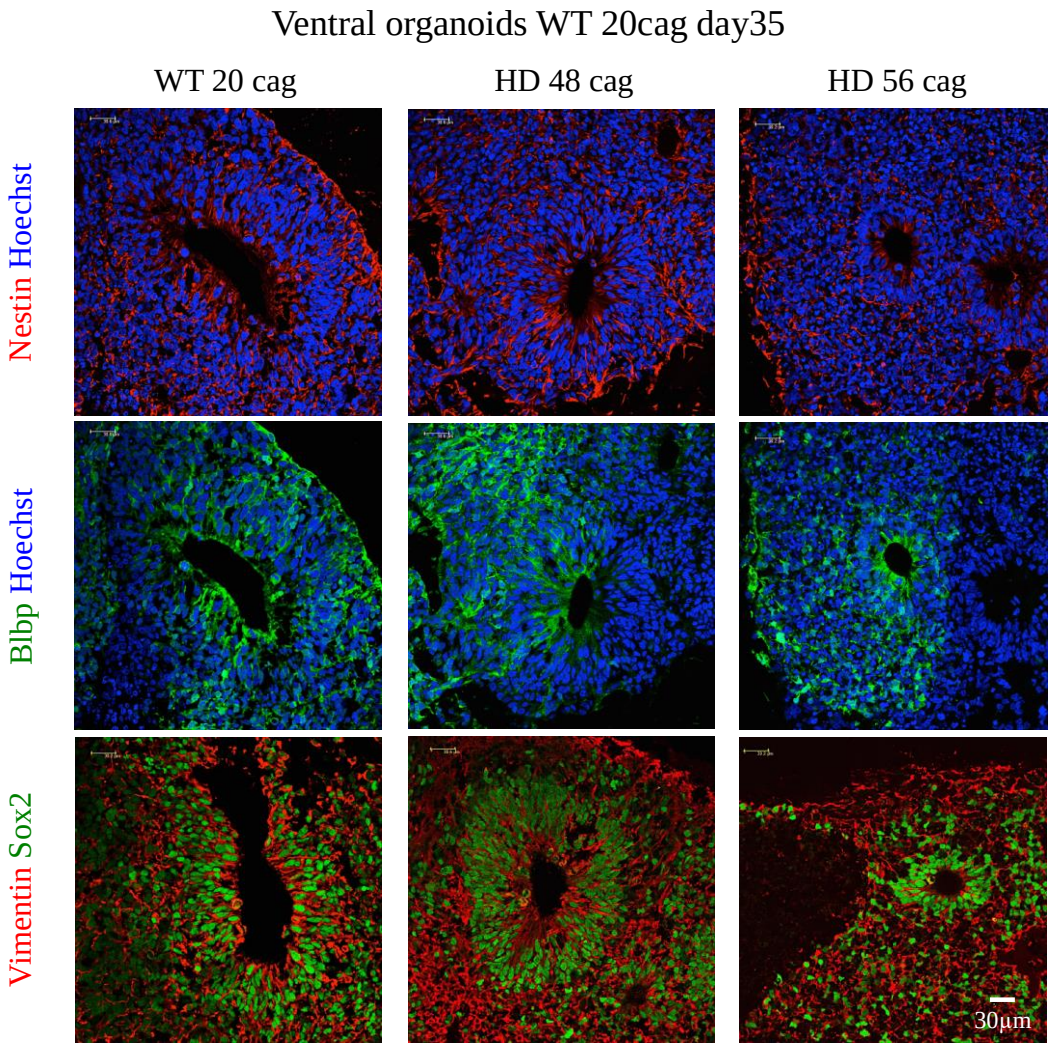

Fig 2.14: Immunohistochemistry analyses of WT (20CAG) and HD (48 and 56 CAG) ventral organoids at day 35 of differentiation for NESTIN, BLBP, VIMENTIN and SOX2. Scale bar 30µm at the bottom right.

We then looked at the amount of VZ like structures in ventral organoids and found that they were reduced in HD lines compared to control ones, both at day 35 and 60 of differentiation (Fig 2.15). Using antibodies against apical proteins enriched in lumens such as PALS1, N-CADHERIN or ZO1 we were able to quantify the number of these structures over the total area of the organoids' slice for

each slice of the ventral organoids. Although there is a bit of variability in terms of numbers, it is possible to appreciate that only organoids from CTRL lines (mean of 2 WT lines: the un-edited parental line and the edited with 20CAG) show high numbers of structures and that the mean is significantly higher in CTRL compared to HD lines (48, 56 and 72 CAG). This data could indicate a difficulty of HD organoids in forming organized VZ like structures (Fig 2.15).

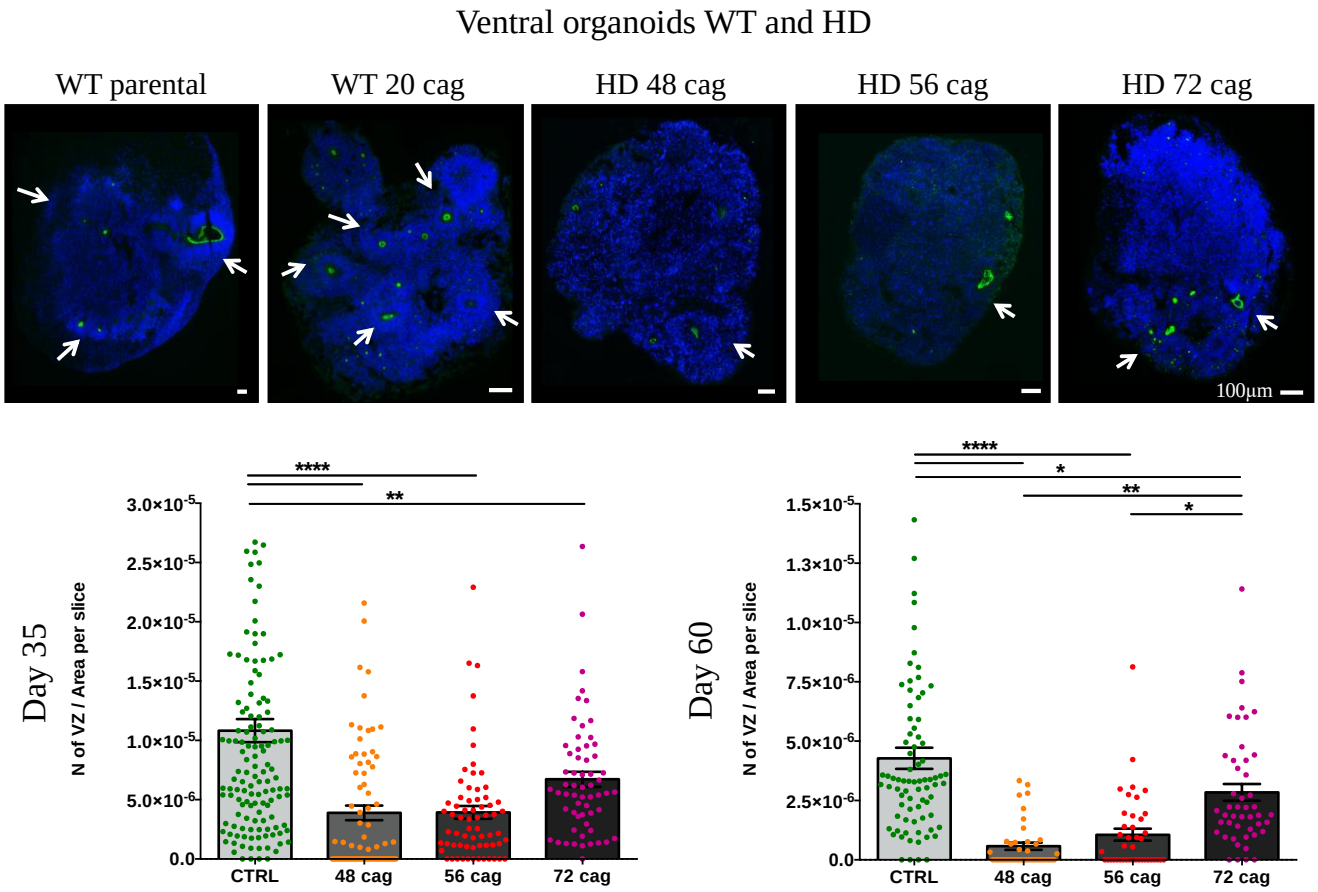

*Fig 2.15: Immunohistochemistry analyses of CTRL (parental and 20CAG) and HD (48, 56 and 72 CAG) ventral organoids at day 35 and 60 of differentiation for PALS1 or N-CADHERIN. Scale bar 100µm. Quantification of numbers of VZ like structures per area of each organoids' slice, at the bottom. (Manual counting. Anova One Way, Bonferroni post test. *p< 0,05; **p<0,01; ****p< 0,0001. N ≥ 30 slices for each cell line, from 10 organoids of 4 biological replicates at each time points.)*

In Huntington disease, the number of CAG repeats is inversely related to age of onset such that the higher the number, the earlier the onset (Lee et al. 2012; Nopoulos 2016). For this reason we were surprised to see a less severe defect in the HD line with longer poly Q repeats, the72 CAG (Fig 2.15). Although in the following paragraphs I will describe some phenotypes with a sort of CAG-length

dependent trend, it is usually hard to demonstrate phenotypes with this regulation because of the intrinsic variability between each cell line. Moreover, the inverse correlation between CAG repeats and age of onset accounts for roughly 50% to 70% of the variance. This variance between different individuals may be due to both environmental elements and modifying gene factors (Langbehn et al. 2004; Wright et al. 2019). The "take home message" of these analyses on VZ like structures concerns a difficulty of the HD organoids in self-organization plausibly related to defects in polarity, migration and cell cycle regulation that will be discussed in following chapters.

2.2.2 Cell cycle regulation in control and HD ventral organoids

The measurement of the total area for each organoid's slice quantified previously in Fig 2.15 revealed a significantly reduced size of HD organoids with longer CAG tracts both at da 35 and 60 of differentiation (Fig 2.16). We decided to investigate the initial organoids' growth. By the quantification of WT and HD ventral organoids' area in the first period of differentiation, it is possible to notice that WT and HD organoids start with similar dimension with only a slight tendency of bigger area in WT samples (Fig 2.17). Therefore, HD organoids with longer CAG tracts present reduced dimension due to a lower growth during the differentiation and not since the early days of EB stage.

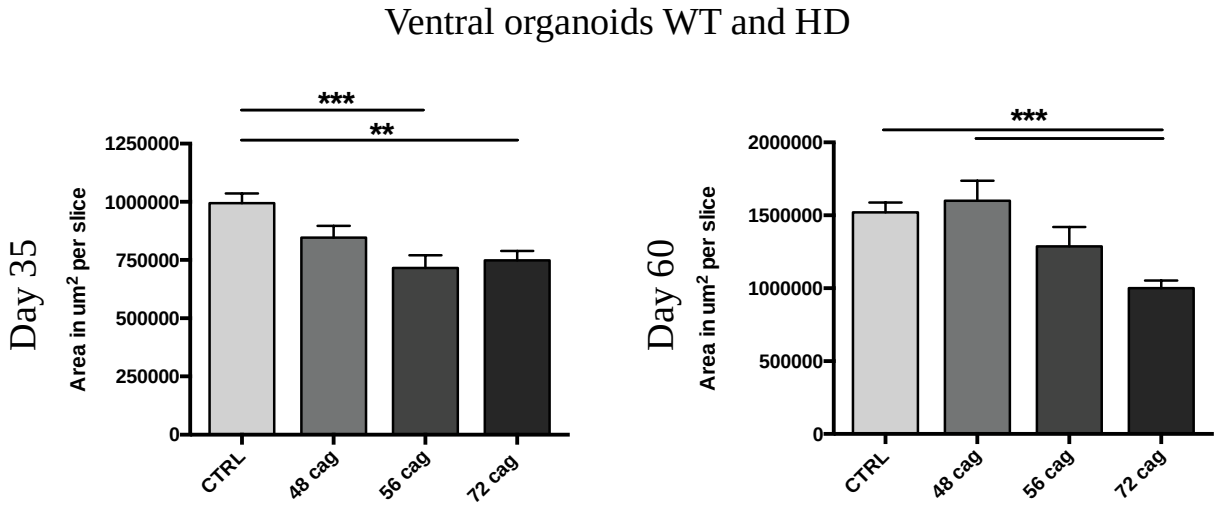

*Fig 2.16: Quantification of the area from each organoids'slice of CTRL (parental and 20CAG) and HD (48, 56 and 72 CAG) ventral organoids at day 35 and 60 of differentiation. (Manual measurement. Anova One Way, Bonferroni post test. **p<0,01; ***p<0,001. N ≥ 30 slices for each cell line, from 10 organoids of 4 biological replicates at each time points.)*

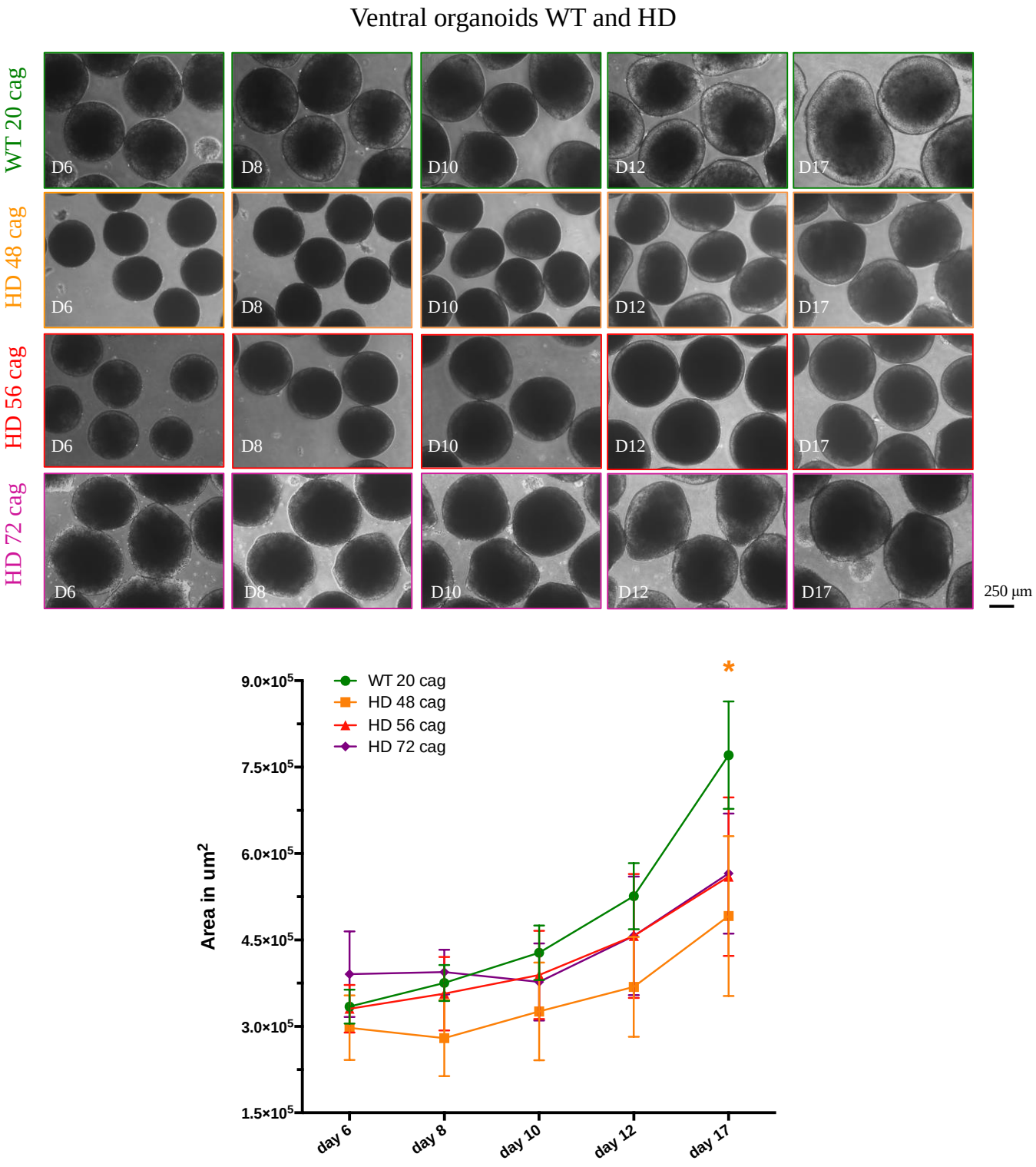

*Fig 2.17: Phase contrast images of WT (20CAG) and HD (48, 56 and 72 CAG) ventral organoids during earliest days of differentiation. Scale bar 250µm on the right. Quantification of organoids area at the bottom (Manual measurement. Anova Two Way, Bonferroni post test, *p< 0,05 between 48CAG and WT at day 17. N=5 biological replicates, each with 4 organoids per time points.)*

Based on the above data, we wonder if the lower growth in linked to different cell cycle regulation. This is an essential aspect because the VZ like structures, like the ventricular zones in embryos, are composed of progenitors in active and tightly regulated cell cycle (Falk et al. 2017; Pilz et al. 2013).

We performed IC analyses on WT and HD ventral organoids in order to measure the percentage of KI67 (active cell cycle) and PH3 (mitosis phase) positive cells at day 35 of differentiation. The data shown in Fig 2.18 demonstrate that HD organoids with 56CAG have a 45% reduction in the percentage of KI67 positive cells. This statistically significant reduction in $KI67^{+}$ cells is not present in $PH3^{+}$ cells, suggesting less cells in other cell cycle phases (e.g. S phase) or more cell cycle exit.

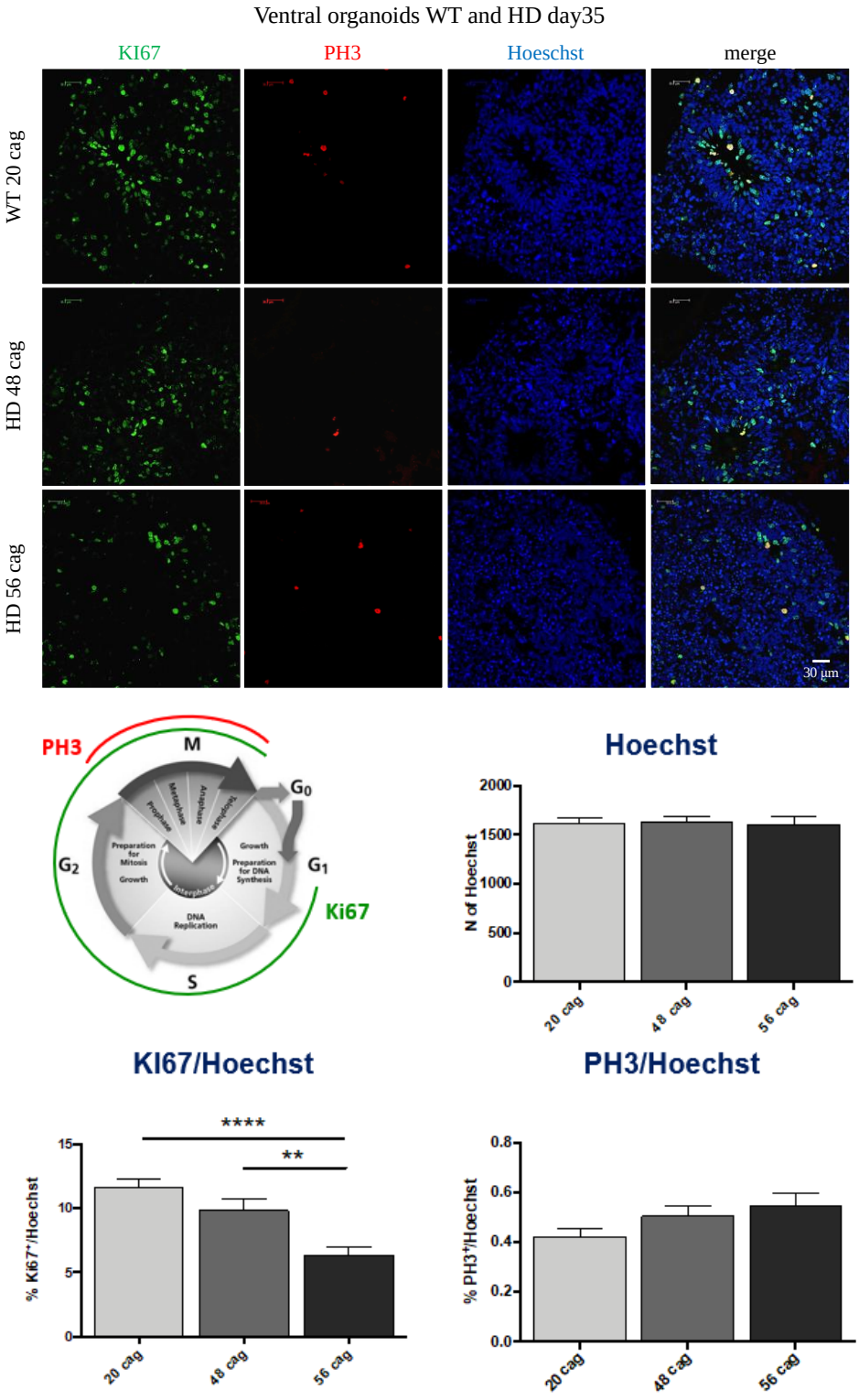

*Fig 2.18: Immunohistochemistry analyses of WT (20CAG) and HD (48 and 56 CAG) ventral organoids at day 35 of differentiation for PH3 and KI67 on the top. Scale bar 30µm. Quantification of numbers of cells positive for each marker on the bottom. (Automatic counting with Cell Profiler. Anova One Way, Bonferroni post test, ** p< 0,01, *** p<0,001. N=30 images, from 12 oranoids of 3 biological replicates, 2 z stacks for each image and 5 images for replicate.)*

As these results point out a defect in cell cycle progression, we investigated in our model the known role of HTT in mitotic spindle orientation (Godin et al. 2010; Lopes et al. 2016; Molina-Calavita et al. 2014; Ruzo et al. 2018a). Both the absence of HTT or the presence of mHTT affects the cell divisions leading to abnormal neurogenesis with more asymmetric divisions and reduced progenitors' expansion, similarly to what occur in some neurodevelopmental diseases (Microcephaly and Lissencephaly).

In our analyses we coupled phosphorylated VIMENTIN (that marks cells in mitosis) with PERICENTRIN (that marks the centrosomes). With ImageJ it was possible to measure in cells in mitosis, the angle between the lumen and the direction of division (drawn in white in the scheme on the top right of Fig 2.19). We applied this analysis on ventral WT and HD organoids at day 35, to quantify the three type of mitotic divisions: horizontal (0°-30°), oblique (30°-60°) or vertical (60°-90°). This data revealed that HD ventral organoids with longer CAG repeats (56CAG and 72CAG lines) have higher percentage (from 22% to 45%) of horizontal divisions compared to control organoids (white bar in the graph in Fig 2.19). This significant increase in the asymmetric type of division could suggest a difficulty in expanding the pool of progenitors inside the VZ like structures and an earlier cell cycle exit towards faster differentiation.

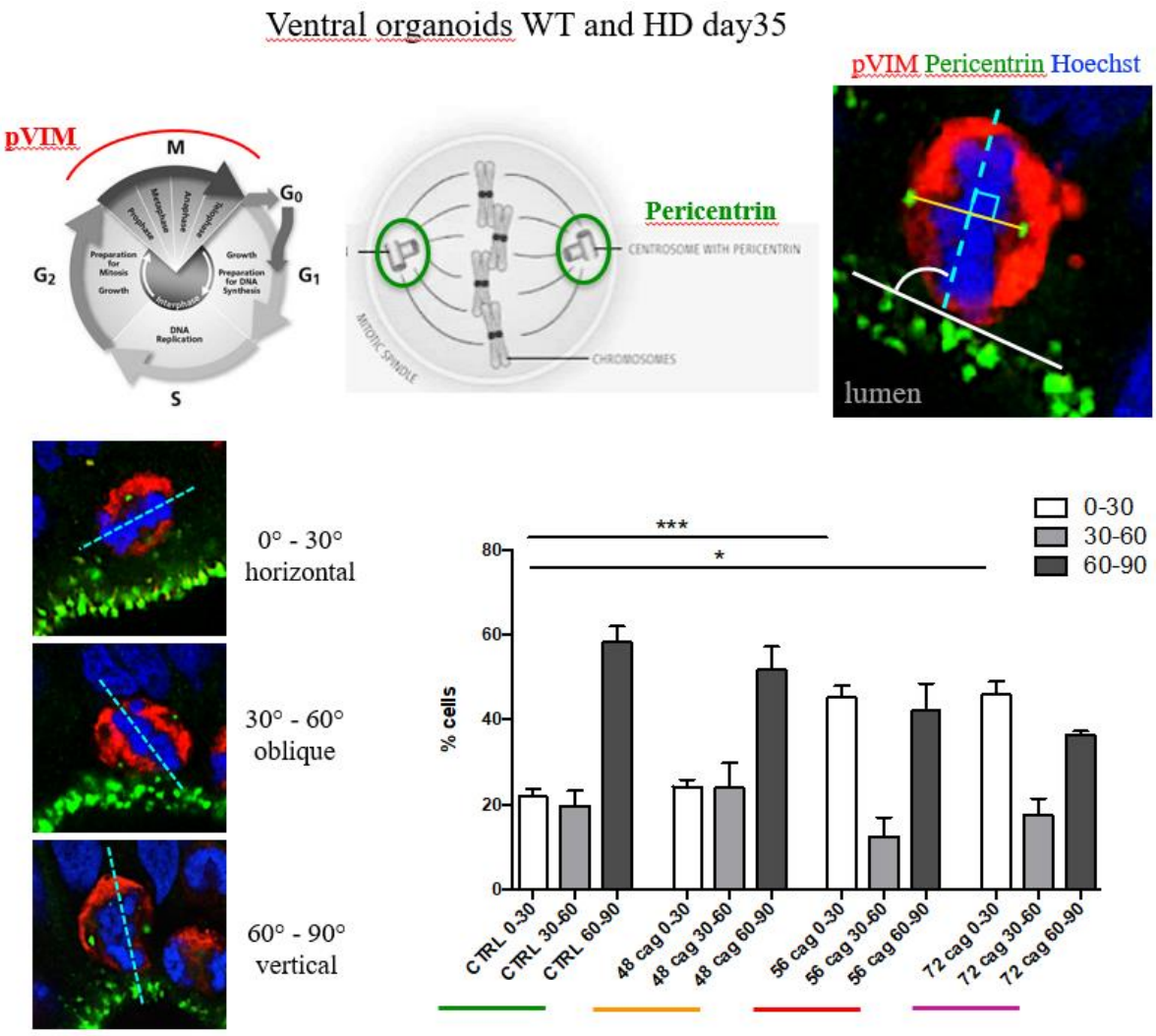

*Fig 2.19: Scheme of the analysis on the top. Immunohistochemistry analyses of CTRL (parental and 20CAG) and HD (48, 56 and 72 CAG) ventral organoids at day 35 of differentiation for pVIMENTIN and PERICENTRIN. Example of different types of division: horizontal (angle between 0 and 30 degree), oblique (angle between 30 and 60 degree) and vertical (angle between 60 and 90 degree). Quantification of percentage of cells in each type of division on the bottom right. (Manual measurement with ImageJ. Unpaired t test, Welch's correction,*p<0,1; ***p<0,001. N ≥ 100 cells from 10 organoids of 4 biological replicates (2 replicates for 72CAG line) for each cell line.)*

The mitotic spindle orientation is one of the multiple functions in which HTT is involved through the formation of complexes with many different interactors. Normal HTT interacts with HAP1, dynein and dynactin and this complex localizes in mitosis at the spindle poles where it helps the accumulation of NUMA and LGN, guiding the orientation of mitotic division in a evolutionarily conserved manner (Elias et al. 2014; Godin et al. 2010; Gutekunst et al. 1995). In our data the mitotic spindle misorientation is not as strong as in microcephaly models (it is present only in cell lines with longer

CAG track and is not correlated with a huge reduction of the symmetric divisions: 60°-90° degree) suggesting a milder phenotype in HD. Indeed, HD patients born with an apparent normal brain but in terms of dimensions there are few publications reporting a smaller intracranial volume in HD children (Lee et al. 2012; Nopoulos et al. 2011).

A further parameter linked to mitotic spindle is the ratio of cell cycle exit, which is explored in the following analysis to understand if HD cells with more asymmetric divisions are actually becoming postmitotic earlier. The cell cycle exit ratio was already studied in brain organoids, such as in the publication from Jaenisch's group (Li et al. 2017) to compare proliferation rates of control and mutated (PTEN deletion) organoids.

To investigate this aspect in our model, we used EdU assay at day35. EdU is incorporated in DNA of cell in S phase and kept in the daughter cells. Analysing immunohistochemistry for KI67 and EdU at later time points it is possible to quantify the percentage of cells that have exit the cell cycle. These cells out of the cell cycle are marked only by the EdU, they were cycling at day 35 when we gave them EdU but are now KI67 negative (orange arrows in the scheme on the top right of Fig 2.20). Instead the one that are still cycling in the moment of the analyses are marked by both EdU and KI67 (orange triangle in the scheme on the top right of Fig 2.20). At day 35 we noticed many double positive cells, while at day 60 there are almost no double positive cells, meaning that the ones that receive EdU have exit the cell cycle. The quantification of these cell populations disclosed a significant 15% increase in cell cycle exit ratio at day 45 in HD organoids compared to WT ones. At day 60 there are no differences because the exit ratio is reaching his plateau also in control cells (Fig 2.20). For this reason, in our future analyses we will include also day 40, as an earlier read out to confirm this data.

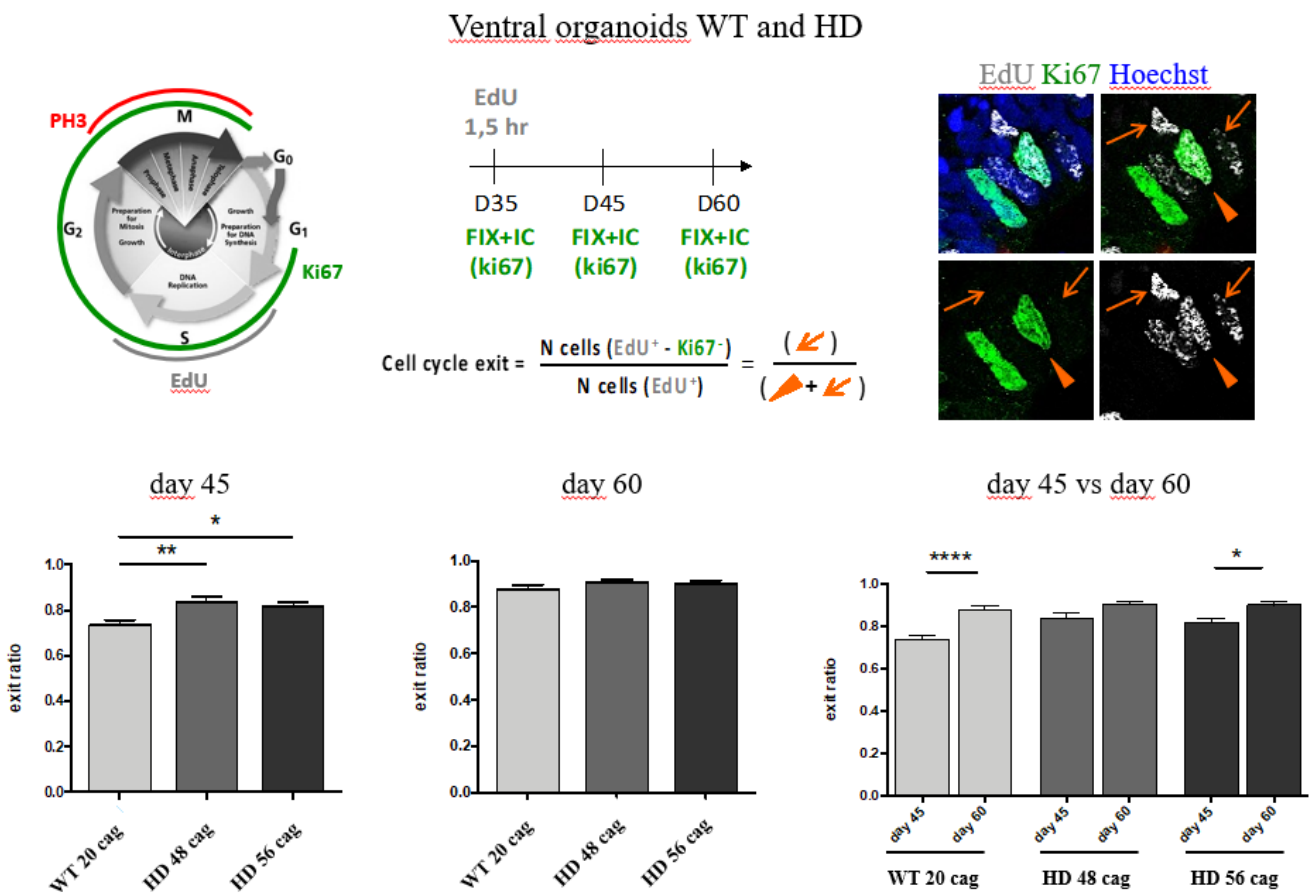

*Fig 2.20: Scheme of the experiment on the top. Immunohistochemistry analyses of WT (20CAG) and HD (48 and 56 CAG) ventral organoids at day 45 and 60 of differentiation for EdU and KI67 after EdU treatment performed at day 35. Example of cells still in active cell cycle (orange arrows) and cells that have exit the cell cycle (orange triangle). Quantification of cell cyle exit ratio is described in the formula on the top. Graphs on the bottom. (Automatic quantification with Cell Profiler. Anova One Way, Bonferroni post test, *p<0,05, **p<0,01. N = 24 images from 8 organoids of 2 biological replicates (1 for 48CAG line) with 2z stacks for each image and 6 images for each replicate.)*

It is important to notice that this result is in line with the misoriented mitotic spindle, shown above in Fig 2.19. Together our data on cell cycle regulation from $KI67^+$ cells to spindle orientation and exit ratio could explain the reduced growth and the lower amount of VZ like structures in HD organoids compared to control ones.

The EdU assay can be used also to study interkinetic nuclear migration (INM), a stereotypical nuclear movement characteristic of progenitors in division in embrional VZ. Mitotic divisions occur at the apical surface of the VZ whereas the nuclei of cells in S phase are located on the basal side of the VZ (Willardsen and Link 2011). This type of assay was already performed on brain organoids to prove

their ability in recapitulating some physiological aspects of neurogenesis during neural development (Kadoshima et al. 2013; Lancaster et al. 2013). In organoids cells in M phase, marked by PH3, are localized closer to the lumen (that resemble the physiological ventricle) while cells in S phase, marked by EdU, displaced far from the lumen. The EdU treatment (described in materials and methods) is the same used for cell cycle exit ratio but in this case the organoids are fixed for the analyses at day 35 just after the EdU removal. Although this analysis is still ongoing on additional replicates to have a wider and clearer interpretation, in our preliminary test both WT and HD organoids present cells correctly localized (Fig 2.21). Indeed there are no proved evidences of INM defects in Huntington's disease, neither from *in-vivo* or *in-vitro* studies, distinguishing this pathology from Lissencephaly in which LIS1 mutation seems to impair both the mitotic spindle orientation and the INM (Faulkner et al. 2000; Vallee and Tsai 2006).

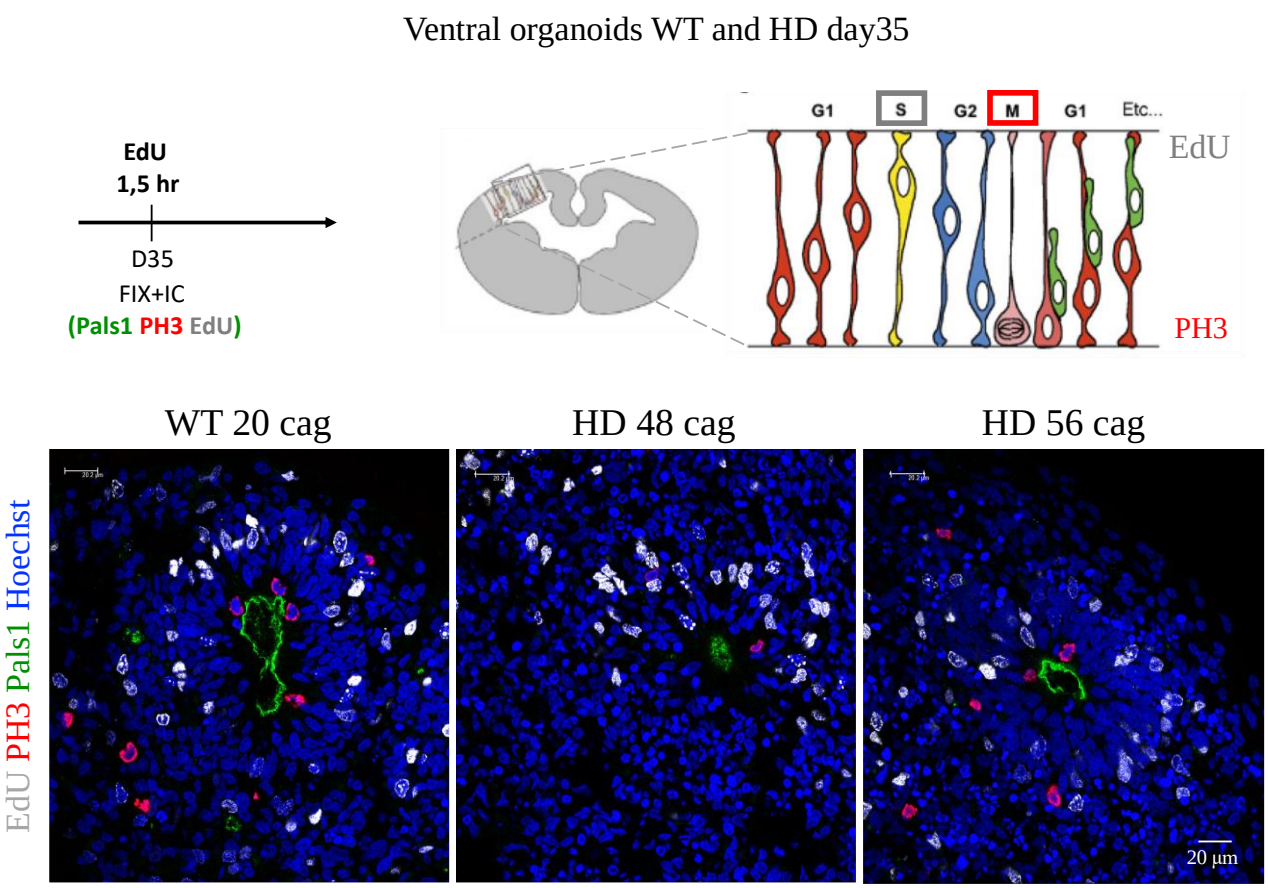

Fig 2.21: Scheme of the experiment and representation of INM during neuraldevelopment on the top. Immunohistochemistry analyses of WT (20CAG) and HD (48 and 56 CAG) ventral organoids at day 35 of differentiation for EdU, PH3 and PALS1 at the end of EdU treatment on the bottom. Scale bar 20µm.

2.2.3 Polarity in control and HD ventral organoids

Beside the cell cycle regulation, we also focused on polarity arrangement because this mechanism plays essential roles in VZ like structures organization and is affected by mHTT. Effects of mHTT in polarity, indeed have been documented from reduced cell adhesion in HD-iPSC neural progenitors, neural tube defects in HD zebrafish embryos, randomization of epithelial polarity in the skin of frog embryos and reduced rosette formation in mouse and human HD cells (Conforti et al. 2018; Haremaki et al. 2015; Ruzo et al. 2018; Lo Sardo et al. 2012).

We focus on lumens organization by analysing their morphology, dimensions and internal localization of primary cilia through immunohistochemistry of PALS1 (apical polarity protein) and ARL13B (marker of ciliary membrane). Ventral organoids at day 35 present lumens that can be considered *"empty"* (arrows) or *"full"* (arrowheads) by PALS1 staining (Fig 2.22). We noticed a different proportion between them in control and HD ventral organoids. The control samples are characterized by a majority of "empty" lumens, the HD line with 48CAG presents similar distribution between the two lumens' forms and the HD line with 56CAG display a majority of "full" lumens (Fig 2.22).

Ventral organoids WT and HD day35 Pals1 Hoechst

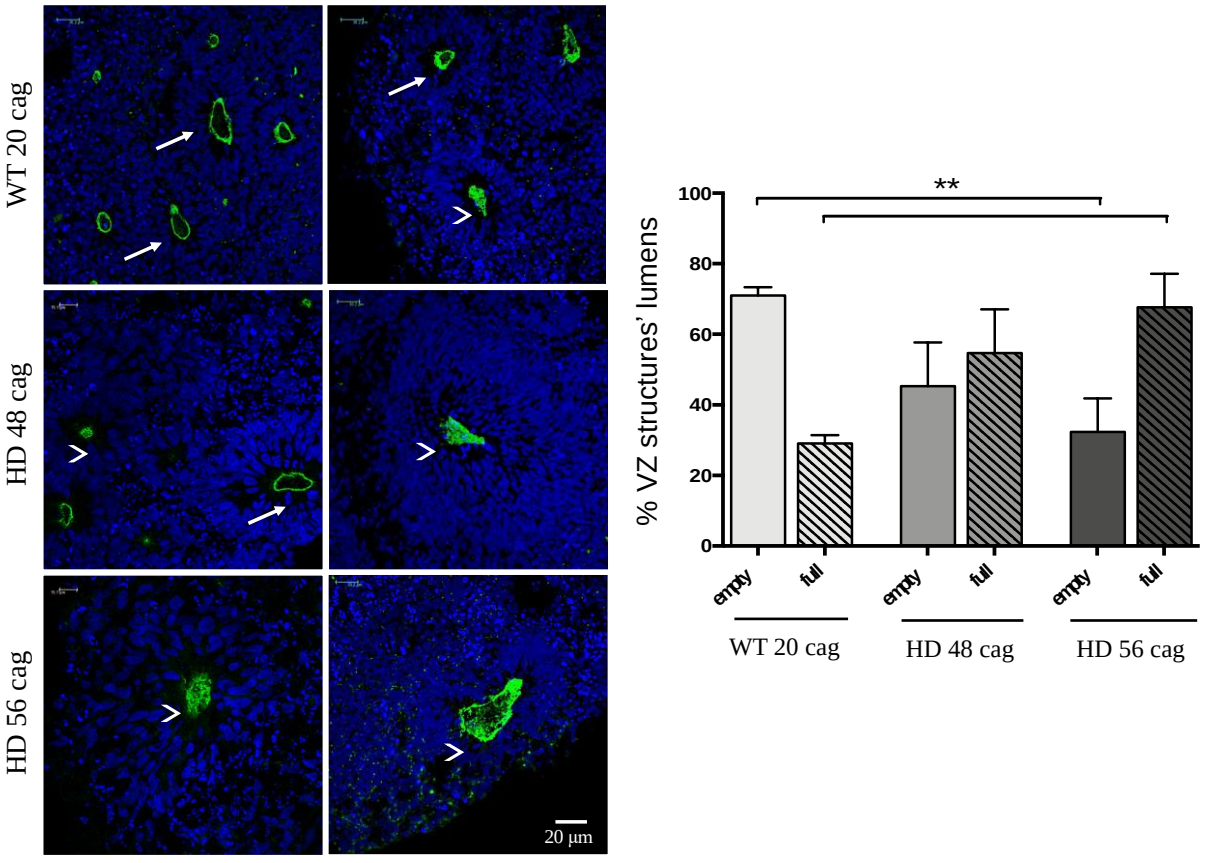

*Fig 2.22: Immunohistochemistry analyses of WT (20CAG) and HD (48 and 56 CAG) ventral organoids at day 35 of differentiation for PALS1. Example of "full" lumens (arrowheads) and "empty" lumens (arrows). Scale bar 20µm. Quantification of percentage of the two types of lumens on the right. (Manual quantification. Mann Whitney test, ** p< 0,01. N ≥ 70 rosettes, from 12 organoids of 3 biological replicates for each cell line.)*

A plausible interpretation is that the HD organoids exhibit defects in organizing polarity proteins, leading to structures with a sort of collapsed lumens. This interpretation is supported by the reduced lumen's size both in terms of area and perimeter (Fig 2.23), that we found in HD organoids with 56CAG compared to the control counterparts (mean of parental line and 20 CAG line). Similar data were previously reported in literature with models of 2D neuronal rosettes (Conforti et al. 2018; Ruzo et al. 2018a; Xu et al. 2017).

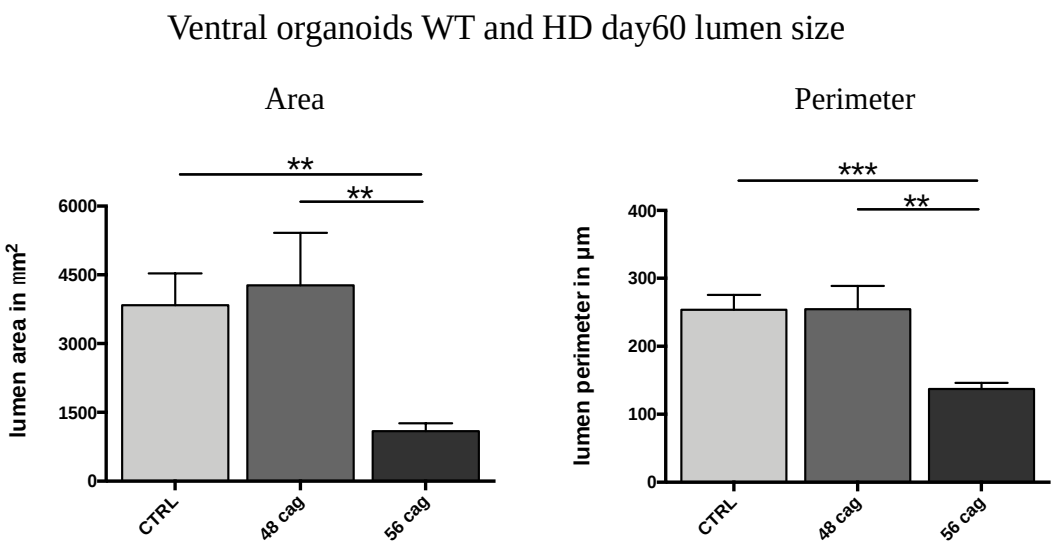

*Fig 2.23: Area and perimeter quantification of lumens of VZ like structures in CTRL (parental and 20CAG) and HD (48 and 56 CAG) ventral organoids at day 60 of differentiation. (Manual measurement. Anova One Way, Bonferroni post test, **p<0,01, ***p<0,001. N ≥ 30 rosettes, from 12 organoids of 3 biological replicates (2 for 48CAG line) for each cell line.)*

To characterize the lumens organization and understand which material could be localized inside them we looked at primary cilia. These signaling organelles of most mammalian cells, are critical for the development of the vertebrate nervous system, playing either essential or modulatory roles in specific neurodevelopmental signaling pathways such as Sonic hedgehog (SHH) and WNT (Gazea et al. 2016; Han et al. 2008). Cilia also play important roles in regulation of stem cells and regeneration

in the adult nervous system, where cell cycle and specific morphogen pathways recapitulate developmental functions to facilitate cell fate decisions in ciliated tissues (Guemez-Gamboa, Coufal, and Gleeson 2014). Interestingly WT HTT regulates ciliogenesis by interacting through huntingtin-associated protein 1 (HAP1) with pericentriolar material 1 protein (PCM1). Pathogenic polyQ expansion led to centrosomal accumulation of PCM1 and abnormally long primary cilia in mouse striatal cells (Keryer et al. 2011). Longer cilia lead in alteration of the cerebrospinal fluid flow, therefore primary cilia could contribute in HD pathogenesis: perhaps by disrupting signalling pathways, propagating toxic misfolded polyQ-HTT fragments, and exacerbating disease progression (Kaliszewski et al. 2015; Liu and Zeitlin 2011).

We found presence of primary cilia marked by ARL13B inside the apical side of the VZ like structures in control ventral organoids at day 60 (Fig 2.24).

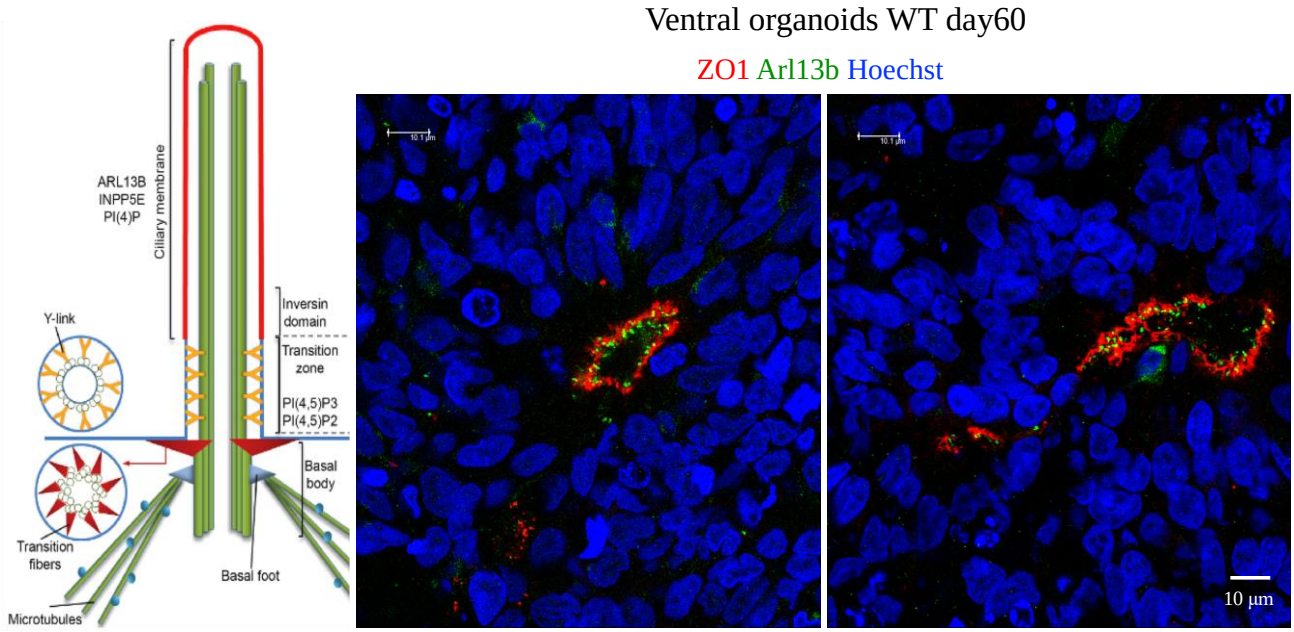

Fig 2.24: Schematic representation of primary cilia. Immunohistochemistry analyses of WT ventral organoids at day 60 of differentiation for ZO1 and ARL13B. Scale bar 10µm on the bottom right.

We then wondered if it is possible to reveal a phenotype in primary cilia morphology as previously described in mouse models (Keryer et al. 2011). The quantification with NIS software after images deconvolution is reported in fig 2.25 showing that the HD organoids and particularly the 56CAG and

72CAG lines, showed higher ciliogenesis. They present bigger primary cilia both in terms of length and perimeter and higher ciliary density (measured as the ratio between the area occupied by primary cilia, or their number, and the total area of the lumen marked by ZO1). This data is in line with the one in literature suggesting the involvement of HTT in cilia's morphology and function.

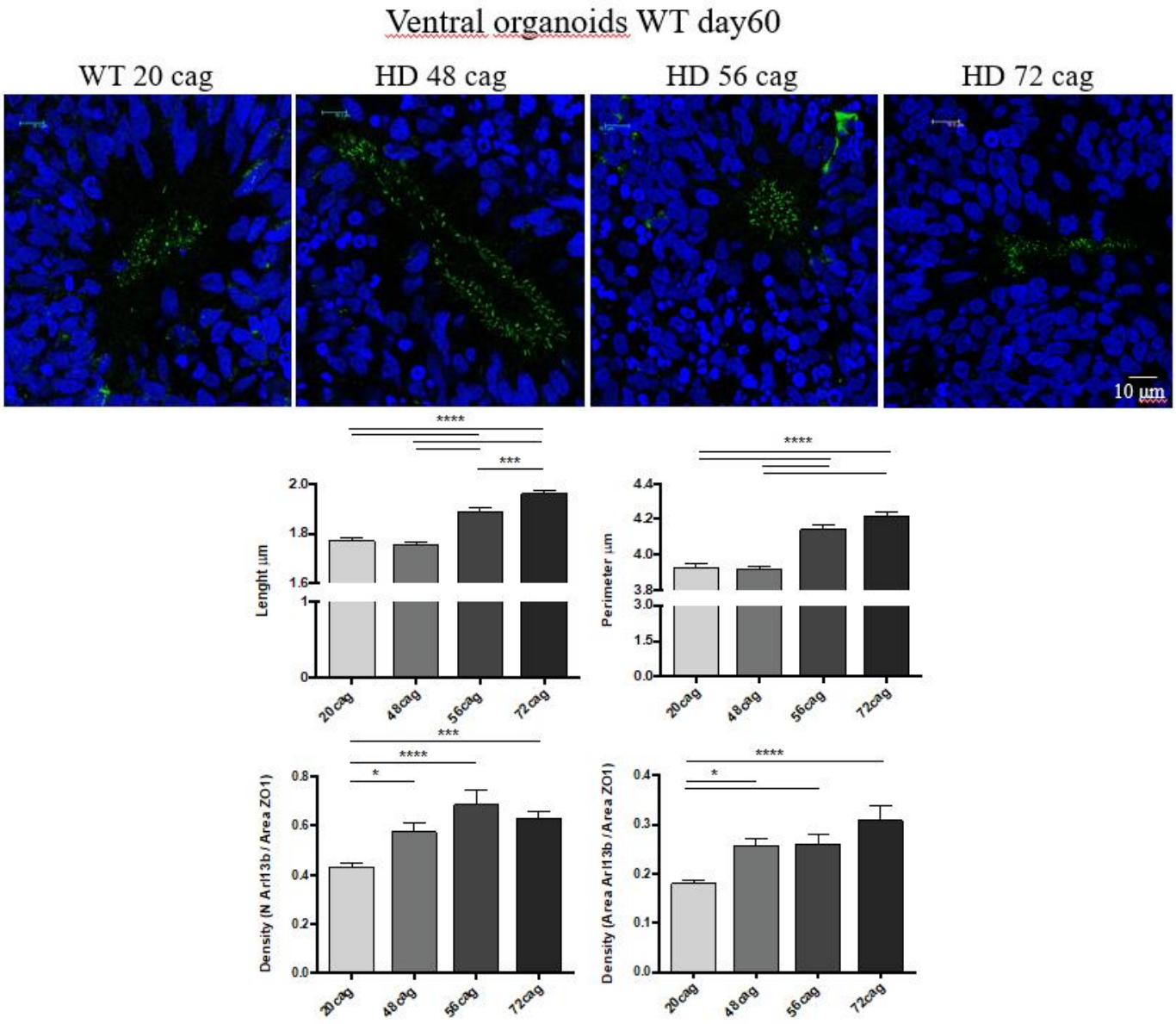

*Fig 2.25: Immunohistochemistry analyses of WT (20CAG) and HD (48, 56 and 72 CAG) ventral organoids at day 60 of differentiation for ARL13B. Scale bar 10µm. Quantification of parameters for primary cilia: length, perimeter and density, both in terms of cilia's numbers or cilia's area over the area of the lumens.(Automatic quantification with NIS software. Anova One Way, Bonferroni post test, *p<0,05; ***p<0,001; ****p<0,0001. N = 14 rosettes from 8 organoids of 2 biological replicates for each cell line.)*

In our future analyses, we planned to perform a high-throughput rt-PCR (The BioMarkTM HD System from Fluidigm) that allows reliable gene expression analyses, investigating in parallel up to 96 different transcripts in 96 different samples. For this analysis, organoids samples generated from WT lines (Rues2 parental and 20 CAG) and HD lines (48, 56 and 72CAG) have been collected from different biological replicates at days 17, 35 and 60. Among transcripts involved in neuronal differentiation and ventral specification, we will look also for components of primary cilium structure, cell cycle regulators, and SHH signaling to unravel possible DEGs between WT and HD samples. Promising transcripts candidates can be derived from our ongoing analyses of scRNAseq on three singular WT (20cag) organoids and three singular HD (56cag) organoids. In this case, we can go deeper in our investigation looking for DEGs that can be peculiar of specific cellular subpopulations (such as early progenitors or neurons) while in the Biomark system we will validate the more general ones because this bulk analysis will be performed on a larger cohort of samples compared to the scRNAseq that concerns only three organoids per genotype.

Once we find interesting transcripts involved in the regulation of primary cilium and cell cycle that show different expression levels in HD samples compared to controls, we could ideally test rescue effects by overexpressing key genes that were downregulated in HD organoids for a final mechanistic comprehension and validation. An example is the recent work on organoids (Zhang et al., 2019) in which the mutation in WDR62 gene responsible for microcephaly causes the reduction of KIF2A's basal body localization. The authors demonstrated how enhanced KIF2A expression partially rescued deficits in cilium length, progenitors' proliferation and organoids size. Another interesting candidate for our attention could be the dynein binding protein Nde1 that regulates the G1-S transition by acting on cilia and able to interact with Lis1 (other dynein partner and mutation associated with Miller-Dieker lissencephaly syndrome). Depletion of Nde1 lengthened cilia and delayed cell cycle re-entry through the G1–S transition, supporting a general link between elongated cilia and a block to S phase entry (Kim et al. 2011).

Considering the role of primary cilia in SHH signaling, to understand if HD ventral organoids present defects in dorsoventral telencephalic patterning that could depend on alterations at the level of cilia, in our future Biomark and scRNAseq analyses we will investigate transcripts involved in Shh pathways such as Gli, PTCH1, Nkx2,1, Pax6, and Gsx2. It would be fundamental to verify defects in the acquisition of ventral identity in HD samples and see if they are linked to primary cilia. Indeed, we found fewer $Gsx2^{+}$ and $Ascl1^{+}$ cells in the VZ-like-structures of HD ventral organoids compared to controls and we are currently analyzing the scRNAseq results from HD e and WT organoids also for dorsoventral markers. One possible hypothesis could be that the HD cells are less able to activate the Shh targets genes due to alteration on primary cilia. These aspects would represent the connection

between lower differentiation efficiency and cell cycle alterations both displayed in HD ventral organoids.

2.2.4 Ultrastructure and morphology of ventral organoids by Electron Microscopy

We used electron microscopy to analyze ultrastructure and neuronal morphology of ventral organoids at day 60 of differentiation. Particularly, transmission electron microscopy (TEM) specifically demonstrated the presence of excitatory and inhibitory synapses in our organoids. Figure 2.26 shows representative images of two synapses from control organoids, revealing a clearly visible presynaptic terminal with synaptic vesicles (SV) and a mitochondrial profile (M). Moreover, based on morphological features we distinguished between excitatory synapse, characterized by the presence of postsynaptic density (PSD) in the postsynaptic compartment and inhibitory symmetric synapse where the PSD is absent. In the insets we can appreciate synaptic vesicles, with a mean diameter of 20 nm, the pre- and postsynaptic membrane (harrow heads) and the synaptic cleft (asterisk).

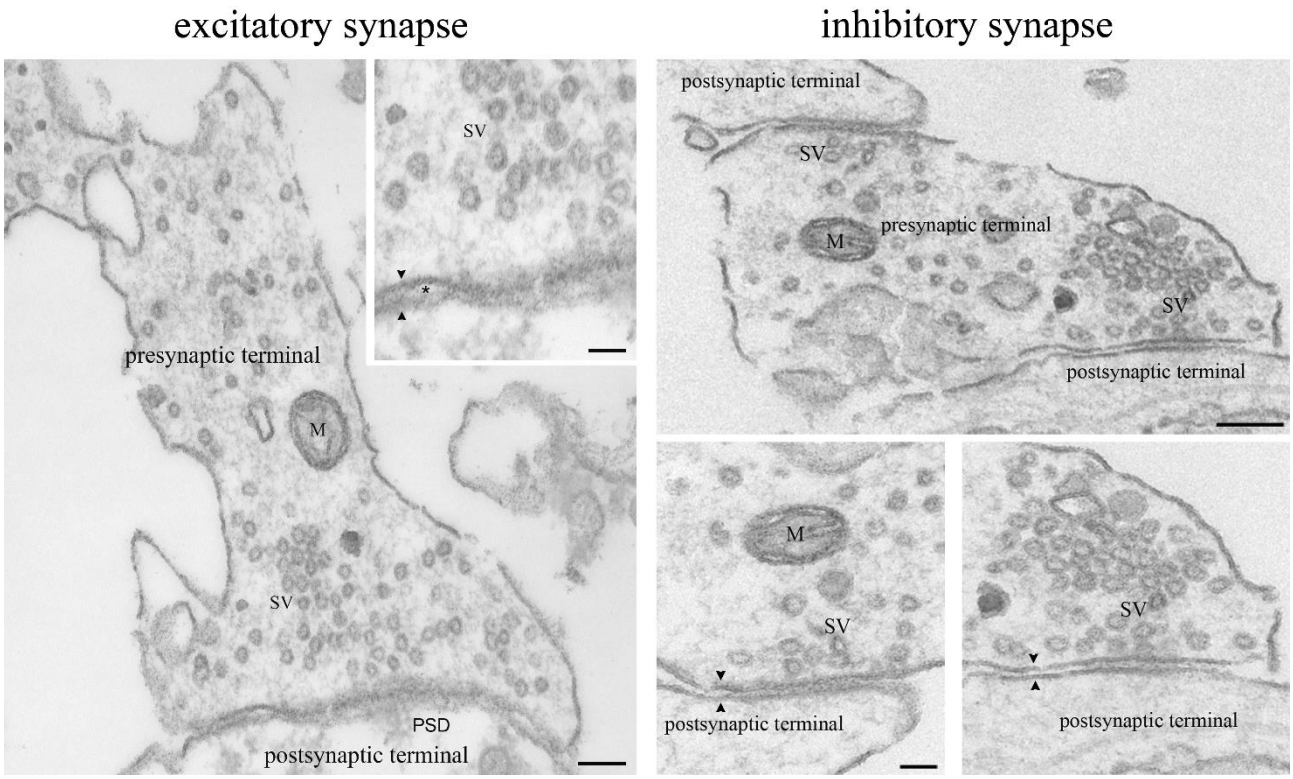

Fig 2.26: TEM images of a WT (20 CAG) organoid cross section showing mature synapses, both excitatory (left) and inhibitory (right) after 60 days of differentiation. (N=6 organoids from two independent replicates. Scale bars images at low magnification = 100 nm; scale bars insets = 50 nm).

These data indicate that 60-day-old WT ventral organoids can reach functional neuronal maturation, which was not visible in the HD counterparts. One of the simplest hypothesis may be that the HD organoids are unable to make mature synapses, but we can't completely exclude that analyzing many more samples or at many other time points, this conclusion could change.

Further analysis with scanning electron microscopy (SEM) showed the overall organoid architecture, confirming the presence of the key features of neuronal cells in both WT and HD ventral organoids. SEM revealed surface details of the whole organoid structure that is composed by two different layers: the external surface and the internal part. Inside the internal part of the organoids (Fig 2.27) it is possible to notice the cellular organization and the complexity of the cellular processes. Representative images at high magnification (right panels of Fig 2.27) display cells with neuronal-like morphology with multiple processes both in WT and HD samples. The comparison of WT and HD organoids highlights a lot of cellular debris in HD organoids, suggesting a possible higher cellular stress. Moreover, neuronal processes of HD organoids seem less defined with bigger diameter (mean diameter = 1 µm, n = 100 processes, in 2 independent organoids) respect to the corresponding WT ones (mean diameter = 600 nm, n = 100 processes, in 2 independent organoids). Taking together this data with immunohistochemistry results previously shown and the presences of synapses, we could consider these cells as neural type, but to be precise the cells with many processes that exit from the cell body could be also glial types so we won't refer to them as neurons.

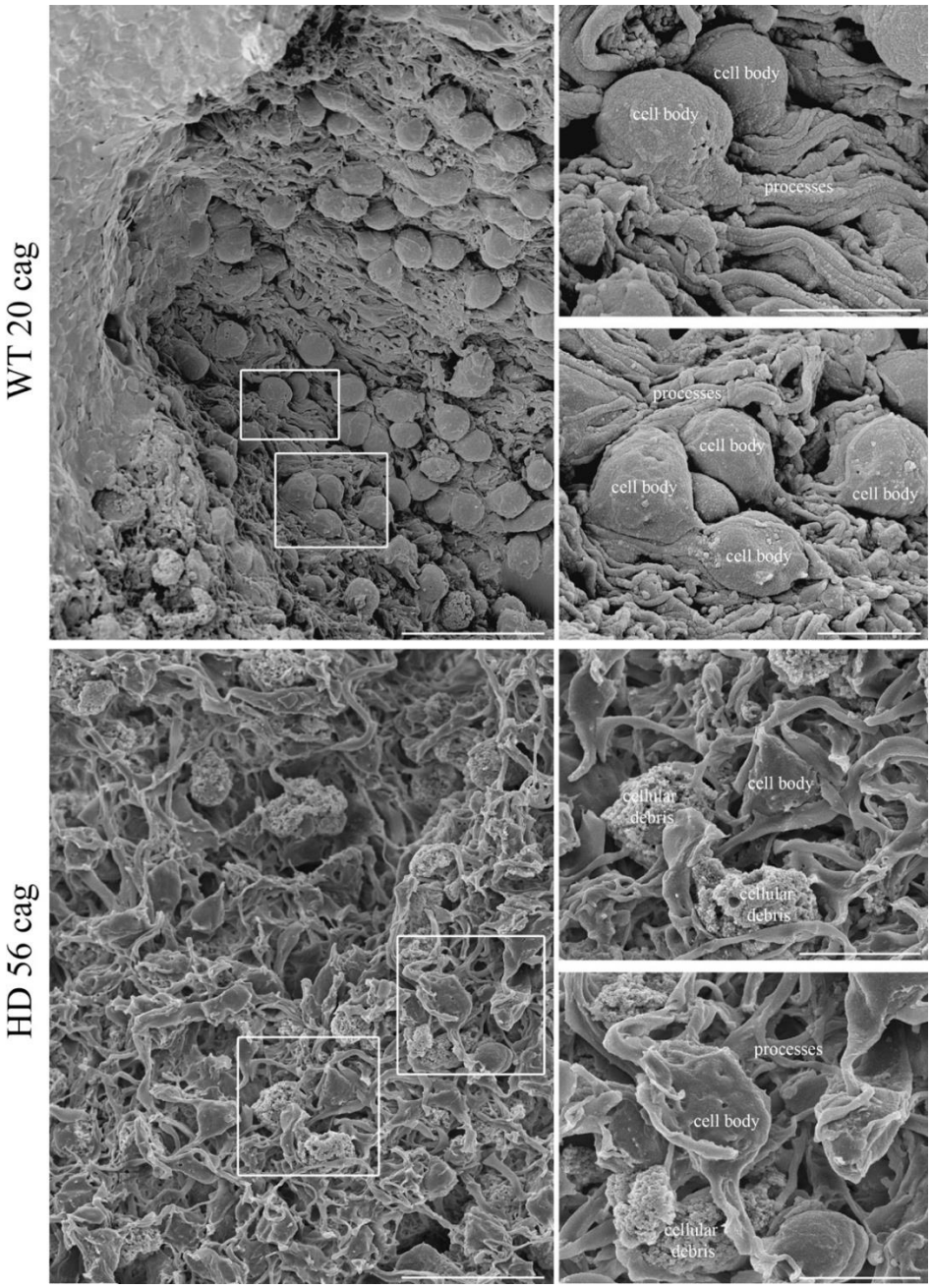

Fig 2.27: SEM images of opened WT (20CAG) and HD (56CAG) organoids at 60 day of differentiation, to reveal the internal part. (Scale bars of images at low magnification = 10 µm; scale bars of images at high magnification = 5 µm).

It is possible to visualize also the external surface of the organoids (Fig 2.28). This part usually presents cell body more flatted then the internal one, maybe due to the mechanical forces or to the contacts with the external environment. Even in this case the neuronal like morphology seems to be more define and organized in the WT organoids.

These differences could mean a diverse level of maturation or a defect in neuronal self-organization between WT and HD organoids, that will be interesting for future investigation. Our results shed light on a quite unusual way to investigate organoids morphology pointing out the advantages of electron microscopy for studies like this.

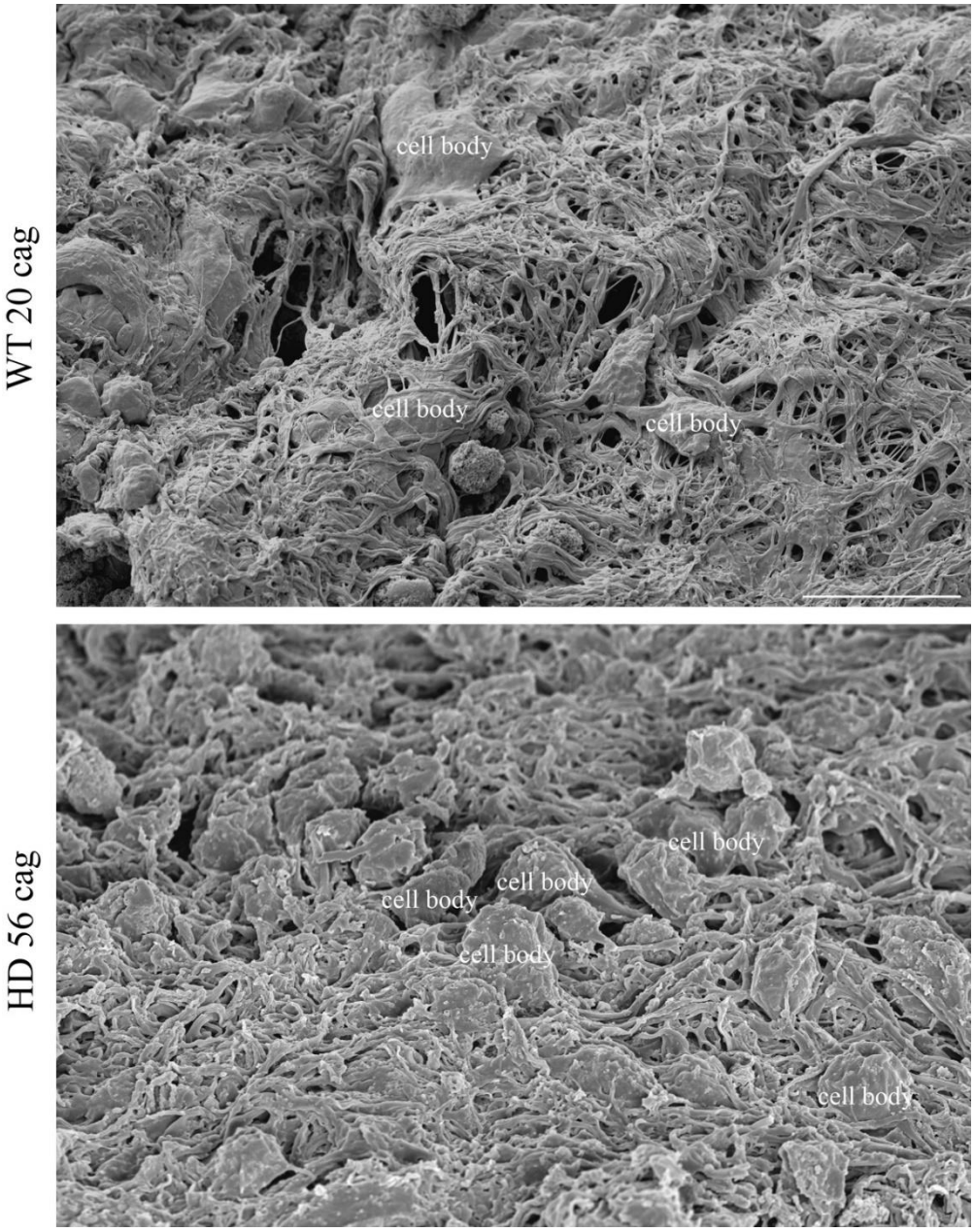

Fig 2.28: SEM images of the external surface of WT (20CAG) and HD (56CAG) organoids at 60 day of differentiation. (Scale bar = 10 µm).

2.2.5 Preliminary conclusion on HD modelling in ventral organoids

Considering the data obtained in control and HD ventral organoids, we can derive the following conclusions:

i. The radial distribution of ventral progenitors positive for GSX2 and MASH1 is absent in HD organoids. Moreover, these are less efficient in differentiation showing fewer $GSX2^+$ and $MASH1^+$ cells in each ventricular area.

ii. HD organoids present fewer proliferating structures that resemble ventricular zones (VZ) of developing embryos, both at early and mature stages.

iii. Compared to controls, the HD ventral organoids with longer CAG repeats present reduced number of $KI67^+$ cells, defects in mitotic spindle orientation with increased horizontal asymmetric divisions and higher cell cycle exit ratio at day 45.

iv. Analyses of lumens' morphology of ventral organoids shows different organizations between "empty" and "full" ones, with the majority of 56CAG characterized by "full" form. Both WT and HD organoids present primary cilia, with higher ciliogenesis in HD organoids.

v. EM analyses allowed to investigate the ultrastructure and the morphology of ventral organoids, revealing differences in neuronal architecture of WT and HD samples.

It would be crucial to understand if these phenotypes are due to different gene expression profiles in the same pathways and which transcripts are the most de-regulated suggesting a sort of phenotype-driver behavior. For these reasons, we are collecting organoids samples from different biological experiments at day 17, 35 and 60 for high throughput rt-PCR (BioMarkTM HD System). We are interested in understanding the dynamics of HD alterations distinguishing which transcripts differ in a specific moment and which are dis-regulated continuously in a sort of unidirectional way. Moreover, by using HD lines with different CAG length (48, 56 and 72CAG), we could ideally appreciate transcripts that have a CAG-length-dependency regulation.

2.3 Cellular interactions between WT and HD cells in co-culture organoids

The third aim of this project that is still under construction concerns a system to study some underlying molecular dynamics. During this thesis writing, the experiment is ongoing and the samples are under collection. The experimental plan is based on chimeric mixed organoids with co-culture of WT and HD cells to investigate cellular interactions, distributions and particularly unravel cell-autonomous or non-autonomous mechanisms in HD. Achieving this goal could improve the comprehension of pathological mechanisms in HD with clinical implications that are crucial to define efficacy therapies especially in the case of transplantations. Considering the huge atrophy of striatum in HD and the fact that there are no effective therapies to slow or arrest the progression of the disease, several neural transplant trials have been initiated with the hope of translating promising preclinical research into clinical therapies (Bachoud-Lévi et al. 2000; Peschanski, Cesaro, and Hantraye 1995; Reuter et al. 2008).

Post-mortem analyses have revealed grafted cell survival at 18 months and 6 years post-transplantation while analyses at 10 years after cell implantation demonstrated clear grafted cell degeneration with a pattern similar to the disease itself, including preferential loss of grafted striatal projection neurons (Cicchetti, Soulet, and Freeman 2011). These data suggest that some of the pathogenic events underlying neuronal death in HD are also responsible for the long-term degeneration of genetically unrelated transplanted cells. Potential causes include an insufficient amount of grafted tissue (Barker et al. 2013), allograft immunoreactivity, microglial responses to grafted cells, and cell-to-cell neurotoxicity (Cicchetti et al. 2011). This latter cause is due to non-cell-autonomous (or extrinsic) mechanisms in which the HD cells affect the WT ones in a toxic way, while the opposite scenario concerns a beneficial effect of the WT cells towards the HD ones. Our analyses will focus on the identification of these mechanisms to unravel pathways and transcripts involved in these complex regulations (Fig 2.29).

Firstly, we will compare the chimeric Co-culture organoids (composed of WT and HD cells, in orange in Fig 2.29) with the Mono-culture (entirely WT or entirely HD, in blue in Fig 2.29) looking for gene expression levels alterations. We could identify cell-autonomous (or intrinsic) aspects by transcripts that do not change between Co-cult or Mono-cult conditions (Fig 2.29 on the left) whereas non-cell-autonomous transcripts in case of a change (Fig 2.29 on the right). Furthermore, analyzing deeper these latter extrinsic mechanisms, the beneficial effects (dashed circles in the right of Fig 2.29) should be revealed by transcripts that are similar between WT cells of the Mono-cult and HD cells of the Co-cult but different from HD cells of Mono-cult. In parallel, the toxic effects (continuous circles in the

right of Fig 2.29) should be visible in transcripts that are similar between WT cells of the Co-cult and HD cells of the Mono-cult but different from WT cells of Mono-cult.

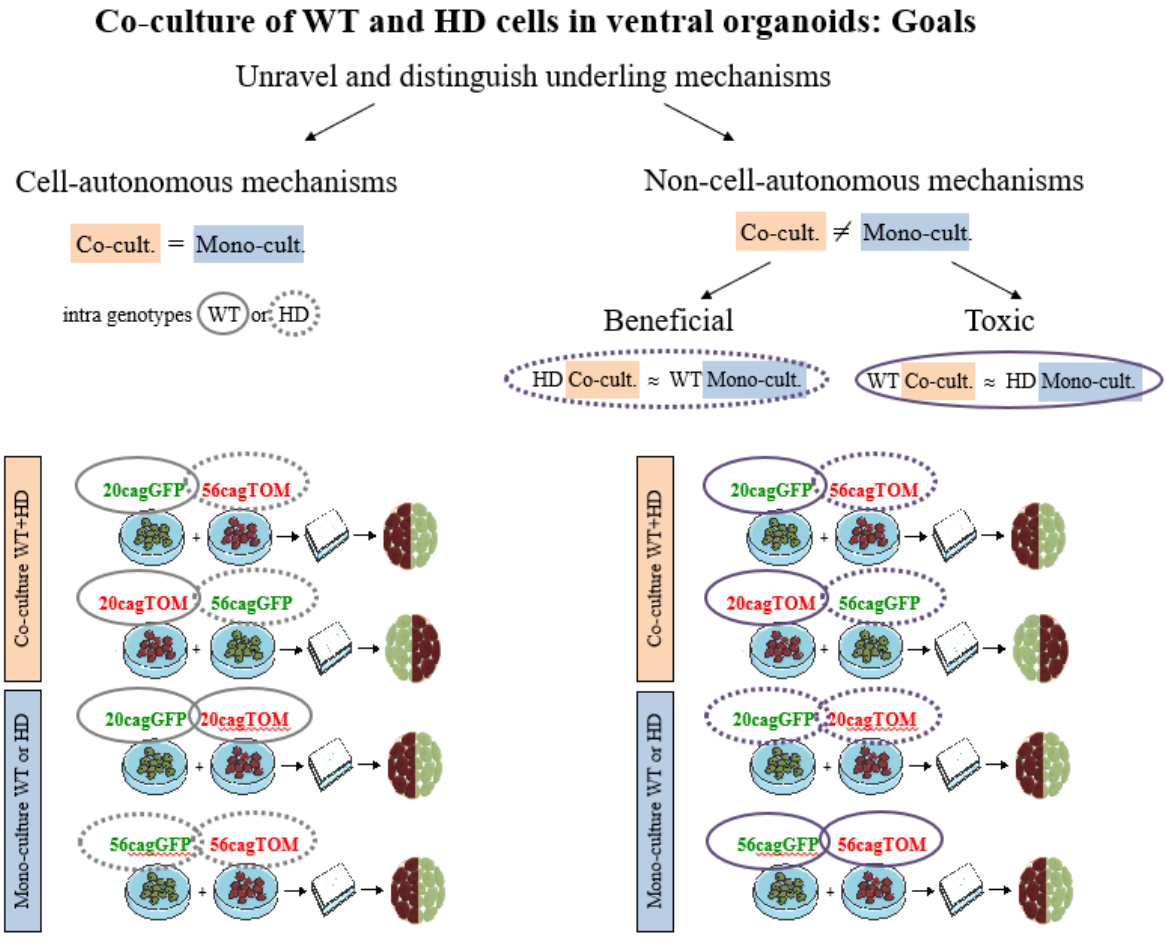

Fig 2.29: Diagram with the experimental scheme of chimeric co-culture organoids approach and relative goals. On the left are represented the cell-autonomous or intrinsic mechanisms in which there are no differences between co-culture (highlighted in orange) and mono-culture (highlighted in blue) intra genotypes (continuous circles for WT and dashed circles for HD). On the right are represented the non-cell-autonomous or extrinsic mechanisms with differences between co-culture and mono-culture. These latter ones are further subdivided into beneficial mechanisms (dashed circles) where the HD cells from co-cultures are beneficially influenced by the presence of the WT cells and toxic mechanisms (continuous circles) where the WT cells from co-cultures are negatively influenced by the presence of the HD cells.

We will investigate gene expression alterations at the level of single-cell resolution because the transcripts of interest (with a cell-autonomous or non-cell-autonomous regulation) could be specific for precise cellular subpopulations and not for the entire composition of the organoid. The power of this technology gives the possibility of focusing on different clusters of cells to specifically investigate the effect of mutant Huntingtin (mHtt) in different cases such as progenitors, intermediate

populations or neurons. Considering that Htt has multiple roles and can act at multiple levels (Saudou and Humbert 2016), it is fundamental to investigate how its effects can vary in different biological environments. Indeed, transcripts peculiar of cell cycle regulation or apical-basal polarity play pivotal roles in early progenitors thus it is plausible to appreciate an influence on their expressions within that specific population, whereas transcripts involved in synapses and firing activity are characteristic of neuronal populations and will be investigated within them. Following this approach we hope to avoid the risk of losing interesting genes that could be masked in a bulk analysis because of their specific cellular expression (Fig 2.30).

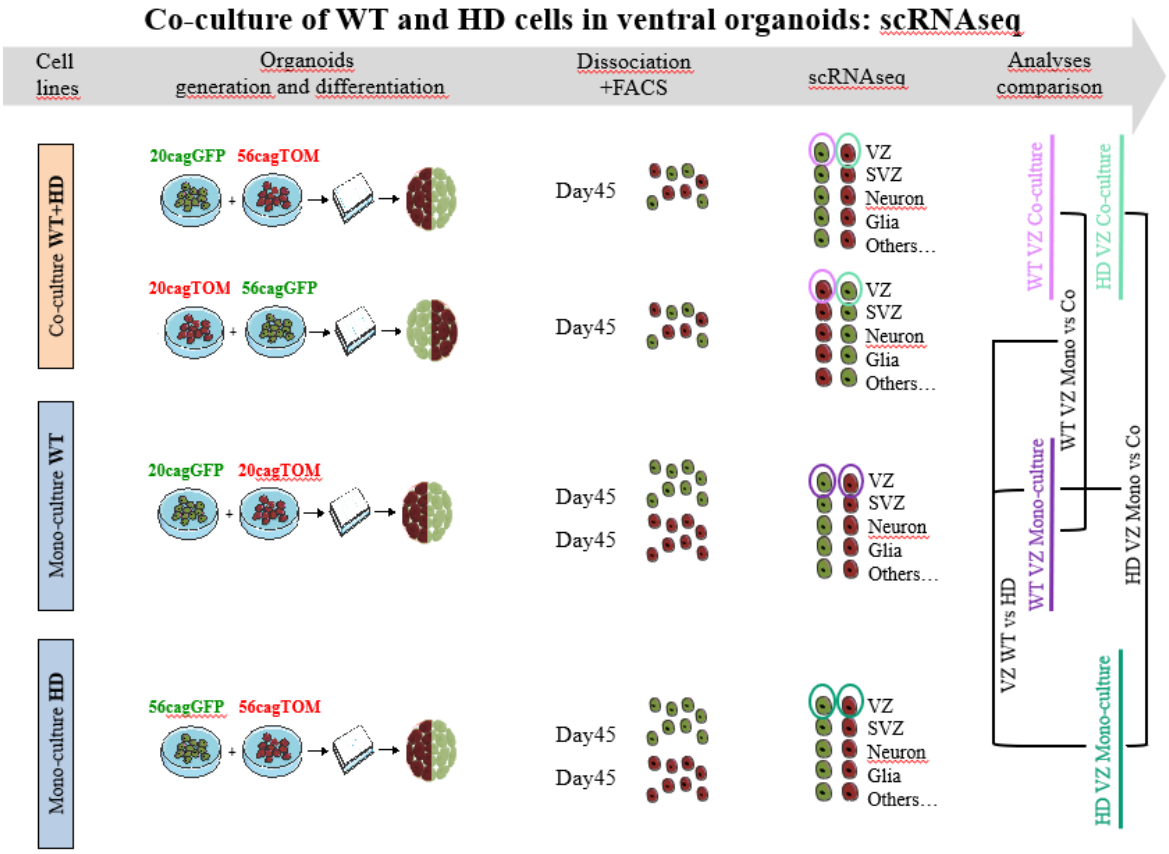

Fig 2.30: Experimental scheme of the scRNAseq of chimeric co-culture organoids. The organoids will be generating mixing the cells at pluripotent stage, differentiated until day 45, dissociated in single cell suspension and FACS sorted for GFP and TOMATO to separate WT and HD populations. The following bioinformatic analyses will define cluster of cellular subpopulation and compared them between co-culture and mono-culture looking for specific transcripts. Here is schematized the comparison for the cluster of progenitors that mimic the VZ regions of in-vivo embryos. WT cells of the VZ cluster from the co-culture (highlighted in light purple) will be compared to WT cells of the VZ cluster from the mono-culture (highlighted in dark purple) while in parallel HD cells of the VZ cluster from the co-culture (highlighted in light turquoise) will be compared to HD cells of the VZ cluster from the mono-culture (highlighted in dark turquoise). This comparison will unravel cell-autonomous versus non-cell-autonomous aspects in a single cell perspective.

In a recent article from Hayden's group (Schmidt et al. 2018), cortical and striatal neurons were co-cultured from a transgenic mouse model of HD and WT littermate mice. Reducing the ratio of cortical to striatal neurons in culture was seen to accentuate pathology, leading to a significant loss of dendritic spines and reduced viability of HD striatal neurons. Interestingly they also used chimeric (HD and WT) co-cultures to demonstrate that this instability of dendritic spines was cell-autonomous and driven by expression of the mutant HD transgene within the striatal neurons (Schmidt et al. 2018). The present study not only suggests that neurons in the striatum are exquisitely sensitive to the number of inputs that receive from cortical projection neurons, but their pathogenic response can be cell-autonomous.

On the other, side non-cell-autonomous mechanisms are also implicated when considering other cell types that interact with the affected neurons such as cortical neurons or glia cells. Indeed, toxic processes of diverse neurodegenerative diseases caused by a mutation in a gene that is often widely or ubiquitously expressed, involve damage within the non-neuronal glial cells. This mechanism seems to be non-cell-autonomous, with toxicity derived from glia as a prominent contributor that drives the disease's progression (Lobsiger and Cleveland 2007). Similar investigations led the Goldman's group to established human HD glial chimeras by neonatally engrafting immunodeficient mice with HD human glial progenitor cells (Benraiss et al. 2016), showing that mHTT glia can impart disease phenotype to normal mice and conversely, WT glia can rescue the electrophysiological and behavioral phenotypes in HD mice. These observations highlight a causal role for glia in HD and further suggest a cell-based strategy for disease amelioration through the comprehension of cell-autonomous versus non-autonomous mechanisms that characterize HD pathogenesis.

In parallel to scRNAseq we are also investigating the phenotypes previously showed in HD ventral individual organoids. A focus will be dedicated to the ability of WT and HD cells to interact in the polarized VZ-like-structures. The amount of these structures will be quantified at day 35 and 60 and the lumens' area will be measured, as it was previously done in individual organoids (reported in chapter 2.2 of this thesis) to understand if the co-culture organoids show differences compared to mono-culture. We will also look for the proportion of WT and HD cells in the structures and if this is linked to the dimension of the structures. We could postulate non-cell-autonomous beneficial mechanisms in which the presence of WT cells is helpful for the HD component's organization. Concerning the cell cycle regulation, we will investigate the number of $Ki67^+$ cells in HD and WT in the co-culture compared with the mono-culture and the area of the organoids. At the moment, a plausible hypothesis is that the phenotypes more related to polarity and distribution will be extrinsically affected in the co-culture while the phenotypes in the cell cycle will present a cell-autonomous regulation. It will be interesting, in particular in light of the transplantation data discussed

above, to study also apoptosis and cell death to investigate if these aspects could contribute to the reduced growth of HD organoids and if they are cell-autonomously or non-cell-autonomously regulated.

2.3.1 Co-culture ventral organoids of WT and HD cells

The isogenic Rues2 lines were infected with lentivirus that promotes the expression of GFP or TOMATO under the pCAG promoter with puromycin selection. To avoid any bias, we generated the two combinations of colours for each line: WT 20CAG GFP and TOMATO, HD 48CAG GFP and TOMATO and HD 56CAG GFP and TOMATO.

Our idea is to first focus on the co-culture of 20CAG and 56CAG, as these two cell lines were well characterized and showed a broader difference in each phenotype described in this thesis. We have to mix the cells at the time of aggregation and growth the co-culture organoids until day45. We will dissociate the organoids in a single cell suspension for following FACS sorting and RNAseq to compare the different cellular combinations.

In our pilot experiment, organoids were monitored to check the presence of the right combination of cells and to investigate the competition between the two cell lines. The proportion between these lines needs to be equal at least at initial stages because it could also occur that one line takes over the other one, due to different growth rate or diverse levels of apoptosis. An example at day 15 is shown in Fig 2.31 in which it is possible to appreciate the GFP and TOMATO expression in the organoids by both live image and immunohistochemistry (Fig 2.31).

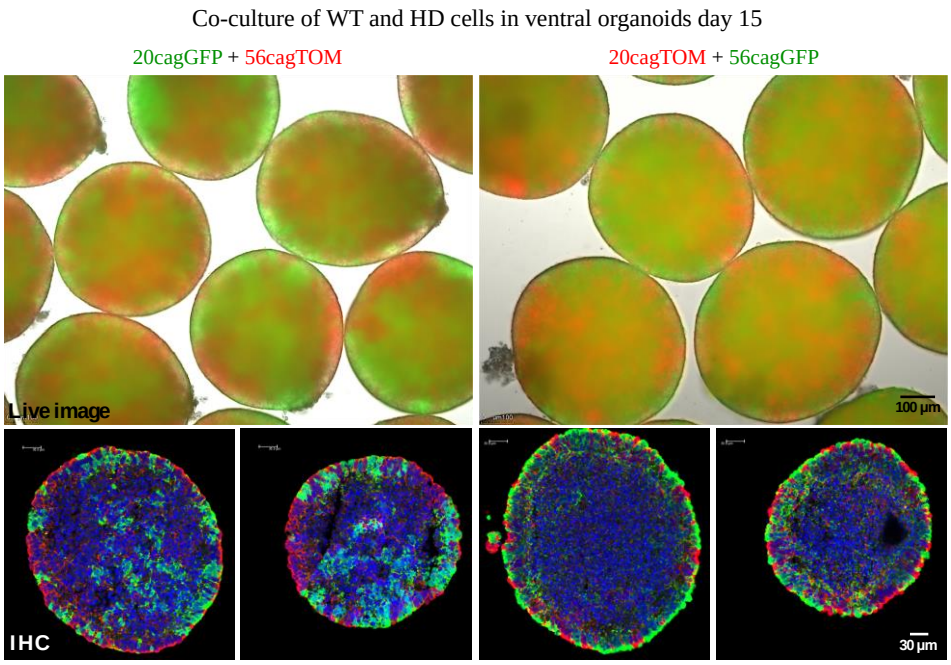

Fig 2.31: Live image on the top and immunohistochemistry on the bottom of co-culture WT and HD cells inside ventral organoids at day 15 for the two colours combinations: 20CAGGFP+56CAGTOMATO on the left and 20CAGTOMATO+56CAGGFP on the right. Scale bar 100µm on the top and 30µm on the bottom.

2.3.2 FACS sorting of GFP and TOMATO cells and clearing approach

We are setting different types of analyses for the co-culture, including FACS sorting after dissociation in single cells suspension for further RNAseq analyses and clearing techniques to investigate cellular distribution.

We tested the FACS sorting strategy in a pilot experiment to verify the feasibility and set the conditions. Moreover, it is crucial to check the viability of the cells after dissociation and sorting, because they may suffer during this process (described in paragraph 4.8.2). Our test was performed on ventral organoids at day 30 of differentiation with the following combination: mono-culture WT 20CAGGFP; mono-culture HD 56CAGTOM and co-culture WT-HD 20CAGGFP-56CAGTOM. The dissociated cells were FACS sorted, allowing the exclusion of dead cells or duplets based on their morphology and the isolation of WT and HD cells based on their fluorescence. After the sorting, we checked the cell viability with Trypan blue and Countess Automated Cell Counter obtaining around 93-99% of live sorted cells. This preliminary result is encouraging for our plan of future experiments with the complete set of fluorescence combinations.

To investigate cellular distribution and connections between control and HD cell lines, 3D imaging with volume reconstruction of the organoids will be helpful. This technic is possible by clearing the organoid through protocols such as X-clarity, iDiSCO and SWITCH with subsequent acquisition at light-sheet microscopy or 2-photon microscopy (Birey et al. 2017; Renner et al. 2017). In collaboration with San Raffaele Alembic facility, we are testing the X-clarity approach. Although the protocol still needs adjustments to preserve the entire organoid volume, we were able to visualize both GFP and TOMATO endogenous signals at the 2-photon microscopy (Fig 2.32). It will be interesting to see if control and HD cells distribute equally or segregate between each other and if there are differences in their morphology.

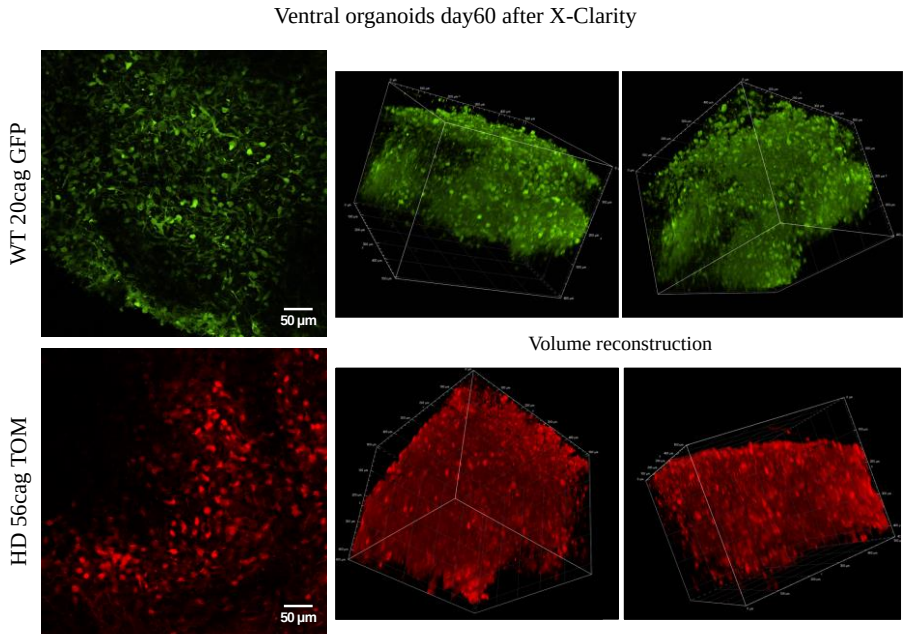

Fig 2.32: Two-photon acquisition at Alembic after X-clarity of WT (20CAG-GFP) and HD (56CAG-TOMATO) ventral organoids at day 60 of differentiation.

3. Conclusions and future perspectives

In this project, we used the organoids approach to mimic the human telencephalon development and create a three-dimensional model of Huntington's disease *in vitro*. We generated a new unpublished protocol for the generation of ventral telencephalic organoids. During differentiation, these organoids displayed markers of neural induction at early time points and acquired a GABAergic identity at later time points, resembling the cellular composition of ventral telencephalic nuclei. These data are supported by single cell RNAseq analyses on three individual organoids that highlight the presence of ventral neuronal sub-populations with no contamination from neurons of dorsal identity. A variety of protocols have been published to model many brain regions, but only few for ventral telencephalon (Bagley et al. 2017; Birey et al. 2017; Xiang et al. 2017) that mainly focused on fusion between two organoids of cortical and MGE identity to study interneurons migration. Our protocol will be useful to recapitulate human ventral telencephalon development in a 3D system.

By comparing our organoids with dorsal ones, it was possible to verify the correct acquisition of ventral and dorsal identity. In both protocols, the neural progenitors self-organized in concentric areas around the lumens of VZ-like structures mimicking the radial distribution of LGE and the multi-layers organisation of the cortex. Interestingly these structures are present in both types of organoids in equal number but with different thickness suggesting intrinsic diversity in the regulation of ventral and dorsal progenitors' expansion.

The comparison of control and HD ventral organoids derived from isogenic cell lines revealed defects in the self-organization of the VZ-like structures. Control organoids present typical radial distributions of $GSX2^+$ and $MASH1^+$ progenitors that are absent in HD counterparts. Moreover, these latter show a 50% reduction in the number of VZ-like structures, both at early and mature stages. To derive more information about the organoids' morphology, we analyzed them with electron microscopy revealing differences in the organization of neural architecture of HD samples, pointing out the advantages of this technic.

As VZ-like structures are populated by progenitors in active cell cycle, we investigate its regulation founding some defects in HD organoids. They present fewer $KI67^+$ cells, increased horizontal asymmetric divisions and higher cell cycle exit ratio at day 45. These data on cell cycle are in line with the reduced growth of HD organoids and could explain the decreased amount of VZ-like structures. Interestingly the absence of HTT or the presence of mHTT affects the spindle orientation

leading to abnormal neurogenesis with more asymmetric divisions and reduced progenitors' amplification (Godin et al. 2010; Lopes et al. 2016; Molina-Calavita et al. 2014; Ruzo et al. 2018). Future analyses will focus on the consequences of mitotic spindle misorientation in our samples; for the moment we can only speculate that HD organoids may present mature neurons earlier or reduced number of progenitors or even higher levels of cell death.

HTT is also known to play critical roles in polarity (Conforti et al. 2018; Ruzo et al. 2018a; Lo Sardo et al. 2012) which is fundamental for the VZ-like structures' formation. The analysis on lumens' morphology show different organization between "empty" and "full" ones, with the majority of 56CAG characterized by "full" form. This cell line is also characterized by VZ structures with decreased lumen dimensions and bigger primary cilia with higher ciliary density. These results pointed out abnormalities in polarity's organization in HD samples. Considering the role of primary cilium both in mitotic spindle orientation and in SHH signaling, future perspectives include deeper investigation of cilia's structure components and dorsoventral telencephalic patterning.

This 3D model has therefore provided a way to better understand the potential neurodevelopment aspects of HD and may provide a platform for disease modelling applications. We plan to investigate the gene expression profiles in organoid samples from different biological experiments at day 17, 35 and 60. By using HD lines with different CAG length (48, 56 and 72CAG), we could ideally appreciate transcripts with a CAG-length-depend regulation. Moreover, preliminary analysis of scRNAseq on HD samples are currently ongoing to understand if there are misrepresented sub-populations when compared to controls. For example, in HD organoids compared to WT we could find an unbalanced composition between progenitors and mature neurons or the presence of mis-differentiated cells towards unwanted identities. A further level of investigation will aim to investigate cellular interactions between HD and control cells and to discriminate cell-autonomous versus non-cell autonomous defects within co-culture organoids. We hope to reveal some new interesting pathways involved in the pathogenesis of HD and relevant for clinical purposes in regenerative medicine.

4. Materials and methods

4.1 Culture of human pluripotent stem cells (hPSCs)

The Rues2 lines used in this thesis were regularly tested and maintained mycoplasma-free. Karyotype for each cell line was monitored every 3 months during the passages by Q-banding analyses (by ISENET group). Cells are kept in monolayer on 120-180 ug/ml Geltrex (Thermo Fisher Scientific) coated cell culture dishes and in feeder-free conditions in mTeSR1 medium (StemCell Technology). Once a week, when cells are at 80% confluence, hESC colonies are splitted with a ratio of around 1:10 onto new plates using ReleSR (StemCell Technology) dissociation without centrifugation and replating them in mTeSR1. Cells are kept in culture for 3 months and then a new batch is thawed.
Cells are freezed in cold KSR (Knockout serum, Life technologies) containing 10% DMSO, 0,1% RI (Rock Inhibitor) in 1ml freezing vials at -80°C using the freezing boxes (CoolCell, Bicision). The following day the vials are moved to -150 °C. For thawing the vials are kept in thermal bath at 37 °C few minutes and then the cells are centrifugated and quickly resuspended in mTeSR1 with RI for plating. The day after the media is changed with normal mTeSR1.

4.2 Generation of ventral organoids

This protocol was developed in the lab, taking inspiration from the protocol published in 2013 (Delli Carri et al. 2013) for adherent cell culture to get MSN. The mechanical steps are similar to the cortical organoids protocol, described later, but the media are different. In particular the neural induction in achieved with dual SMAD inhibition strategy by SB and LDN (Chambers et al., 2009) while the ventral identity is pushed by SHH (Gaiano et al. 1999; Rallu et al. 2002) and inhibiting WNT with Dkk1 (Backman et al. 2005; Suzuki and Vanderhaeghen 2015).

Day 0

hESC are kept in a 6cm dish until they are 70-80% confluent. The cells should be no more than 70-80% confluent to form good aggregates, otherwise they will fail to form homogeneous aggregates. The cells are detached with 1ml of Accutase for 4-5’ until round cells are visible. The detached cells are resuspended with 3volumes of mTeSR and transfer to a 15-ml conical tube. The dish is washed with 1ml of mTeSR to recover all the cells. The cells are centrifugated at 1000rmp for 3’ at room temperature and then resuspended in 3ml of Neural Induction Medium. Using a multichannel the cells are seeded at a $2x10^4$ cells/well concentration in a V-bottom non-treated 96well plate with 150 µl/well of Neural Induction Medium. The 96well plate with the cells is centrifugated at 800rmp for 1’ at room

temperature. At the microscope is possible to check the presence of a dense aggregate of single cells at the bottom of the plate. The 96well plate is placed back in the incubator.

Day 3

At day3 the cells receive a partial change of the medium by taking out 50µl of Neural Induction Medium and adding 50µl of fresh medium without Rock inhibitor.

Day 6

Around day 6 the aggregates can be transferred to a 6cm dish, in the fresh medium (6ml) containing Shh and Dkk1 at a final concentration of 200ng/ml and 100ng/ml, respectively (Patterning Medium). A cut 1000-ml pipette tip is used, being careful not to disrupt the aggregate. The dish is then placed back into the 37° incubator, on an orbital shaker, at 70rpm.

From day 8 to day 15

The aggregates in the 6cm dish are fed every other day by gently pipetting approximately half of the medium (2 o 3 ml) without disturbing the aggregates. An equal volume of fresh Patterning Medium is added to the dish.

Day 15-17

Around day15-17 the aggregates should be ready for embedding in Matrigel. The Matrigel is thawed on ice at 4° for 1-2h. Using the dissecting microscope, under biological safety cabinet, 12 aggregates with a similar morphology and dimensions are selected and placed one by one using a cut 200µl pipette tip (set at 25µl) on an upside down 6cm dish lid. With a 20µl pipette tip is possible to remove the medium around each aggregate. Excess medium is quickly removed to avoid the tissues dry out and then 25µl of liquid Matrigel are immediately added to each aggregate. Using a 10µl pipette tip, the aggregate is positioned in the centre of the Matrigel droplet. It is important to use a pre-wetted 10µl pipette tip, to avoid the aggregates sticking to the tip. After waiting 3-5 min, the lid with the aggregates is carefully flipped and put on a 6cm dish. The dish is placed back into the 37° incubator and incubated for about 15’ to allow the Matrigel to polymerize. Before proceeding, it is possible to test with a 10µl pipette tip if the Matrigel is solidified. Using a 1000µl pipette tip, the Matrigel/aggregates are flushed forcing them to deposit in the dish plate with 6ml of Neuronal Differentiation Medium. The dish is placed back on the orbital shaker into the 37° incubator.

From day 18 to day 60

Half of the medium is changed two days a week and the dish is kept on the orbital shaker at 37°.

Media composition:

Neuronal Induction Medium

- DMEM/F12:
- N2 supplement 1:100
- B27-RA supplement 1:50
- NEAA 1:100
- Heparin: 1mg/ml
- Insulin: 5ng/ml
- SB: 10µM
- LDN: 500nm
- Rock Inhibitor: 10µM (only for 48 hours)

Patterning Medium (From day5 to day15)

- DMEM/F12
- N2 supplement 1:100
- B27-RA supplement 1:50
- NEAA 1:100
- Heparin: 1µg/ml
- Insulin: 5ng/ml
- SB: 10µM
- LDN: 500nM
- Shh 200ng/ml
- Dkk1 100ng/ml

Neuronal Differentiation Medium

- DMEM/F12:Neurobasal 1:1
- N2 supplement 1:100
- B27 supplement (complete) 1:50
- NEAA: 1:100
- Insulin: 5ng/ml
- Laminin: 200ng/ml
- BDNF: 10ng/ml

- 2-Mercaptoethanol: 50µM
- Ascorbic acid: 100µM

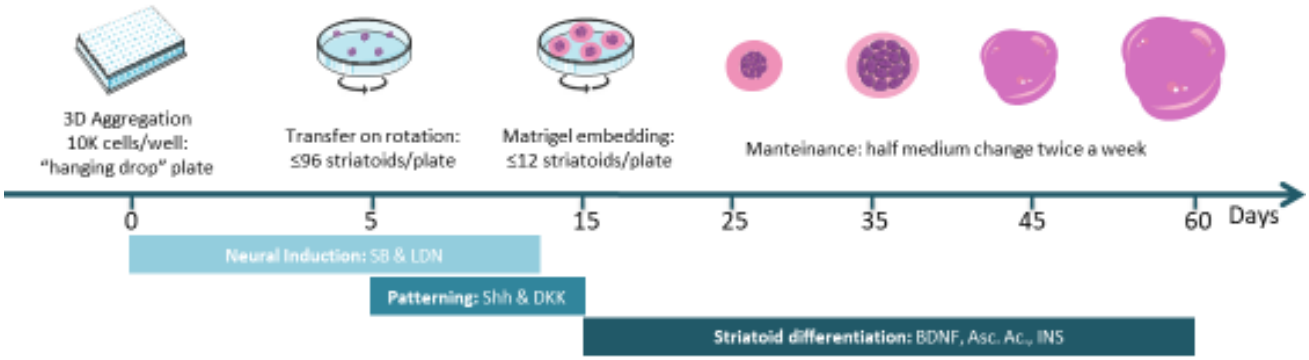

Fig 4.1: Scheme of the protocol for ventral organoids

4.3 Generation of dorsal organoids

This protocol is an adaptation from Lancaster's original one (Lancaster et al. 2013; Lancaster and Knoblich 2014). The major differences consist in using hanging drop plates, putting the aggregates on rotation since day5 and maintaining them in rotation after matrigel embedding instead of bioreactors while the media are the same from the published protocols. These little modifications make possible to obtain cortical organoids quite similar without bioreactors.

Day 0

hESC are kept in a 6cm dish until they are 70-80% confluent. The cells should be no more than 70-80% confluent to form good aggregates, otherwise they will fail to form homogeneous aggregates. The cells are detached with 1ml of Accutase for 4-5' until round cells are visible. The detached cells are resuspended with 3volumes of mTeSR and transfer to a 15-ml conical tube. The dish is washed with 1ml of mTeSR to recover all the cells. The cells are centrifugated at 1000rmp for 3' at room temperature and then resuspended in 4ml of Aggregation medium. Cells are counted to have $1x10^4$ cells/well of a 96well plate. Using a multichannel the cells are seeded using the $1x10^4$ cells/well concentration in a Perfecta3D hanging drop plate (HDP1096, 3D Biomatrix) in 35µl/well of Aggregation medium. The 96well hanging drop plate is then placed back in the incubator.

Day 1

5µl of Aggregation medium are added to each well of the plate.

Day 3

The bottom tray is removed from the Hanging Drop Plate assembly and the spheroid-containing Hanging Drop Plate is placed on top of a receiving 15cm dish plate, already containing about 10ml of Aggregation medium. 50µL of medium are slowly dispensed through the access holes to transfer the hanging drops to the receiving 15cm dish plate. With a 10ml pipette it is possible to transfer the spheroids to a 15ml conical tube, allowing the EBs to deposit at the bottom of the conical tube. The EBs are then gently resuspended in 6ml of Aggregation medium and the dish is placed back into the 37° incubator, on an orbital shaker, at 70rpm.

Day 6-8

When the EBs are about 500-600µm in diameter it is time to change the Aggregation medium with Neural Induction Medium. Until day 13-16 the aggregates need partial medium change by gently pipetting approximately half of the medium without disturbing the aggregates and adding an equal volume of Neural Induction medium.

Day 13-16

Between day 13-16 the EBs should be brighter around the outside, indicating neuroectodermal differentiation, and ready to be transferred into Matrigel droplets. The Matrigel is thawed on ice at 4° for 1-2h. Using the dissecting microscope, under biological safety cabinet, 12 aggregates with a similar morphology and dimensions are selected and placed one by one using a cut 200µl pipette tip (set at 25µl) on an upside down 6cm dish lid. With a 20µl pipette tip is possible to remove the medium around each aggregate. Excess medium is quickly removed to avoid the tissues dry out and then 25µl of liquid Matrigel are immediately added to each aggregate. Using a 10µl pipette tip, the aggregate is positioned in the centre of the Matrigel droplet. It is important to use a pre-wetted 10µl pipette tip, to avoid the aggregates sticking to the tip. After waiting 3-5 min, the lid with the aggregates is carefully flipped and put on a 6cm dish. The dish is placed back into the 37° incubator and incubated for about 15’ to allow the Matrigel to polymerize. Before proceeding, it is possible to test with a 10µl pipette tip if the Matrigel is solidified. Using a 1000µl pipette tip, the Matrigel/aggregates are flushed forcing them to deposit in the dish plate with 6ml of Neuronal Differentiation medium. The dish is placed back on the orbital shaker into the 37° incubator.

From day 16 to day 60

Half of the medium is changed two days a week and the dish is kept on the orbital shaker at 37°.

From day 30 until the end of differentiation, the Neuronal Differentiation medium is changed in Final Differentiation medium by substitution of B27-RA with B27 compete to support neuronal differentiation and maturation.

Media composition:

Aggregation medium

- DMEM/F12
- KSR 20%
- NEAA 1:100
- 2-Mercaptoethanol: 100nM
- FGF2: 4ng/ml
- Rock Inhibitor: 50µM (only for 48 hours)

Neural induction medium

- DMEM/F12
- N2 supplement: 1:100
- NEAA 1:100
- Heparin: 1µg/ml

Neuronal Differentiation medium

- DMEM/F12:Neurobasal 1:1
- N2 supplement: 1:200
- NEAA 1:200
- Insulin: 2,5ng/ml
- B27-RA supplement 1:100
- 2-Mercaptoethanol: 100nM

Final Differentiation medium

- DMEM/F12:Neurobasal 1:1
- N2 supplement: 1:200
- NEAA 1:200
- Insulin: 2,5ng/ml

- B27 supplement (complete) 1:100
- 2-Mercaptoethanol: 100nM

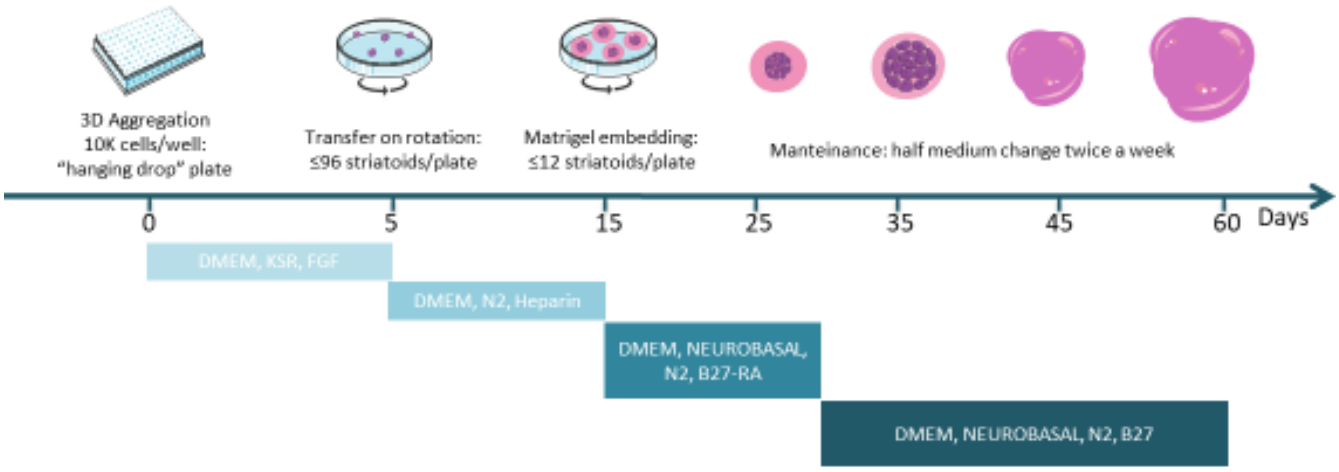

Fig 4.2: Scheme of the protocol for cortical organoids

4.4 EdU Click-iT assay

We took advantage from EdU assay to study interkinetic nuclear migration (INM) and cell cycle exit ratio in our ventral organoids from control and HD cell lines.

Usually proliferation-detection methods are based on the incorporation and measurement of nucleoside analogues in newly synthesized DNA, with bromodeoxyuridine (BrdU). BrdU-labeled DNA is visualized using anti-BrdU antibodies following DNA denaturation by harsh methods (HCl, heat, or enzymes) to expose the BrdU molecules. The harsh treatment can adversely effect sample integrity and quality, by destroying cell morphology or antigen recognition sites which makes co-staining with other antibodies challenging. EdU (5-ethynyl-2'-deoxyuridine), a nucleoside analogous of thymidine, is incorporated into DNA during active DNA synthesis and is fluorescently labelled with a bright, photostable Alexa Fluor dye. With Click-iT EdU, mild fixation and detergent permeabilization is sufficient for the detection reagent to gain access to the DNA, giving useful alternative to BrdU.

At day 35 EdU (10uM, Life Technologies) is added through a partial change of the medium for ventral organoids. EdU medium is kept for 90 minutes in rotation, after which organoids are collected immediately and fixed, or washed with PBS and fed with fresh medium until later collection for other time points. EdU click-it assay is then performed on fixed organoids sections following manufacture's

instruction (Invitrogen) and immuno-staining. Briefly the detection is based on a click reaction, a copper-catalyzed covalent reaction between an azide and an alkyne. In this application, the EdU contains the alkyne and the Alexa Fluor® dye contains the azide.

4.5 Immunohistochemistry and imaging

Organoids were fixed in 4% (vol/vol) paraformaldehyde for 1h at 4 °C. They were washed 3 times in PBS and transferred in a 15% sucrose solution at 4 °C for 24h and 30% sucrose solution for the following 24h. Next, organoids were transferred into Tissue-Tek OCT compound, frozen immediately on dry ice and stored at −80 °C. Sections of 12/15-μm thickness were then generated using a cryostat. Cryo-sections were left overnight at room temperature to dry and then washed with PBS, permeabilized with 0.5% Triton X-100 in PBS for 15 min and blocked with 10% NGS, 0.1% Triton X-100 in PBS at room temperature for 1h. Primary antibodies used (see below) were diluited in solution containing 5% NGS and 0.1% Triton X-100 in PBS at 4 °C overnight. The following day sections were washed three times in PBS at room temperature. Secondary antibodies conjugated to Alexa fluorophores 488, 568 or 647 (Molecular Probe, Life Technologies) were used 1:500 in solution containing 5% NGS and 0.1% Triton X-100 in PBS at room temperature for 1 h mixed with Hoechst 33258 (5 μg/ml; Life Technologies) to visualize nuclei. Then the sections were washed two times in 0.1% Triton X-100 in PBS, once in PBS and finally mounted with Dako Glycergel (Aqueous Mounting Medium, Agilent) at room temperature overnight. The following day the section were dry enough to be visualize at the microscope and then conserved at 4 °C.

Images were acquired with either fluorescent widefield microscope (Leica AF6000) or confocal microscope (Leica SP5) and analysed with software for imaging (Fiji , CellProfiler v.2,2 and NIS-Elements AR v5.11).

Primary antibodies and concentrations were as follows:
ARL13B (Rabbit, 1:500, Abcam), ASCL1 (mouse, 1:500; Becton Dickinson), BLBP (rabbit, 1:500; Millipore), βIII-TUBULIN (rabbit, 1:1,000; Promega), CASPASE3 (Rabbit, 1:200, Cell Signalling), CTIP2(rat, 1:500; Abcam), DARPP32 (rabbit, 1:200; Abcam), EBF1 (mouse, 1:1000; Santa Cruz), FOXG1 (rabbit, 1:500; StemCulture), GAD67 (Mouse, 1:1000, Millipore), GFP (chicken, 1:1000, Abcam), GSX2 (rabbit, 1:200, GeneTex), ISLT1T1/2 (mouse, 1:1,000; Hybridoma Bank), KI67 (rabbit, 1:500; Abcam), MAP2a/B (mouse, 1:500; Becton Dickinson), N-CADHERIN (1:800; Becton Dickinson), NESTIN (mouse, 1:300; Millipore), NKX2.1/TTF1 (rabbit, 1:200; Abcam), PALS1 (rabbit, 1:500; Santa Cruz), PAX6 (rabbit, 1:300; Covance), PERICENTRIN (Rabbit, 1:1000, Abcam), PH3 (Mouse, 1:1000, Cell Signaling), RFP (Rabbit, 1:500, MBL), SOX2 (rabbit, 1:200;

Millipore), TBR1 (rabbit, 1:1,000; Abcam), TBR2 (rabbit, 1:100; Abcam), VIMENTIN (mouse, 1:100; Hybridoma Bank), p-VIMENTIN (Mouse, 1:1000, MBL international), ZO-1 (Mouse, 1:100, Thermo Scientific),

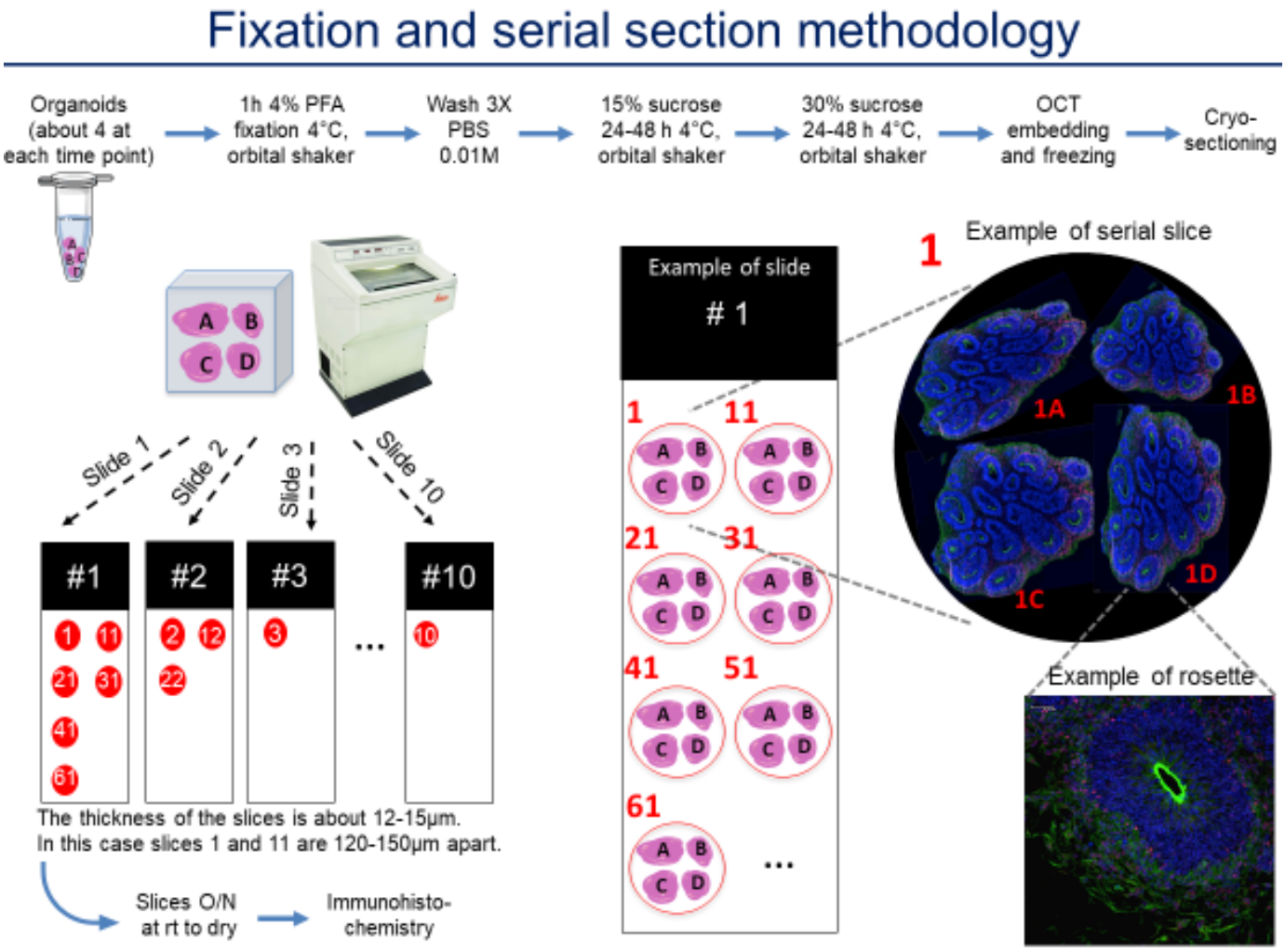

Fig 4.3: Scheme of the protocol used for fixation, freezing and cryo-sectioning the organoids sample before proceeding with IHC.

4.6 Quantitative assessment of organoids:

Overall size of organoids is measured in pictures collected at 4X bright field microscope. The total organoid area in µm2 is determined by using Fiji ImageJ software.

The masks of concentric areas for VZ like structures is drawn with ImageJ in a semi-automatic approach through a manual drawing of the lumen‘s perimeter and then an automatic enlargement of this mask in three or four concentric areas with similar path.
For quantification of nuclear markers (such as GSX2, ASCL1, KI67, PH3; EdU) the cells are counted with CellProfiler platform or manually with Fiji ImageJ and percentage quantification is based on positive cells over total nuclei marked by Hoechst staining.
Mitotic spindle is manually drawn and quantified with Fiji ImageJ software in VZ like structures by p-VIMENTIN (to identify dividing cells) and PERICENTRIN (a centrosome marker used to visualize the plane of dividing cells). The orientation of the mitotic spindle is investigated in relation to the prospective ventricular surface in at least 40-50 VZ like structures per cell lines. Quantification of primary cilia, based on ARL13B staining, is done with NIS element. The confocal images taken at 63x with 4zoom factor are deconvolved with NIS to increase the resolution and then quantified by the general analyses tool following a pipeline set on parameters such as background, dimensions and circularity.
Statistics and graph are done with PRISM software v. 6. Data derived from different independent biological replicates are represented with mean and SEM.

4.7 Electron microscopy

The samples were processed and acquired for electron microscopy by Elena Vezzoli and Andrea Falqui. The acquisitions were taken in the laboratory of Prof. Falqui at the KAUST (King Abdullah University of Science and Technology) Institute.

4.7.1 TEM sample preparation

Organoids were fixed using 2.5% glutaraldehyde (Electron Microscopy Sciences, catalog 16220), and 2% paraformaldehyde (Electron Microscopy Sciences, catalog 19200) as fixatives, both in sodium cacodylate buffer 0.15 M (pH 7.4) (Electron Microscopy Sciences, catalog 12300). organoids were manually cut in small pieces (1 mm^3) and post-fixed for an additional 24 hours at 4°C. Samples were then washed with 0.1 M cold sodium cacodylate buffer and then postfixed in a reduced osmium solution (i.e., 1.5% potassium ferrocyanide, Electron Microscopy Sciences, catalog 20150) with 2% osmium tetroxide (Electron Microscopy Sciences, catalog 19170) in 0.15 M cacodylate buffer for 1 hour on ice. After the first heavy metal incubation, the samples were washed with ddH2O at room temperature and then placed in the 0.22 μm Millipore- filtered 1% thiocarbohydrazide (TCH) (Electron Microscopy Sciences, catalog 21900) in ddH2O solution for 20 minutes at room

temperature. Organoids were then rinsed again in ddH2O and incubated in 2% osmium tetroxide in ddH2O for 30 minutes at room temperature. After several washings at room temperature in ddH2O, they were then placed in 1% uranyl acetate (aqueous) and left overnight at 4°C. Samples were washed once again and then immersed en bloc in Walton's lead aspartate solution (0.066 gr lead nitrate, Electron Microscopy Sciences, catalog 17900) dissolved in 10 ml of 0.003 M aspartic acid solution, pH 5.5) at 60°C for 30 minutes. The organoids were washed and then dehydrated stepwise through an ethanol series and finally placed in anhydrous ice-cold acetone for 10 minutes. Infiltration was performed with an acetone (Sigma-Aldrich, catalog 179124)/Epon812 (Electron Microscopy Sciences, catalog 14120) mixture with 3:1 volume proportions for 2 hours, then 1:1 overnight. The samples were left for 2 hours in pure resin and then embedded in Epon812 resin and placed in a 60°C oven for 48 hours for polymerization. For TEM imaging, ultrathin sections 70 nm thick were prepared by an UltraCut E Ultramicrotome (Reichert) and placed on TEM copper grids, which were then observed using a LEO 912AB microscope (Carl Zeiss) equipped with a thermionic W electron source and operating at an acceleration voltage of 100 kV. The images were acquired using a bottom mount Esivision CCD-BM/1K system (ProScan Camera) with a final resolution of 1024 × 1024 pixels.

4.7.2 SEM sample preparation

The organoids were removed from the differentiation medium, washed with NaCacodylate 0.1 M and fixed with glutaraldheyde 2% in NaCacodylate 0.1 M for 24 hours at 4°C. After the fixation they were washed three times with NaCacodylate 0.1 M for 10 minutes, and post-fixed with osmium tetroxide (OsO4) 2% in NaCacodylate 0.1 M for one hour at room temperature. After removing the OsO4 solution and rinsing twice with bi-distilled water, the samples were gradually dehydrated by consecutive 10 minutes' incubations 20%, 30%, 40%, 50%, 70%, 80% and 90% ethanol-H2O, and 100% ethanol, followed by 50% (v/v) ethanol–hexamethyldisilazane (HMDS) and 100% HMDS that was air-dried overnight at room temperature. All of the reagents were purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Organoids were opened to reveal the internal structure with 0.5-mm tungsten needles using a Leica stereomicroscope (Wien, Austria) and mounted onto 12-mm specimen stubs. Before SEM imaging, the samples were coated with 20 nm-thick film of Pt-Pd using a Cressington 208HR sputter coater (Watford, UK) operated at a current of 40 mA. The scanning electron microscopy (SEM) imaging was performed by a Zeiss Merlin field emission gun (FEG)-SEM (Oberkochen, Germany), working at an acceleration voltage of 5 kV, a beam current of 200 pA and a working distance of 10 mm, and acquiring the secondary electron (SE) signal by an Everhart-Thornley (ET) in-chamber detector. 4.7.1 SEM sample preparation

4.8 single-cell RNA-seq

4.8.1 Rationale

scRNAseq, although with different technics, is been widely used in organoids field because of their heterogeneity (Quadrato et al. 2017; Velasco et al. 2019) giving benefits for comparison with human cortical development (Camp et al. 2015; Giandomenico et al. 2019) or for evolutionary studies (Mora-Bermúdez et al. 2016; Pollen et al. 2019)This powerful approach allowed to clearly address one of the organoids' limits when compared to human fetal development, it regards the higher cell stress or glycolysis and the reduced representation of cell types in vitro (Pollen et al. 2019).
We planned scRNAseq analyses to understand at which transcriptomic level WT and HD organoids are different and furthermore to visualize possible misrepresented sub-populations in HD samples that can explain the early defects seen with VZ like structures. For example in HD organoids, compared to WT, we could find an unbalanced composition between progenitors and mature neurons or the presence of mis-differentiated cells towards unwanted identities.

4.8.2 Single-cell dissociation of ventral organoids for scRNAseq

The organoids are transferred in a dish to cut away the excess of Matrigel with a blade and then they are processed by dissociation using Papain Dissociation System with DNase (Worthington) following the manufacturer's recommendations. The incubation time is adjusted, based on size, at 30 min in the orbital shaker at 37°C. Then the samples are centrifugate for 1min at 1000 rpm and resuspended in DMEM+Neurobasal (containing N2 1:100, B27 1:100 and RI 10µM). For the dissociation into single cell suspension, the samples are pipetted up and down first with plastic 1000µl pipettes and following with glass pipettes of increasingly smaller tip diameter (fire-polished). To eliminate any pieces of undissociated tissue the suspension is filtered with disposable filter 20µm (Celltrics sysmex). The cells are counted with Countess Automated Cell Counter to check viability (should be higher than 90% to proceed) and concentration. The suspension is centrifugated for 1 min at 1000 rpm and resuspended at the concentration of $1x10^4$ cells /µl in PBS containing 0.04% weight/volume BSA (400 µg/ml).

4.8.3 Library construction using the GemCode platform

This procedure was performed by Paola Conforti. For experiments using the 10x Genomics platform, the Chromium Single Cell 3′ Library & Gel Bead Kit v2 (PN- 120237), Chromium Single Cell 3′ Chip kit v2 (PN-120236) and Chromium i7 Multiplex Kit (PN-120262) were used according to the manufacturer's instructions in the Chromium Single Cell 3′ Reagents Kits V2 User Guide.

Briefly the procedure can be divided in three steps. The first one is “Gel Bead-In-EMulsions (GEMs) generation and barcoding” to separately index each cell’s transcriptome. The cells lysate is mixed with primers containing (i) an Illumina R1 sequence (read 1 sequencing primer), (ii) a 16 bp 10x Barcode, (iii) a 10 bp Unique Molecular Identifier (UMI) and (iv) a poly-dT primer sequence. The second step is “Post GEM-RT Cleanup & cDNA Amplification” in which barcoded cDNA is amplified by PCR to generate sufficient mass for library. In the third step “library construction” the cDNA amplicon size is optimized by enzymatic fragmentation and size selection. A sample index and R2 (read 2 primer sequence) are added during library construction via End Repair, Atailing, Adaptor Ligation and PCR.

4.8.4 Sequencing and bioinformatics analyses

The following parts regard the sequencing of the library through Illumina and finally the bioinformatic analyses at a scale of hundreds to millions of cells.
In our case samples were sequenced by GATC eurofins company and then the analyses were performed by Vittoria Bocchi including the following steps: alignment, quality controls, clustering, cell type classification and DEGs.
At the moment of writing this thesis the analyses are ongoing for three single ventral organoids WT (line edited with 20CAG) and three HD (line edited with 56CAG) at day 45 of differentiation.

4.9 Co-culture of WT and HD cells inside organoids

4.9.1 Lentivirus production

To achieve stable expression of GFP and TOMATO, the Rues2 hESC line were infected with lentivirus particles containing the fluorescent genes under the control of chicken β-actin promoter and the puromycin selection cassette (Espuny-Camacho et al. 2013).
The lentivirus particles were generated by Ira Espuny Camacho through HEK293 cultured in Dulbecco's Modified Eagle's Medium (Gibco-Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (Gibco Thermo Fisher Scientific), 0,5% Penicillin/Streptomycin (Gibco Thermo Fisher Scientific), 2mM Glutamine (Euroclone), 1mM Sodium Pyruvate (Gibco Thermo Fisher Scientific) and 1% Non-Essential Aminoacids (Gibco Thermo Fisher Scientific). The cells were transfected in OptiMEM medium with helpers pVSVG (envelope plasmid) and pPAX2 (packaging plasmid). The lentivirus particles were then collected by centrifugation and concentrated by Amicon column.

4.9.2 Generation of GFP and TOMATO stable cell lines

hES Rues2 cell lines were infected with GFP and TOMATO lentiviruses to generate: WT-Rues2-20CAGGFP; WT-Rues2-20CAGTOMATO; HD-Rues2-48CAGGFP; HD-Rues2-48CAGTOMATO; HD-Rues2-56CAGGFP and HD-Rues2-56CAGTOMATO. Cells in self renewal were detached with Accutase and resuspended at the concentration of 100'000 cells/ml in mTeSR containing Rock Inhibitor (RI, 10µM). The viruses (50µl for each ml of cells) were added at this suspension and placed in tubes at 37 °C in the incubator for30min. The infected cells were then plated on geltrex coated plates at 37 °C and after 2,5h the media was change with fresh mTeSR containing RI (10µM). The following day the medium were changed without RI and after 3 days Puromycin (300ng/ml) was added for selection. After one week in selection with daily medium change the colonies were picked with the help of a dissecting microscope and pipette p10 and plated in presence of RI. Due to the difficulty of having colonies made of pure clones with homogeneous levels of fluorescent proteins, the cells were sub-picked in a second round in the same way. After one week of self renewal in Puromycin selection the cells were freezed and fixed for IF to check levels of fluorescent proteins (with AbI anti-GFP and anti-RFP) and pluripotency (with AbI anti-Oct4). The selected clones were thawed and used for organoids experiment.

4.8.3 Co-aggregation of WT and HD cells

We followed the procedures described before to generate ventral organoids (see paragraph 4.2 of this thesis). For co-aggregation at day0 it is important that the cells in self-renewal receive same manipulation in terms of split ratio and show similar growth rate. The two cell lines (WT and HD) are detached, counted and resuspended in parallel. The cells are plated in three 96well V bottom plate: one plate for each cell line and the third plate with a mixture of the two lines in a ratio 1:1. The following steps are the same of ventral organoids protocols (see paragraph 4.2). Around day 8 the EBs are check at the microscope for endogenous levels of fluorescent proteins in live.

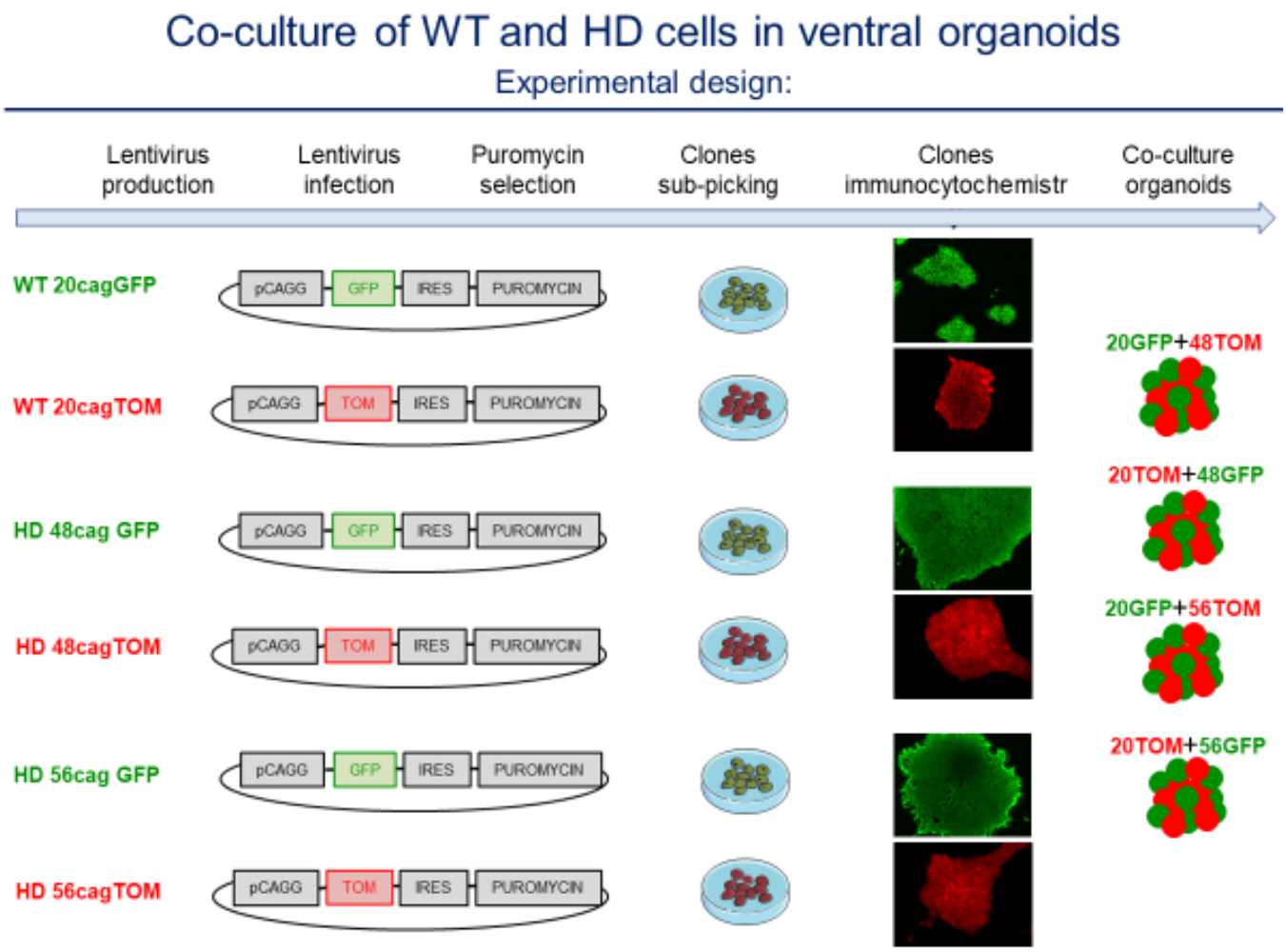

Fig 4.4: Experimental design for the generation and co-aggregation of GFP and TOMATO stable cell lines

4.9.4 Dissociation ad FACS sorting of co-culture organoids

The co-culture ventral organoids will be differentiated until day 45 and then they will be dissociated in single cells suspension, following the same procedure tested for scRNAseq (see paragraph 4.8.2 of this thesis). This suspension will be FACS sorted to isolate WT and HD populations inside the co-culture by their GFP or TOMATO signal. The sorting is performed by the FACS facility of INGM Institute with the BD FACSAria cell sorter and analyzed by the FACS Diva v. 6.1.3 software.
We will extract RNA from the sorted cells for future analyses of RNAseq in bulk to compare gene expression of the different combinations (see paragraph 2.3.1 of this thesis for the experimental plan).

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6. Appendix

6.1 Contributions to published articles

"Differentiation of human telencephalic progenitor cells into MSNs by inducible expression of GSX2 and EBF1"

Andrea Faedo, Angela Laporta, Alice Segnali, Maura Galimberti, Dario Besusso, Elisabetta Cesana, Sara Belloli, Rosa Maria Moresco, Marta Tropiano, Elisa Fucà, Stefan Wild, Andreas Bosio, Alessandro E. Vercelli, Gerardo Biella, and Elena Cattaneo

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The aim of this work was to established human embryonic stem (hES) cell-inducible lines that express specific transcription factors (GSX2 and EBF1) to improve medium spiny neuron (MSN) differentiation protocol and to study human striatal development in vitro. My specific goals in this project was to understand the effect of GSX2 and EBF1 overexpression, alone or combined, in regulating early patterning genes, promoting maturation towards MSN identity and regulating cell cycle kinetics. In details I collaborated in experiments of 2D differentiation of the inducible hESC lines towards MSN, with or without Doxycyclin treatment, and their analyses through BrdU-IddU assays, immunocytochemistry, cell counts and western blots. All results and analyses are under the paragraphs: "GSX2 and EBF1 Regulation of Patterning Genes", "GSX2 and EBF1 Regulate Cell-Cycle Kinetics" and "GSX2 and EBF1 Overexpression Differentially Regulates Early Neuronal Differentiation". The figures produced by this work are: Fig1, FigS2 (from A to H), Fig2, FigS3 (from H to M) and Fig3. (from A to K).