Digital Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Medicine 2019

Role of *MYCN* in retinoblastoma

From carcinogenesis to tumor progression

HANZHAO ZHANG

ACTA UNIVERSITATIS
UPSALIENSIS
2024

ISSN 1651-6206
ISBN 978-91-513-2041-0
urn:nbn:se:uu:diva-523595

UPPSALA
UNIVERSITET

Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjölds Väg 20, Uppsala, Wednesday, 10 April 2024 at 09:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Professor Johan Holmberg (Department of Molecular Biology, Umeå University).

Abstract

Zhang, H. 2024. Role of *MYCN* in retinoblastoma. From carcinogenesis to tumor progression. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 2019. 51 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-2041-0.

Retinoblastoma, a pediatric malignancy of the retina, is primarily driven by the bi-allelic inactivation of the *RB1*gene. However, a subset of cases are characterized by proficient *RB1* functions but with *MYCN* copy number mutations, suggesting an alternative oncogenic mechanism in the absence of *RB1* mutations. The aim of this thesis is to investigate the intricate molecular and cellular pathways implicated in retinoblastoma, with a particular focus on the role of *MYCN* expression and its interplay with the cell cycle and apoptotic pathways.

In Paper I, we explored the regulatory mechanisms underpinning *MYCN*-induced retinoblastoma using a*RB1*-proficient *MYCN*-overexpressing *in vivo* model in embryonic chicken retina and *MYCN*-transformed cells in culture. Our findings revealed that *MYCN* overexpression led to a significant upregulation of E2F levels, thereby dysregulating the cell cycle and mimicking the mechanistic phenotype of *RB1*-deficient tumors. Inhibition on E2f DNA-binding activity efficiently normalized growth and apoptosis in *MYCN*-transformed cells in culture. Despite *RB1* proficiency, the elevated E2F levels induced a neoplastic behavior in retinal cells, indicating a novel mechanism of retinoblastoma carcinogenesis independent of *RB1* inactivation.

Paper II employed single-cell RNA sequencing to dissect the cellular composition of *MYCN*-driven retinoblastoma in chicken *in vivo* model, revealing a predominant origin in cone photoreceptor progenitors. This finding suggested a cell-type-specific vulnerability to *MYCN*-induced transformation. The research further identifies a notable heterogeneity within the *MYCN*-transformed cells, with a subset of cells exhibiting non-cone photoreceptor features but features of other neurons like ganglion cells. A cluster was also identified withelevated expression of genes related to malignancy and tumor progression, including *UBE2C* and *TOP2A*. This suggested a link between *MYCN* overexpression and tumor development, potentially mediated through the E2F pathway.

In Paper III, the focus shifted to the interplay between *MYCN* expression, E2f activity, and the p53 pathway in human retinoblastoma cell lines exhibiting both *RB1* deficiency and *MYCN* amplification. By modulating E2f and p53 pathway activities using chemical inhibitors, we demonstrated the essential role of *MYCN* expression level in mediating p53-driven growth inhibition and highlighted the independent effects of E2f inhibition and p53 activation by a Mdm2 inhibitor.

Together, these studies illuminate the intricate molecular pathways involved in *MYCN*-amplified retinoblastoma, emphasizing the pivotal role of *MYCN* in disrupting cell cycle regulation and promoting tumorigenesis. These insights not only advance our understanding of retinoblastoma pathogenesis but also provide potential therapeutic targets within the MYCN-E2F axis, offering novel treatment strategies in *MYCN*-amplified retinoblastoma.

Keywords: MYCN, retinoblastoma, RNA sequencing, cancer, avian, E2F, carcinogenesis

Hanzhao Zhang, Department of Immunology, Genetics and Pathology, Neurooncology and neurodegeneration, Dag Hammarskjölds väg 20, Uppsala University, SE-751 85 Uppsala, Sweden.

ISSN 1651-6206
ISBN 978-91-513-2041-0
URN urn:nbn:se:uu:diva-523595 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-523595)

To family and friends

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Zhang, H**., Konjusha, D., Rafati, N., Tararuk, T., Hallböök, F. (2023). Inhibition of high level E2F in a RB1 proficient MYCN overexpressing chicken retinoblastoma model normalizes neoplastic behaviour. *Cell Oncol (Dordr)*

II. **Zhang, H**., Konjusha, D., Rafati, N., Tararuk, T., Hallböök, F. Single-cell RNA sequencing reveals cellular properties and carcinogenesis of a *RB1*-proficient *MYCN*-overexpressing retinoblastoma model from embryonic chicken retina. *Manuscript*

III. **Zhang, H**., Tararuk, T., Hallböök, F. Characterization of MYCN amplified retinoblastoma lines with respect to effects of E2f and p53 activity. *Manuscript*

Other related work by the author

Blixt, M. K. E*., Hellsand, M*., Konjusha, D*., **Zhang, H**., Stenfelt, S., Akesson M., Rafati, N., Tararuk, T., All-Eriksson, C., Ring, H., Hallbook, F. (2022) MYCN induces cell-specific tumorigenic growth in RB1-proficient human retinal organoid and chicken retina models of retinoblastoma. *Oncogenesis*, 11(1), 34

*Authors contributed equally to the work

Contents

Abbreviations

AC	Amacrine cell
ARR3	Arrestin 3
ATM	Ataxia-telangiectasia mutated
ATR	ATM and Rad3-related
bHLH-LZ	Basic helix-loop-helix leucine zipper
BP	Bipolar cell
CDK	Cyclin-dependent kinase
CKI	CDK inhibitor
cPR	Cone photoreceptor
DEG	Differentially expressed gene
E	Embryonic day
GC	Ganglion cell
GCL	Ganglion cell layer
GO	Gene ontology
GSEA	Gene set enrichment analysis
HC	Horizontal cell
hESC	Human embryonic stem cell
IHC	Immunohistochemistry
INL	Inner nuclear layer
MAX	MYC-associated protein X
MG	Müller glia
ONL	Outer nuclear layer
PR	Photoreceptor
RNA seq	RNA sequencing
RPC	Retinal progenitor cell
RPE	Retinal pigment epithelium
rPR	Rod photoreceptor

Retinoblastoma

Etiology and genetics

Retinoblastoma is a malignant pediatric cancer originated in developing retina. The incidence rate of retinoblastoma worldwide is 1 in 17,000 live births [1]. Despite its relative rarity compared to other pediatric cancers such as leukemia or lymphoma, retinoblastoma is the most common intraocular malignancy among children [2, 3]. As a malignant tumor, retinoblastoma develops fast from intraocular tumor to metastasis to other sites including brain via the optic nerve. Now the survival rate of retinoblastoma is reaching near 100% in high-income countries but the tumor is fatal and the mortality can be 100% within 48 months if left untreated upon diagnosis [1, 4, 5]. The prognosis in low-income countries, however, is much poorer due to late diagnosis and lack of treatment [1, 6].

The earliest documented case of retinoblastoma dates to 1597, although it was not recognized as a retinal tumor until 1809 when Scottish surgeon James Wardrop contested the predominant hypothesis that it was an unknown type of fungus [7]. The etiology of retinoblastoma is complex, involving genetics in hereditary forms and other factors such as environmental and lifestyle factors in sporadic forms [8]. Knudson's "Two-Hit" hypothesis, presented in 1971, delineated the oncogenesis of both hereditary and sporadic retinoblastoma and was supported by later case studies. According to the hypothesis, patients with hereditary retinoblastoma have a first germline mutation from parents and acquire a second mutation during retina development. In the end, this results in bilateral hereditary retinoblastoma. In sporadic retinoblastoma, however, it requires two distinct somatic mutations of retinoblastoma gene in the same retinal cell during retina development without germline mutation and this results in unilateral sporadic retinoblastoma [9, 10]. There are around 30% retinoblastoma cases are bilateral while 70% are unilateral. The mean age at diagnosis is around 24 months for unilateral cases and 12 months for bilateral cases [11]. About 6% of bilateral retinoblastoma patients develop also a primary intracranial tumor usually in pineal gland in addition to the intraocular tumor [12]. This is referred as “trilateral retinoblastoma”. Patients with trilateral retinoblastoma have poorer prognosis and rarely survive after treatment [13].

The etiological basis of retinoblastoma is predominantly attributed to the biallelic inactivation of the *RB1* gene [14]. In addition to *RB1* inactivation, other

mutations such as *MYCN* amplification, *BCOR* deletion, *OTX2* amplification have also been implicated in the tumorigenesis and progression of retinoblastoma [15]. It is noteworthy that a minority of retinoblastoma cases (approximately 2%) are initiated by *MYCN* amplification in the absence of *RB1* gene mutations [16].

Cell-of-origin of retinoblastoma

The cell-of-origin of retinoblastoma remains a subject of active research and debate within the field. Various studies suggested retinoblastoma arises from either a retinal progenitor cell or a postmitotic cell that re-enters cell cycle [17-19]. In the former hypothesis, the Rb protein encoded by *RB1* gene is essential for regulating the transition of G1 to S phase during cell cycle in progenitor cells, crucial for the formation of postmitotic neurons. Alternatively, in the latter hypothesis, the role of Rb protein is to prevent newly born postmitotic cells from re-entering cell cycle [20]. However, the two hypotheses are not mutually exclusive, considering that different retinal progenitor cells and postmitotic cells have different propensity to form tumors upon *RB1* inactivation. For instance, mouse studies suggest that progenitors or postmitotic cells predisposed towards amacrine and horizontal fate are more likely to form retinoblastoma when *RB1* is inactivated [17, 19, 21]. This indicates that intrinsic features of retinal cells influence their likelihood of becoming tumorigenic.

Identifying retinoblastoma's cell-of-origin is complicated by the tumor's cellular heterogeneity. Tumor analyses indicate a composite nature, consisting of progenitor, precursor, and differentiated cells that re-enter the cell cycle [19, 22]. Like previously mentioned, studies on mouse models implicated possible cell types from the inner nuclear layer of retina to be the origin of retinoblastoma, including horizontal cells, amacrine cells and Müller glia cells [17, 21, 23]. In human studies, however, several subtypes were identified with different possible cell-of-origin. The initiation of pediatric cancers like retinoblastoma are believed to require fewer genetic alterations than adult cancers, potentially due to the intrinsic tumor-like characteristics of the cell-of-origin [20, 24]. Based on the differentiation status and gene expression, two subtypes of retinoblastoma have been suggested. In one subtype, tumor cells remain at a less differentiated status and more recurrent genetic alterations other than *RB1* inactivation are found, including *MYCN* amplification. Due to the mixed cell types in this subtype, retinal progenitor cells are suggested to be the cell-of origin. In the other subtype, tumor cells are found to retain many cone photoreceptor features and have higher overall genomic stability. Fewer genetic alterations are found other than *RB1* inactivation compared to the former subtype. Committed cone precursors are therefore suggested to be the cell-of-origin [25-27].

RB1 and MYCN

RB1 gene

As previously mentioned, Knudson's "Two-Hit" hypothesis, formulated in 1971, laid the groundwork for understanding retinoblastoma's genetic basis. Ever since Kundson's "Two-Hit" hypothesis, studies on retinoblastoma have found the tumor development depends on disposing recessive mutations on human chromosome 13 and specific mitotic events [28, 29]. It was not until 1986 that the *RB1* gene, responsible for predisposition to this cancer, was isolated and identified as the first tumor suppressor gene [14, 30].

The *RB1* gene has a size of 180 kb and is located on chromosome 13q14.2 with 27 exons [31]. *RB1*, when mutated in both alleles, can initiate retinoblastoma in most cases [32]. *RB1*'s product, Rb protein, belongs to retinoblastoma protein family and retinoblastoma protein family also includes p107 (encoded by *RBL1* gene in human) and p130 (encoded by *RBL2* gene in human) [33, 34]. Rb protein functions to regulate cell division by controlling the G1 to S phase transition in the cell cycle via its interaction with the E2F transcription factor [35, 36]. In retinoblastoma, dysfunctional Rb fails to restrain E2F, leading to uncontrolled cell proliferation and cancer. While the inactivation of both alleles of *RB1* is necessary for tumorigenesis, additional secondary mutations often occur, furthering tumor growth and progression [32]. Loss of *RB1* alone typically results in retinoma, a benign retinoblastoma variant [37]. Subsequent genomic changes post-*RB1* mutation include chromosomal gains on 1q, 2p and 6p and losses on 16q, notably the amplification of *MYCN* on chromosome 2p [38].

MYCN gene

The *MYC* transcription factor family includes *MYCN*, *MYC*, and *MYCL*, encoding Mycn, c-myc and Mycl protein, respectively. While *MYC* is expressed universally in proliferating cells during development, expression of *MYCN* and *MYCL* are more restricted. Deregulation of *MYC* family genes is associated with a variety of cancers [39]. The very first identification of *MYC* amplification was in leukemia cell line in 1982, soon after which *MYCN* amplification was found in neuroblastoma and *MYCL* amplification in small cell lung cancer [40-42]. Because MYCN is restrictedly expressed in neural tissues, amplification

of MYCN is found preferentially in cancers arising from immature nervous system, such as neuroblastoma, medulloblastoma and retinoblastoma [43].

Proteins encoded by MYC family genes are distinguished by their basic helix-loop-helix leucine zipper (bHLH-LZ) domain, which is on the C-terminal and is crucial for DNA binding and dimerization [44]. The bHLH-LZ domain enables Myc proteins to form heterodimers with a co-factor, MYC-associated protein X (MAX), allowing them to specifically bind to E-box sequences (CACGTG) in the DNA and regulate gene expression [45]. On the N-terminal of Myc proteins there is a highly conserved N-terminal transcriptional activation domain, which is essential for the recruitment of transcriptional coactivators and chromatin remodelling complexes, thereby modulating gene transcription [46]. The regulation of Myc proteins is sophisticated, involving transcriptional, post-transcriptional and post-translational mechanisms through various regulatory pathways. This includes control by the RAS/MAPK and PI3K/Akt signalling pathways affecting MYC expression levels and stability through post-translational modifications such as phosphorylation and ubiquitination [47]. Another example is the interaction with Aurora Kinase A encoded by *AURKA* gene. This interaction has been shown to phosphorylate Myc proteins and influence the protein stability and transcriptional activity [48].

The oncogenic mutation of *MYCN* has long been found as a common event in primary retinoblastoma [49]. Although previous studies indicated that *MYCN* amplification was independent of retinoblastoma tumorigenesis but only reflected the embryonic tumor origin, more recent research has identified that the occurrence of *MYCN* amplification in retinoblastoma extends beyond cases with *RB1* loss, presenting in tumors without *RB1* inactivation as well [16, 50]. The retinoblastoma cases with *MYCN* amplification, recently delineated by its gene expression and phenotypic profile, exhibits less differentiation and a more aggressive, metastatic nature [26]. This thesis work predominantly addresses retinoblastomas initiated by *MYCN* amplification absent of *RB1* loss. Such instances are rare, constituting less than 2% of retinoblastoma cases. These tumors are usually sporadic and formed by somatic mutation of *MYCN* gene [51]. In a recent case study with two patients, retinoblastomas harboring *MYCN* amplification without *RB1* mutation display resistance to conventional chemotherapy, with metastasis to the orbit and lymph nodes [52].

MYCN target genes

Gene copy number mutations of *MYCN, MYCN* amplification has been identified in various types of cancers, both pediatric and adult cancers [53]. Its downstream targets participate in various cellular processes, such as cell cycle regulation, apoptosis, and differentiation [54]. The role of *MYCN* can be paradoxical, promoting cell proliferation while also predisposing cells to apoptosis. In neuroblastoma, ectopic expression of *MYCN* causes cells to re-enter the cell

cycle but also increases their susceptibility to apoptosis when subjected to DNA damage [55-58]. In fact, neuroblastoma cell lines with *MYCN* amplification exhibit a greater propensity for apoptosis than non-*MYCN*-amplified cell lines [59]. Investigating the targets of *MYCN* is critical to demystifying its dualistic role in tumorigenesis.

Various methods have been applied to identify MYCN target genes, from knockdown in early days to nowadays large-scale approaches like microarray and RNA sequencing [57, 60, 61]. Part of experimentally identified *MYCN* downstream target genes are listed in Table 1.

Targeting *MYCN* as therapeutic strategy

Historically, *MYCN* has been considered "undruggable" due to its absence of enzymatic activity, its complex network of protein interactions, and its DNA binding properties [62]. The N-terminal transcription activating domains of Mycn protein are intrinsically disordered, and the C-terminal domain lacks the defined hydrophobic pockets typically needed for small molecule interactions [63-65]. The close similarity between *MYCN* and its family members, *MYC* and *MYCL*, adds to the difficulty of targeting *MYCN* specifically [66]. Nevertheless, researchers continue to explore innovative strategies to circumvent these challenges and influence *MYCN*'s activity through indirect means [53, 67].

Targeting *MYCN* transcription

The transcription of *MYCN* can be targeted through different mechanisms. Retinoic acid indirectly suppresses *MYCN* without targeting a specific DNA element, impacting multiple regulatory regions within the *MYCN* promoter [68]. Cis-regulatory elements like super-enhancers, clusters of enhancers associated with oncogenic activity, are also targeted to disrupt *MYCN* expression [69, 70]. BRD4 inhibitors such as JQ1 prevent Brd4, a protein that reads acetylated histone marks and activates *MYCN* transcription, from binding to chromatin, thereby reducing *MYCN* levels [71]. Additionally, inhibiting CDKs involved in the transcription process like CDK7 or CDK9 can also suppress *MYCN* [72, 73].

Targeting Mycn protein stability

Mycn protein has a half-life of around 30 minutes and its degradation is regulated by ubiquitination and following proteasomal degradation [74]. Targeting the degradation of Mycn provides a potential therapeutic strategy for tumors with *MYCN* amplification. One example is to inhibit *AURKA*, whose gene product forms a complex with Mycn and protect it from ubiquitination and degradation [75]. Treatment with AURKA inhibitors decreased Mycn level and suppressed tumor growth in neuroblastoma [76]. Another approach is to inhibit PI3K. Inhibition on PI3K/Akt axis increases GSK3ß kinase activity and results in increased Mycn degradation [77].

Other approaches include to inhibit key enzymes in metabolic pathways such as *ODC1* [78]. Interfering the dimerization of Mycn to its coactivator MAX with small molecules also results in decreased *MYCN* level and tumor growth [79]. In the meanwhile, the development in chemical genomics creates possibilities in developing direct targeting on *MYCN*, with technologies like proteolysis targeting chimeras (PROTACs) induced protein degradation [80].

Table 1 Part of experimentally identified MYCN downstream targets

Biological process	**Gene symbol**	**Reference**
Cell cycle	*SKP2*	E. Bell, J. Lunec and D. A. Tweddle, Cell Cycle 6 (10), 1249-1256 (2007).
	DKK3	
	E2F1	C. W. Woo, F. Tan, H. Cassano, J. Lee, K. C. Lee and C. J. Thiele, Pediatr Blood Cancer 50 (2), 208-212 (2008).
	ID2	
	MDM2	A. Slack, Z. Chen, R. Tonelli, M. Pule, L. Hunt, A. Pession and J. M. Shohet, Proc Natl Acad Sci U S A 102 (3), 731-736 (2005).
	ODC1	T. Ben-Yosef, O. Yanuka, D. Halle and N. Benvenisty, Oncogene 17 (2), 165-171 (1998).
	NLRR1	M. S. Hossain, T. Ozaki, H. Wang, A. Nakagawa, H. Takenobu, M. Ohira, T. Kamijo and A. Nakagawara, Oncogene 27 (46), 6075-6082 (2008).
	ALK	M. K. Hasan, A. Nafady, A. Takatori, S. Kishida, M. Ohira, Y. Suenaga, S. Hossain, J. Akter, A. Ogura, Y. Nakamura, K. Kadomatsu and A. Nakagawara, Sci Rep 3, 3450 (2013).
Apoptosis	*TP53*	L. Chen, N. Iraci, S. Gherardi, L. D. Gamble, K. M. Wood, G. Perini, J. Lunec and D. A. Tweddle, Cancer Res 70 (4), 1377-1388 (2010).
	CDKN2A (p14ARF)	S. Amente, B. Gargano, D. Diolaiti, G. Della Valle, L. Lania and B. Majello, FEBS Lett 581 (5), 821-825 (2007).
	H-TWIST	S. Valsesia-Wittmann, M. Magdeleine, S. Dupasquier, E. Garin, A. C. Jallas, V. Combaret, A. Krause, P. Leissner and A. Puisieux, Cancer Cell 6 (6), 625-630 (2004).
	TOMM20	M. Szemes, Z. Melegh, J. Bellamy, J. H. Park, B. Chen, A. Greenhough, D. Catchpoole and K. Malik, Cancers (Basel) 13 (4) (2021).
	PDK1	

Differentiation	*CDC42*	L. J. Valentijn, A. Koppen, R. van Asperen, H. A. Root, F. Haneveld and R. Versteeg, Cancer Res 65 (8), 3136-3145 (2005).
	NME1	
	NME2	
	PAX3	R. G. Harris, E. White, E. S. Phillips and K. A. Lillycrop, J Biol Chem 277 (38), 34815-34825 (2002).
	EZH2	M. Vanden Bempt, K. Debackere, S. Demeyer, Q. Van Thillo, N. Meeuws, C. Prieto, S. Provost, N. Mentens, K. Jacobs, O. Gielen, D. Nittner, S. Ogawa, K. Kataoka, C. Graux, T. Tousseyn, J. Cools and D. Dierickx, Blood 140 (23), 2463-2476 (2022).

Retina development

Neural differentiation

The development of retinoblastoma is intricately linked to retinal development, a finely orchestrated process unfolding in a temporally precise manner. In mature vertebrate neural retina, there are six types of neurons—rod and cone photoreceptors, bipolar cells, horizontal cells, amacrine cells, and ganglion cells—alongside a glial cell type, the Müller glia, all of which are derived from the multipotent retinal progenitors. Moreover, another two types of glial cells, microglia and astrocytes, also exist in mature retina [81]. Microglia enter the retina via the ciliary body, retinal vasculatures and optic nerve head during development and are located in the ganglion cell layer [82]. Astrocytes are also immigrants from the optic nerve to the retina and carry out various glial homeostatic functions [83, 84]. These cells are structured into three distinct nuclear layers: outer nuclear layer (ONL) with rod and cone photoreceptors; inner nuclear layer (INL) with horizontal cells, bipolar cells and amacrine cells; ganglion cell layer (GCL) with ganglion cells. Müller glia cells have their cell bodies spanning the whole retina and with the outer segments in the photoreceptor side. (Fig. 1). Early investigations by Holt CE et al. posited a stochastic model for retinal cell fate, independent of lineage or timing, hinging instead on the cell's position and microenvironment [85]. Subsequent mouse model research, however, suggested that all retinal cell types arise sequentially from a common pool of retinal progenitor cells (RPCs) [86]. The prevailing model now is the “competence model” which suggests RPCs undergo mitosis and then exit the cell cycle at certain time point to begin differentiation, with two waves of neuronal differentiation during retinal neurogenesis. In the early wave RPCs produce early-born retinal neurons, including ganglion cells, horizontal cells, cone photoreceptors and amacrine cells. Late-born retinal cells include rod photoreceptors, bipolar cells and Müller glia and are produced in the second wave from RPCs (Fig. 1) [87]. The timing of RPCs' cell cycle exit is crucial; an early exit can deplete RPCs for later stages, whereas a late exit can lead to an overabundance of late-born retinal cells. This balance is maintained by a synergy of intrinsic and extrinsic signals that govern RPC behavior [88, 89]. Typically, retinal neurons perform interkinetic nuclear migration with terminal mitosis on apical side, followed by cell cycle exit and migration to proper cell layer; however, horizontal cells are noted for their atypical non-apical terminal mitosis [90].

A B

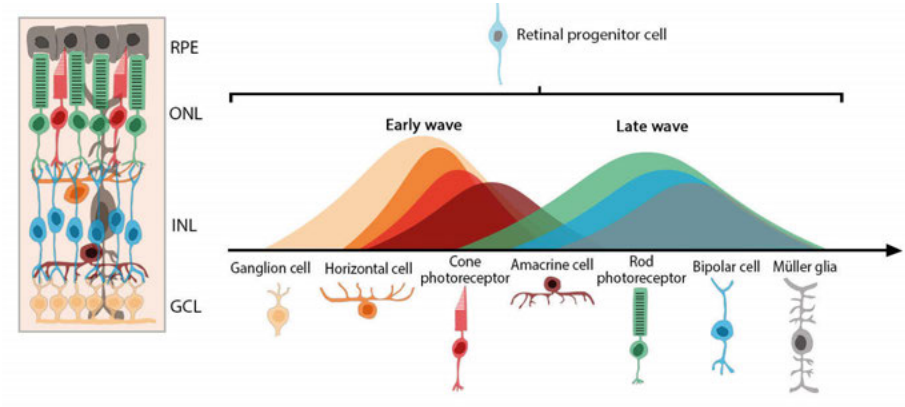

Fig. 1 Retina anatomy and waves of cell generation

A Retina anatomy with different cell layers. **B** Retinal neural differentiation with two waves. RPE retinal pigment epithelium, ONL outer nuclear layer, INL inner nuclear layer, GCL ganglion cell layer.

Naturally occurring cell death

Another factor to ensure the correct number and distribution of retinal cells is the programmed cell death, which naturally occurs in several distinct phases during retina development. Initially, morphogenic death shapes the optic cup and optic vesicle. Subsequent early neural death affects proliferating cells and newly born neurons. Later neurotrophic death targets differentiated neurons during synaptogenesis, mostly due to competence in neurotrophic support [91]. Naturally occurring cell death has been studied across various species. In chicken, for example, although it is difficult to clearly distinguish between morphogenic and early neural cell death due to the temporal overlap, naturally occurring cell death sequentially affects ganglion cell layer at embryonic day 9 (E9) and then inner nuclear layer at E11 [92]. These programmed deaths are also regulated by intrinsic and extrinsic signals within the retinal environment [93].

Cell cycle regulation

Cyclins, CDKs and CKIs

Uncontrolled cell cycle is one of the hallmarks of cancer cells. In mammalian cells, the progression through the cell cycle is strictly regulated by the interplay of cyclins and cyclin-dependent kinases (CDKs). Each phase transition within the cell cycle is modulated by distinct cyclin-CDK complexes. During G1 phase, CDK4/6, upon activation by cyclin D in early G1 phase, and the cyclin E-CDK2 complex in late G1 phase, phosphorylate the Rb protein and release the E2F transcription factors. This release of E2F enables the transcription of pivotal genes for S phase entry. Progression through S phase and into G2 phase requires the cyclin A-CDK2 complex, while transitions from G2 to M phase and the completion of mitosis are directed by cyclin A-CDK1 and cyclin B-CDK1 complexes (Fig. 2). The activities of specific cyclin-CDK complexes are temporally constrained to their respective cell cycle stages and are subjected to stringent regulation through targeted degradation of the complexes or downregulation of their encoding genes [94].

Cell cycle regulation also involves the role of CDK inhibitors (CKIs), which can block the kinase activity of CDK to guarantee the correct progression of cell cycle. Two families of CKIs have been identified: the INK family (p15, p16, p18 and p19) binding cyclin D- CDK complexes and Cip/Kip CKIs (p21, p27 and p57) that bind cyclin D- CDK complexes and cyclin E- CDK complexes [95] (Fig. 2). Studies on CKIs during retinal development found that CKIs play an important role in cell cycle exit and cell fate decision for RPCs. For instance, research in rodent retinas reveals a heterogeneity in CKI expression among RPCs, particularly during retinal histogenesis [89]. There are distinct RPC populations expressing different CKIs ($p27^{Kip1}$ and $p57^{Kip2}$) and the expression of these CKIs is upregulated at different phases of cell cycle, indicating that RPCs possibly use different mechanisms to exit cell cycle and to determine retinal cell fate [96]. Notably, the expression of $p27^{Kip1}$ is found to have different roles in different retinal cell types. It is required to maintain quiescence in bipolar cells, Müller glia and cone photoreceptors while in other cell types, it is not necessary for preventing cells from re-entering cell cycle. The expression of $p27^{Kip1}$ is also important for survival and differentiation of cone photoreceptors, linking it to the cell-of-origin of retinoblastoma [97].

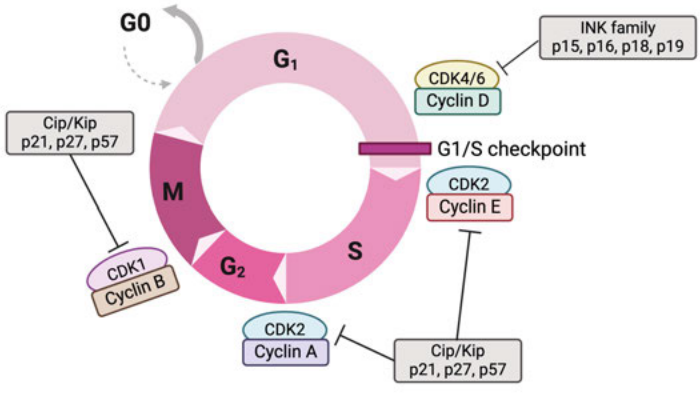

Fig. 2 Illustration of cell cycle regulation

Role of E2F transcription factor

In **paper I**, we discovered the effect of *MYCN*-induced high level E2F on retinoblastoma carcinogenesis. The E2F family of transcription factors comprises eight distinct proteins, each encoded by a corresponding gene (*E2F1* - *E2F8*). This family plays a critical role in cell biology, orchestrating a range of processes including proliferation, apoptosis and other vital biological processes [98, 99]. The first E2F was identified by transcription control mediated by adenovirus E1A [100]. From then on, systematic, large-scale investigations have unveiled hundreds of E2F-regulated genes, many of which are pivotal in cell cycle progression, DNA replication, cell cycle checkpoints, DNA damage response and apoptotic pathways (Table 1) [101]. One important pathway regulated by E2F in cancer is the p53 pathway. E2F directly activates the expression of p14ARF, which is a tumor suppressor protein that interrupts the binding of Mdm2 and p53 to prevent the degradation of p53, playing a role in protecting uncontrolled cell division [102].

The E2F family of transcription factors, controlled by Rb protein interaction, plays a critical role in cell cycle entry as mentioned in previous section. When Rb is phosphorylated by CDK4/6, active E2F is released. Dysregulation of this CDK-RB-E2F pathway, leading to increased E2F activity, is a common early event in cancerous proliferation [103, 104]. Moreover, during S phase, a second E2F complex is found involving E2f and another family member of retinoblastoma protein family, p107, which is independent from E2F-Rb complex [105, 106]. Based on the role of E2F in tumorigenesis, comprehensive understanding of E2F regulation is essential for decoding the mechanisms underlying tumorigenesis. Regulation of E2F occurs at transcriptional, post-transcriptional (mRNA stability), and post-translational (protein binding and protein stability) levels [103]. In retinoblastoma with *RB1* deficiency, the regulatory

constraint on E2F through Rb protein is lost due to dysfunctional Rb. The regulatory mechanisms in retinoblastomas with proficient *RB1*, however, remained less understood until our investigation, detailed in **Paper I**, revealed that *MYCN* overexpression results in elevated *E2F1* expression even when *RB1* is functional. The study found that targeting E2F binding with small molecule HLM006474 reduced proliferation, induced cell cycle arrest and following apoptosis in chicken model, uncovering a potentially new mechanism in the pathogenesis of *MYCN*-amplified retinoblastoma.

Table 2 Part of important E2F target genes in different biological processes [101].

Biological process	**Gene symbol**	**Biological process**	**Gene symbol**
Cell cycle	*MYC*	DNA synthesis	*CDC45L*
	MYCN		*CDC6*
	CCND1		*TK1*
	CCND3	Checkpoints	*BRCA1*
	CCNE1		*BRCA2*
	CCNE2		*BUB1B*
	CDC25A		*CHK1*
	CDK2	DNA damage response	*MGMT*
	TFDP1		*UNG2*
	AURKB		*PRKDC*
	CDC20		*RAD51*
	CCNA1	Apoptosis	*APAF1*
	CCNA2		*BAD*
	PLK		*BCL2*
	KI67		*CASP3*
	CDKN2A (p14ARF)		*BID*
DNA synthesis	MCM family		*CASP7*
	PCNA		*CASP8*
	TOP2A		*TP73*

DNA damage response and apoptosis

Another hallmark of tumor cells is the resistance to DNA damage response and apoptosis, effectively disregarding genetic insults to continue proliferation. The DNA damage response consists of DNA damage checkpoint pathways and DNA repair mechanisms [107]. The DNA damage checkpoints, including G1 checkpoint, S phase checkpoint and G2 checkpoint, are signal transduction pathways involving three types of proteins: sensors, signal transducers and effectors. For instance, in G1 checkpoint, sensor proteins ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) detect DNA damage and are recruited to the damage site. Next ATM and ATR phosphorylate signal transducers Chk1 and Chk2. The phosphorylation result in the activation of two effectors: Cdc25a for rapid G1/S cell cycle arrest and p53 for maintenance of the arrest and apoptosis if necessary [108]. Thus, the DNA damage checkpoints serve to detect damage and provide time for its repair before cell division proceeds.

Apoptosis, or programmed cell death, serves as a defense mechanism against malignancy [109]. Therefore, the ability of cancer cells to resist apoptosis is pivotal in carcinogenesis. This resistance can manifest through compromised death receptor signaling, mutations in the p53 pathway, an imbalance between proapoptotic and antiapoptotic proteins such as those in the Bcl-2 family, or reductions in caspase expression and activity [110]. In the realm of retinoblastoma, there's an ongoing debate about how the cell-of-origin manages to avoid apoptosis. Early research posited that these cells might be inherently resistant to p53-mediated apoptosis, circumventing this pathway [17, 111]. However, more recent studies in human tissues suggest that while p53 activity may initially increase following RB1 gene loss, the subsequent upregulation of the *MDMX* gene and MdmX protein functions to inhibit the p53 pathway, promoting the development of retinoblastoma. This indicates that retinoblastoma might not derive from cells that are intrinsically p53 resistant [112, 113]. Additionally, it has been discovered that *MDM2* amplification within cone progenitors, which are the potential cell-of-origin, can drive proliferation and *MYCN* expression through a p53-independent mechanism, contributing to tumor formation [114].

p53 pathway

In the previously discussed section, the p53 pathway emerges as a crucial effector in the DNA damage response. This tumor suppressor, encoded by the *TP53* gene, is instrumental in cell cycle regulation and oncogenesis prevention [115]. The role of p53 in cancer has been heavily studied. In general, p53 is maintained at low intracellular levels by its negative regulator Mdm2/X, but upon sensing stress, it activates and orchestrates a series of cellular defensive responses, including cell cycle arrest and apoptosis, as a safeguard against tumorigenesis [116, 117]. A host of p53 target genes, such as *PUMA* and *NOXA* for apoptosis and p21 for cell cycle inhibition, have been identified [118]. *TP53* gene mutations represent a frequent genetic aberration in various human cancers, often associated with heightened malignancy and poorer prognoses [119].

In retinoblastoma, however, mutation of *TP53* is rarely found initially to induce cancer [120]. Furthermore, it is uncommon to find subsequent *TP53* mutations following the loss of *RB1* or the amplification of *MYCN* in retinoblastoma. However, this has not deterred ongoing research into the p53 pathway's deregulation during the tumorigenesis of retinoblastoma. Elevated levels of *MDM2* and *MDM4*, known negative regulators of p53, have been detected in retinoblastomas, indicating a potential suppression of the p53 pathway during tumorigenesis [121]. This suppression correlates with the observed sensitivity of retinoblastoma to Mdm2/Mdm4 inhibitors, such as Nutlin-3a [122]. In **Paper III**, our studies have also explored how the presence of *MYCN* affects the response to Nutlin-3a in retinoblastoma cell lines Y79 and WERI-Rb1.

The regulation of *MYCN* on the p53 pathway have been observed in studies focusing on neuroblastoma with *MYCN* amplification, where *MYCN* directly regulates *TP53* and *MDM2*, enhancing their expression and activating p53-dependent cell death. Despite this, cells harboring *MYCN* amplification often adopt escape mechanisms from apoptosis, contributing to tumor progression and metastatic potential [54]. In retinoblastoma, analyses of cell lines with *RB1* inactivation and *MYCN* amplification demonstrate a significant correlation between *MDM2* and *MYCN* expressions, implicating them in tumor cell proliferation [114]. A more comprehensive study on retinoblastoma cell lines reveals that, irrespective of the presence or absence of *TP53* mutations, there is an observable increase in *TP53* expression, more pronounced in cells bearing mutations in both *RB1* and *MYCN* [123].

Ubiquitination

Ubiquitination is a post-translational modification of proteins involved in non-lysosomal degradation of proteins. It requires the covalent binding of activated ubiquitin to certain substrate and lead to recognition and following degradation of the substrate by the 26S proteasome complex [124]. The ubiquitination cascade is facilitated by a trio of enzymes: E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin-ligating). Initially, E1 activates ubiquitin, which is then transferred to E2, and in conjunction with E1, E3 ligates the activated ubiquitin to the substrate [125]. The degradation of proteins by ubiquitin-proteasome system regulates a wide variety of cellular processes, including DNA damage repair and cell cycle regulation [126].

Ubiquitination's pivotal role in cell cycle regulation and checkpoint control renders it a process of significant relevance to cancer development. For instance, the E3 ubiquitin ligase Fbw7 is recognized as a tumor suppressor due to its targeting of oncogenic proteins such as cyclin E, c-Myc, and Jun for ubiquitin-mediated degradation [127, 128]. Another well-known example is E3 ligase Mdm2 which regulates the degradation of p53 by ubiquitination. Amplification of *MDM2* is found in various cancers and targeting the interaction of Mdm2 and p53 is also a therapeutic strategy for cancers [129, 130]. Specifically in retinoblastoma, the E2 ubiquitin-conjugating enzyme *UBE2T* has been pinpointed as a prognostic marker, implicated in tumor progression through the activation of the STAT3 signaling pathway [131, 132]. Another E2 enzyme, *UBE2C*, is identified as an indicator of malignancy in multiple cancers, including retinoblastoma [133, 134]. In **Paper II**, we also revealed upregulated expression of *UBE2C* in the most proliferating cluster among *MYCN* overexpressing cells. Moreover, we also proposed a potential mechanism that *UBE2C* is a target of *E2F*.

Retinoblastoma models

Animal models

Early attempts to create retinoblastoma models involved inducing the tumor in animal models using either viruses or primary tumor cells, yet these methods primarily yielded therapeutic insights rather than uncovering the cell-or-origin [135, 136]. Transgenic mice expressing oncogenic viral proteins like HPV E6 and E7 did develop retinoblastoma-like tumors, but the absence of viral DNA in human retinoblastoma samples casted doubt on such model's applicability [137, 138]. Technological advances led to the development of *RB1* knockout mice; however, homozygous knockout of *RB1* was embryonically lethal, preventing model establishment [139]. The Cre-loxP system overcame this by allowing conditional *RB1* gene inactivation. Nevertheless, the murine retina's structural differences from humans, particularly its rod dominance and nocturnal vision, limit the model's relevance. It required a third knockout of another member of *RB* family to finally induce retinoblastoma from inner nuclear layer [17, 21]. This discrepancy underscores the need for *in vitro* and *in vivo* models that more closely mimic human retinoblastoma, especially for studying *MYCN*'s role, which is crucial during retinal development. Studies of *MYCN*-amplified retinoblastoma cell lines in culture, have not demonstrated significant class-specific differences between cells with and without *RB1* inactivation, leading to the hypothesis that the influence of *MYCN* is more pronounced during the developmental stages of retinoblastoma rather than in the final stages observed in cultured cells [123].

Our group has adopted the chicken as a model organism, possessing a cone-driven retina akin to human vision. The chicken embryo, a mainstay in developmental research for over 200 years, offers a rapid 21-day development cycle and notable embryological parallels with humans [140]. Techniques such as creating a window in the eggshell facilitate direct observation and intervention [141]. Utilizing the Hamburger and Hamilton staging system enables precise timing for experimental procedures [142]. In the eye development research, the chicken embryo's comparatively larger eyes are particularly advantageous for intricate ocular manipulations. In our lab we employ electroporation to introduce plasmid constructs, thereby modulating gene expression within the chicken retina. In our previous research, we established a *RB1*-proficient *MYCN*-overexpressing retinoblastoma model with developing chicken

embryonic retina. As the first reported *MYCN*-induced retinoblastoma animal model, we identified cell-of-origin as the progenitor of horizontal cell and cone photoreceptor lineage [143]. Results presented in **Paper I** and **Paper II** are also based on our chicken retinoblastoma model.

Human models

Given that retinoblastoma predominantly occurs in humans, *in vitro* human models are indispensable. The most direct approach is retinoblastoma cell lines derived from patient tumors, with over 100 established lines varying in *RB1* and *MYCN* gene status [123]. These cell lines facilitate preclinical drug response studies. Our own research, documented in **Paper III**, utilized the Y79 and WERI-Rb1 retinoblastoma cell lines to assess the impact of *MYCN* expression levels on Nutlin-3a response. Besides retinoblastoma cell lines, human fetal retinal tissue has also been used to study retina development and retinoblastoma. The retinal tissue obtained from human *retinae* can be cultured *in vitro* for at least 3 weeks preserving the structure and lentivirus transduction can be used to label specific targets such as *RB1* or photoreceptor marker *RXRγ* [144]. Although fetal retinal tissue in culture is considered to better recapitulate retina development *in vivo*, the drawbacks include limited availability and ethical aspects [145]. Moreover, it has been debated how long the retinal tissue can be cultured *in vitro* and whether all the *in vivo* events can be recapitulated. Nonetheless, cell lines or cultured fetal retinal tissue cannot capture the full progression of retinoblastoma, which has led to a growing focus on stem cell-derived retinal organoids.

The initial endeavors in deriving retinal cells from human stem cells involved promoting retinal progenitor gene expression in 2D cultures with key developmental factors like BMP and Wnt antagonists, and IGF1 [146]. Yet, these cultures couldn't fully mimic retinogenesis due to the absence of the extracellular matrix and RPE. The advent of Sasai's 3D culture method marked a significant advance, forming retinal organoids from mouse pluripotent stem cells with greater fidelity to *in vivo* development [147]. This technique that forming retinal organoids from embryoid body-like aggregates has since been refined and applied to human stem cells. More recently, a hybrid approach has evolved that leverages both 2D and 3D cultures, streamlining organoid production without the need for BMP or Wnt antagonists, utilizing endogenous signaling pathways and the later addition of retinoic acid to encourage photoreceptor development [148].

The development of retinal organoids, therefore, provides an avenue to examine retinoblastoma tumorigenesis. With the help of genome editing tools such as CRISPR-Cas9, stem cells are modified with oncogenic mutations and reporters in certain retinal cell types. For example, fluorescent reporters like

CRX-GFP and GNAT2-EGFP enable the track of photoreceptors during retina development [149, 150]. Retinal organoids derived from embryonic stem cells with *RB1* knockout revealed the important role of *RB1* in tumorigenesis and the cancerous origin in retinoblastoma with *RB1* loss [151-153]. Besides embryonic stem cells, patient-derived stem cells are also used to develop retinal organoids for retinoblastoma research [154]. Retinal organoids from patient-derived stem cells maintain genetic fidelity to the original tumors and, when engrafted in mice, replicate human retinoblastoma's molecular and cellular characteristics [155]. Retinal organoids derived from patients with germline *RB1* inactivation also provide evidence for Knudson's "Two-Hit" hypothesis by creating the second hit with CRISPR-Cas9 [156].

Our previous research established a *RB1*-proficient *MYCN*-overexpressing retinal organoid model from human embryonic stem cells by electroporation. We identified a specific time window when *MYCN* is involved in tumorigenesis and the potential cell-of-origin in horizontal cell and cone photoreceptor lineage [143]. In more recent research, *MYCN*-amplified retinal organoids from patient-derived stem cells are also used in drug testing for retinoblastoma, offering promising directions for retinoblastoma treatment strategies [157].

RNA sequencing in retinoblastoma and retina research

With the advent of Next Generation Sequencing, RNA sequencing has become a cornerstone in elucidating gene expression dynamics. The technique has evolved to offer large-scale, high-sensitivity transcriptome analysis, extending to single-cell resolution, revolutionizing the study of heterogeneous samples such as retina [158]. Single-cell RNA sequencing has rendered comprehensive transcriptomic landscapes across diverse species including mouse, chicken and human and even in retinal organoids derived from human stem cells [159-162]. Such RNA sequencing insights are pivotal in pinpointing disease markers and decoding the cellular characteristics of specific tissues. Adding to this, advancements may allow for the integration of spatial transcriptomics, potentially providing a more detailed understanding of tissue architecture and cellular interactions within their native environment [163].

In **Paper I** and **Paper II**, we performed bulk RNA sequencing and single-cell RNA sequencing on our previously established *RB1*-proficient *MYCN*-overexpressing embryonic chicken retina and derived cell cultures. We choose single-cell RNA sequencing for heterogenous developing chicken retina, while bulk RNA sequencing for derived cell cultures since they are already selected in culture that only cells that can keep proliferating remain. Through these transcriptomic studies, we gained valuable insights into how *MYCN* overexpression impacts retinal development and contributes to the pathogenesis of retinoblastoma.

Previous investigation

MYCN induces cell-specific tumorigenic growth in *RB1*-proficient human retinal organoid and chicken retina models of retinoblastoma

The work in this thesis is based on a previous study that the author has participated in [143]. In the previous work, we established both model systems with developing chicken embryonic retina and human stem cell-derived retinal organoids. In both models, we overexpressed *MYCN* using electroporation and found that *MYCN* overexpression drove retinoblastoma carcinogenesis independently with proficient RB1. The main findings of this work are concluded in Fig. 3.

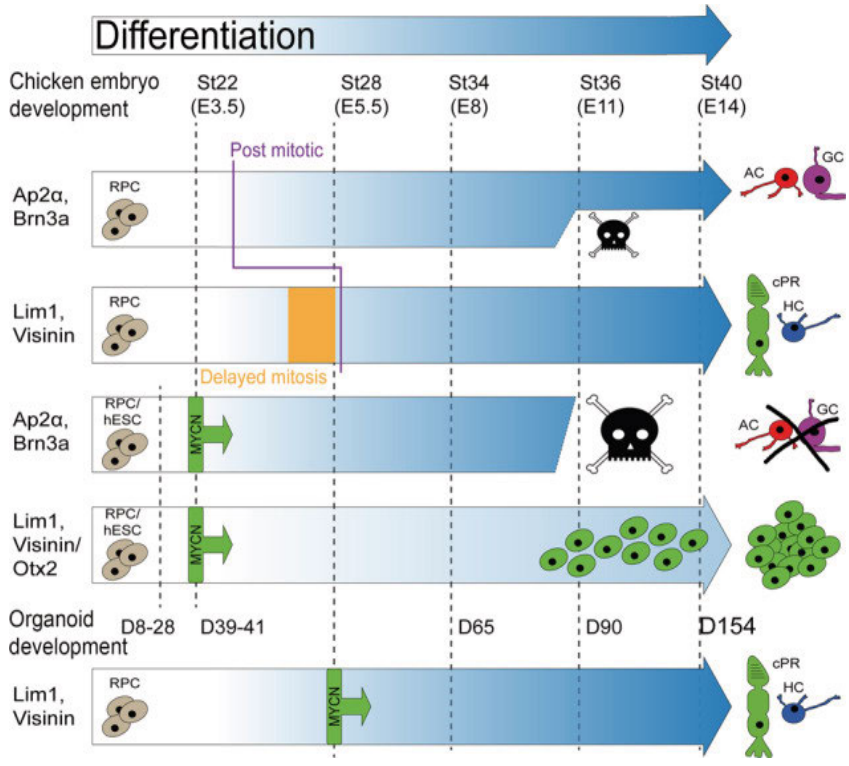

Fig. 3 Graphical abstract of previous work

MYCN overexpression induced neoplastic proliferation in a cell-type specific manner

In order to introduce *MYCN* overexpression, we used a genome-integrating PiggyBac transposon system with a bi-cistronic transcription unit expressing *MYCN* and GFP from the same mRNA.

In chicken retina, *MYCN* overexpression was introduced at st22/E3.5 by electroporation targeting retinal progenitor cells and examined in later developmental stages. By st40/E14, *MYCN* overexpression disrupted retina morphology and formed clusters of GFP positive (GFP+) cells (Fig. 4A). We also examined the cellular and molecular features by staining for cell type-specific markers of the GFP+ cells and from st34/E8 to st40/E14. The results indicated that although retinal progenitor cells were targeted initially, GFP+ cells by st40/E14 featured more photoreceptor/horizontal cell characteristics than amacrine or ganglion cells (Fig. 4B).

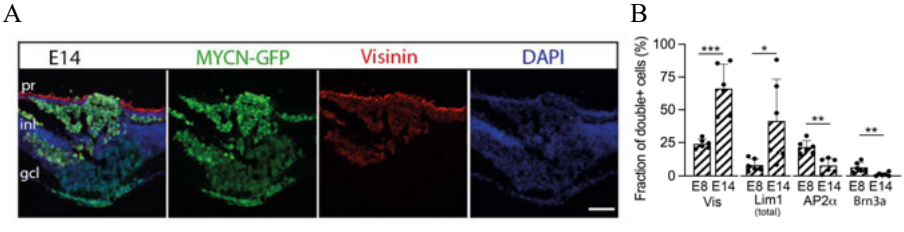

Fig. 4 *MYCN* overexpression in chicken retina

A Double-positive cells with GFP and different cell type markers at E14. **B** Fraction of double positive cells at st34/E8 and st40/E14. pr photoreceptor layer, inl inner nuclear layer, gcl ganglion cell layer, Visinin photoreceptor marker, Lim1 horizontal cell marker, Ap2α amacrine cell marker, Brn3a ganglion cell marker. Scale bars in **A** 10μm.

In human retinal organoids, similarly, *MYCN* overexpression also induced tumorigenic growth, deforming organoid morphology. The organoids was scored based on the size and structure of GFP+ cells and GFP+ cells started to detach from organoids since Day 78 (Fig. 5A, B). Moreover, from Day 78, GFP+Otx2+ and GFP+Lim1+ cells increased while GFP+Ap2α+ and GFP+Brn3+ cells decreased, indicating also cells in the lineage of horizontal cell/photoreceptor. (Fig. 5C).

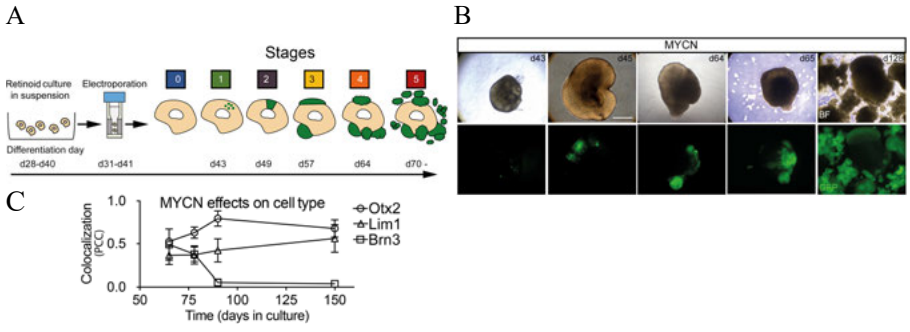

Fig. 5 *MYCN* overexpression in human retinal organoids

A Organoid stages based on sizes and structure of GFP+ cells. **B** Representative micrographs of progressive tumorigenic growth of *MYCN*-transformed cells in organoids over time. **C** Line graph of colocalization of *MYCN*-GFP and Otx2, Lim1 or Brn3. Otx2 photoreceptor marker, Lim1 horizontal cell marker, Brn3 ganglion cell marker, PCC Pearson's correlation coefficient. Scale bars in **B** 200µm.

MYCN-transformed cells grew *in vitro* and maintained photoreceptor features

Dissected GFP+ cells from st40/E14 electroporated chicken retina were transferred into *in vitro* cultures. These *MYCN*-transformed retinal cells *in vitro* maintained the expression of *MYCN*-GFP for long term (Fig. 6A). We performed bulk RNA sequencing on *MYCN*-transformed cells and dissected central area of normal retina at st40/E14. Differentially expressed genes (DEG) included cell cycle related genes and some retinal cell type markers. Compared to normal retina, *MYCN*-transformed cells were more proliferative and expressed more photoreceptor features than features of ganglion cell, amacrine cell and retinal progenitor cells (Fig. 6B). Moreover, gene set enrichment analysis on gene ontology (GO) term revealed an underrepresentation of GO terms related to neuronal differentiation and an overrepresentation of proliferation (Fig. 6C).

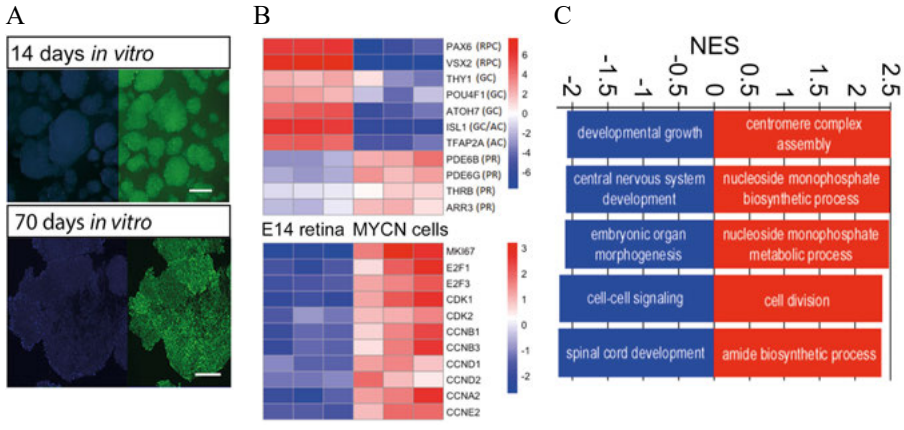

Fig. 6 *MYCN*-transformed cells *in vitro*

A Representative micrographs of *MYCN*-GFP cells dissected from electroporated chicken retina at st40/E14 in culture of two stages. **B** Part of retinal cell type markers and cell cycle related genes among differentially expressed genes (DEG) from bulk RNA sequencing. **C** Top 5 underrepresented and overrepresented gene ontology (GO) terms from DEGs. RPC retinal progenitor cells, GC ganglion cells, AC amacrine cells, PR photoreceptor, NES normalized enrichment score. Scale bars in **A** 300µm.

To conclude, the ability of *MYCN* to drive retinoblastoma tumorigenesis with proficient *RB1* underscores its potent oncogenic role in retinal cells. This research opens up new perspectives on retinoblastoma pathogenesis and highlights the need for therapeutic strategies targeting *MYCN* and its associated pathways. Further investigations are warranted to elucidate the precise mechanisms through which *MYCN* promotes tumorigenesis and to explore its interactions with other oncogenic or tumor suppressor pathways in the retinal cellular context.

Aims

The overall aim of this thesis is to delve into the complex molecular and cellular mechanisms involved in retinoblastoma, emphasizing the impact of MYCN expression and its interaction with cell cycle and apoptosis pathways.

Paper I

To further understand the regulatory mechanisms in *MYCN*-induced retinoblastoma, specifically how high-level E2F expression in *RB1*-proficient *MYCN*-overexpressing cells contributes to retinoblastoma's neoplastic behavior.

Paper II

To investigate the effect of *MYCN* in retina development and analyze *MYCN*-induced retinoblastoma in a developmental perspective, employing single-cell RNA sequencing to also assess the specific intrinsic features and heterogeneity of MYCN-transformed cells in culture.

Paper III

To expand the focus to the interplay between *MYCN*, E2f activity and p53 pathway in human retinoblastoma cell lines harboring both *RB1* deficiency and *MYCN* amplification, by manipulating these molecular pathways using chemical inhibitors.

Present investigations

Paper I

Zhang, H., Konjusha, D., Rafati, N., Tararuk, T., Hallböök, F. (2023). Inhibition of high level E2F in a RB1 proficient MYCN overexpressing chicken retinoblastoma model normalizes neoplastic behaviour. *Cell Oncol (Dordr)*

Introduction

As previously mentioned, retinoblastoma arises predominantly due to *RB1* gene mutations. However, a subset of retinoblastomas exhibits *MYCN* amplification, indicating an alternative oncogenic pathway. This study focuses on understanding the role of *MYCN* in retinoblastoma, particularly how its overexpression influences cell cycle regulation and tumorigenesis in the absence of *RB1* mutations. The introduction underscores the significance of exploring *MYCN*'s interaction with E2F transcription factors, given their pivotal role in cell cycle control and the potential for novel therapeutic targets in *MYCN*-driven retinoblastoma.

Results

Based on the previously established *RB1*-proficient *MYCN*-overexpressing retinoblastoma model from chicken embryonic retina, this study continued the investigation of the *MYCN*-transformed cells *in vitro* (DMC cells). DMC cells grew in suspension and formed clusters, also showing tumorigenic potential and cone photoreceptor (cPR) features. Immunohistochemistry (IHC) further verified the expression and phosphorylation of the retinoblastoma protein (Rb), suggesting an active Rb regulatory system. This indicates that despite *MYCN* overexpression, the Rb protein remained proficient in these cells. Cell cycle analysis on these cells found that the cells exhibited a distorted cell-cycle profile with a noticeable sub-G1 peak, indicative of ongoing apoptosis. However, this cell death did not significantly impact the overall positive growth of DMC cells. Moreover, treatment with Palbociclib, a Cdk4/6 inhibitor, resulted in a partial G1-phase arrest. This is consistent with the presence of a proficient Rb pathway, as Palbociclib's effect on G1-phase arrest aligns with *RB1*'s role in cell cycle regulation.

The bulk RNA sequencing on DMC cells revealed a gene expression profile of both cPR and tumor signature, aligning with the tumor's origin from the cPR lineage, mirroring the cell-of-origin in *RB1*-deficient cancers. Notably, the expression of several activating E2F gene family members including *E2F1* and *E2F3* was found to be upregulated in these cells. The regulation of E2F activity is directly influenced by the Rb. Despite the cells being proficient in *RB1*, indicating functional Rb protein, the elevated levels of E2F appeared to disrupt normal cell cycle regulation, contributing to the neoplastic phenotype of the tumors. Additionally, the results hinted at a degree of resistance to p53 activation within these cells.

Nutlin-3a and Pifithrin-α, targeting p53, were tested on DMC retinoblastoma cells. DMC cells showed no significant change in cell cycle or p21 mRNA levels after treatment, suggesting an altered or insensitive p53 pathway in these cells. On the other hand, the E2f inhibitor HLM006474 effectively induced cell cycle arrest and increased p21 mRNA levels in DMC cells, consistent with its expected action on cell cycle regulation. Long-term treatment with HLM006474 led to apoptosis in DMC cells, indicating its potential therapeutic effect in targeting the elevated E2F levels associated with the neoplastic phenotype in this *RB1*-proficient retinoblastoma model.

Discussion

The study investigates retinoblastoma tumor cell lines induced *in vivo* with *MYCN* overexpression, developed in the chicken embryonic retina, representing an early form of *RB1*-proficient *MYCN*-amplified retinoblastoma. DMC cells, established shortly after initial tumor formation signs, represent an early, pristine form of this retinoblastoma type, contrasting with Type 1 tumors (with few genetic alterations besides *RB1*-inactivation mutations) and Type 2 tumors (characterized by *MYCN*-amplification and heterogeneity). Analysis of DMC cells revealed upregulation of several activating E2F gene family members, suggesting that despite *RB1* proficiency, elevated E2F levels contribute to dysfunctional cell cycle regulation and the cancer phenotype. The established DMC cells, with *MYCN* overexpression but without *RB1* inactivation or other genetic alterations, show an anaplastic and aggressive phenotype, including optic nerve infiltration and extraocular growth. The clear cPR signature and genes indicative of cPR progenitors or immature cPRs reflect early carcinogenesis events. The majority of downregulated GO terms are related to neuronal development, without displaying overt ganglion cell markers seen in Type 2 tumors or after *MYCN* expression in fetal human retina transduced *in vitro*.

Significance

This research provides crucial insights into retinoblastoma, by demonstrating the role of E2F transcription factor in the carcinogenesis of RB1-proficient *MYCN*-overexpressing retinoblastoma. The inhibition of E2f in this model leads to cell cycle arrest and apoptosis, suggesting the potential of targeting E2F proteins as a therapeutic strategy. The results highlight the complex interplay between *MYCN* and E2F in retinoblastoma carcinogenesis and opens up new avenues for targeted therapies in *RB1*-proficient retinoblastomas.

Paper II

Zhang, H., Konjusha, D., Rafati, N., Tararuk, T., Hallböök, F. Single-cell RNA sequencing reveals cellular properties and carcinogenesis of a RB1-proficient MYCN-overexpressing retinoblastoma model from embryonic chicken retina. *Manuscript*

Introduction

The cell-of-origin in retinoblastoma has been proved to be cone photoreceptor or cone photoreceptor progenitors. Oncogenic mutations like *RB1* deficiency or *MYCN* amplification drives neoplastic transformation, particularly within cone photoreceptor lineage cells, signifying cell-type-specific susceptibility to oncogenesis. However, the specific intrinsic features that make these cells potential the cell-or-origin remain to be understood. This study performs single-cell RNA sequencing E8 and E14 embryonic chicken retina with *MYCN* overexpression and cultured *MYCN*-transformed cells, aiming to provide insight to the cellular dynamic during retinoblastoma initiation induced by *MYCN* overexpression.

Results

MYCN overexpression in embryonic chicken retina was achieved using a piggyBac transposon system with co-expression of *MYCN* and GFP, leading to proliferative cell clusters and neoplastic growth in a cell-type-specific manner. Co-localization studies showed MYCN-GFP expression alongside photoreceptor and horizontal cell markers until E10, after which it was restricted to cells with photoreceptor/horizontal cell fate. Cells from GFP-positive regions of E14 retina, when cultured *in vitro*, continued proliferating, indicating neoplastic transformation.

Next, single-cell RNA sequencing was performed on samples from E8 and E14 retina with *MYCN* overexpression and on cultured *MYCN*-overexpressing cells to understand the development and transformation process. Analysis on

E8 and E14 retina revealed heterogeneous cell populations, including neuronal progenitors and post-mitotic neurons, with some cells actively cycling. Clustering analysis identified various retinal cell types and highlighted a significant presence of *MYCN*-overexpressing cells in cone photoreceptor cluster at E14, suggesting a preferential transformation of certain cell types. *MYCN*-overexpressing cells at E14 exhibited features of cone photoreceptors but showed a less differentiated profile compared to normal E14 cone photoreceptors, suggesting that *MYCN* overexpression impedes full photoreceptor differentiation. Heterogeneity was observed within *MYCN*-overexpressing cells both at E14 and cultured cells, with some showing features of ganglion cells and Müller glia markers, indicating a broad differentiation potential.

Moreover, analysis on cultured *MYCN*-overexpressing cells found one cluster enriched of cells at G2/M phase. Gene ontology analysis of this cluster highlighted upregulated genes involved in cell cycle processes and mitosis in specific clusters, indicating active proliferation. Genes related to cell cycle progression and proliferation were also found differentially expressed in this cluster. High level expression of *UBE2C*, a gene associated with cell cycle regulation and tumorigenesis, was also found in this cluster. Treatment with an E2f inhibitor reduced *UBE2C* expression in cultured *MYCN*-overexpressing cells, suggesting its regulation by E2F and potential as a therapeutic target.

Discussion

This study first underscores the efficacy of *MYCN* overexpression in inducing metastatic retinoblastoma within an *RB1* proficient embryonic chicken retina context, pointing to a significant overlap between retinoblastoma carcinogenesis and retinal development. Next, through single-cell RNA sequencing, the research elucidates the expression profiles of *MYCN*-transformed cells at different embryonic stages (E8 and E14), revealing a preference for neoplastic transformation within the cone photoreceptor (cPR) lineage. This suggests a lineage-specific vulnerability to oncogenic transformation, supported by the observation of high endogenous *MYCN* expression in normal cPR progenitors. Moreover, the analysis uncovers considerable heterogeneity among *MYCN*-transformed cells, both *in vivo* and *in vitro*. A notable fraction of these cells retains cone characteristics, while others exhibit markers indicative of various retinal cell types, including ganglion cells and Müller glia. This heterogeneity supports the notion of a cPR progenitor as the cell of origin for retinoblastoma but suggests a broader differentiation range beyond merely cone cells.

The expression profile of cultured *MYCN*-overexpressing cells showed high *UBE2C* levels, a gene associated with cell cycle regulation and tumorigenesis. *UBE2C*'s interaction with the KAT2A/E2F1 complex and its regulation by *E2F1* suggest a mechanism for *MYCN*-induced retinoblastoma. *UBE2C* is involved in transition to G2/M phase during cell cycle. Based on high level

expression of E2f and G2/M phase genes like *UBE2C*, we hypothesize that both G1 checkpoint and G2 checkpoint are set out of play in *MYCN*-induced retinoblastoma.

This study also advocates for the chicken retina as a suitable model for retinoblastoma research due to its cone-driven retina and diurnal vision, contrasting with mouse models that fail to replicate human retinoblastoma accurately due to evolutionary differences in the cPR system.

Significance

The significance of this research lies in its detailed exploration of *MYCN*-overexpressing *RB1*-proficient retinoblastoma, using an embryonic chicken retina model to understand tumorigenesis from a developmental standpoint. The findings reinforce the notion that cone photoreceptor progenitors serve as the origin of this cancer type, highlighting an intrinsic predisposition towards *MYCN*-driven oncogenic transformation in these cells. The study also unveils a critical link between elevated *UBE2C* levels and *MYCN*-induced E2f activity, pointing to a dual deficiency in G1/S and M-phase cell cycle checkpoints. This dual checkpoint deficiency offers a novel explanation for the mechanisms underlying retinoblastoma carcinogenesis, thereby contributing valuable insights into the field and opening new avenues for targeted therapeutic interventions.

Paper III

Zhang, H., Tararuk, T., Hallböök, F. Characterization of MYCN amplified retinoblastoma lines with respect to effects of E2f and p53 activity. *Manuscript*

Introduction

Retinoblastoma, a childhood intraocular malignancy, typically results from biallelic inactivation of the *RB1* gene, leading to deregulated E2F activities and uncontrolled cell cycle progression. The Rb protein, when bound and inhibited by E2f proteins, forms a checkpoint that regulates the G1 to S phase transition in the cell cycle. This process is disrupted in retinoblastoma, resulting in constitutively active E2fs and a dysregulated cell cycle, a major contributor to the malignancy. Besides *RB1* mutations, a subset of retinoblastomas exhibits *MYCN* amplification, presenting a distinct tumor subtype with anaplastic features, histological undifferentiation, early onset, and increased metastatic potential, regardless of *RB1* status. Unlike many cancers, retinoblastomas infrequently harbor *TP53* mutations, suggesting an intrinsic resistance to p53-mediated apoptosis in their cell of origin. This resistance could be attributed to

developmental origins from cells inherently resistant to p53 or apoptosis. The p53 pathway's role is further complicated by the influence of it regulator *MDM2* and *MDM4/X*. This study investigated the relationship between *MYCN* overexpression, E2f activity, and the p53 pathway's response, focusing on human retinoblastoma cell lines Y79 and WERI-Rb1.

Results and Discussion

We first characterized Y79 and WERI-Rb1 cells. The inactivation of *RB1* was confirmed by both gene expression and function analysis by Cdk4/6 inhibitor, Palbociclib. Treatment with Palbociclib failed to induce G1 arrest or apoptosis in both cell lines, consistent with *RB1* gene inactivation. Treatment with Nutlin-3a, an Mdm2 inhibitor, diminished cell viability and induced apoptosis in both cell lines, indicating a functional p53 pathway.

In previous work, it was demonstrated that *MYCN* overexpression in chicken embryonic retina initiates retinoblastoma characterized by elevated E2F levels. This study focused on human retinoblastoma cell lines Y79 and WERI-Rb1, both of which exhibit high *MYCN* expression. Analysis revealed increased *E2F1* mRNA in these cell lines compared to the control Müller glia cells line MIO-M1, suggesting that *MYCN* amplification might also drive the upregulation of E2F expression, contributing to the neoplastic phenotype. Further investigation involved treating Y79 and WERI-Rb1 cells with HLM006474, an E2f DNA binding activity inhibitor, which significantly reduced cell viability, indicating the critical role of E2f in the growth of retinoblastoma cells. The study also explored the effects of additional *MYCN* overexpression through electroporation, which did not elevate E2F1 mRNA levels further, possibly due to cellular apoptosis triggered by excessive *MYCN*. The findings suggest that targeting E2f might be an effective therapeutic strategy in both *RB1*-deficient and *MYCN*-amplified retinoblastoma subtypes.

Moreover, the study examined the interplay between E2f inhibition and p53 activity, finding that HLM006474-induced cell death did not rely on p53 activation, indicating a p53-independent mechanism of action for HLM006474. To further study the effects of E2f inhibition and p53 activation on cell viability, dose-response curves were established for HLM006474 and Nutlin-3a on Y79 and WERI-Rb1 cell lines to identify the half maximal effective concentrations (EC50). The combination of HLM006474 and Nutlin-3a showed nearly additive effects on growth inhibition in both cell lines, suggesting that E2f inhibition and p53 activation operate through parallel and potentially independent pathways. Further investigation revealed that pre-treatment with HLM006474 altered the cells' response to subsequent Nutlin-3a treatment. Specifically, pre-treatment with HLM006474 significantly impacted relative growth, but when followed by Nutlin-3a treatment, the difference in cell viability/growth was not significant. This indicates that Nutlin-3a's effectiveness

requires active E2f, and thus cell cycle progression, for significant growth inhibition. These findings suggest that E2f activity is crucial for p53-mediated cell cycle arrest, and inhibiting E2f with HLM006474 does not primarily involve p53 activity but likely relates to mechanisms at the G1 checkpoint regulated by Rb1 and S-phase progression.

Next, we studied the impact of MYCN expression on p53 activation sensitivity in Y79 cells. Additional MYCN overexpression by electroporation on Y79 sensitized cells to Nutlin-3a, a p53 activator, suggesting a potential vulnerability of tumor cells to p53 pathway activation. We also examined the interplay between *MYCN* and the MDM2-p53 signaling pathways, noting that *MYCN* overexpression can modulate *MDM2* levels and thus affect the p53 response. This observation is further supported by examining the effects of the BET protein inhibitor JQ1 on retinoblastoma cells. JQ1 is known for inhibiting Brd4 and consequently suppressing *MYCN* transcription. Dose-response analysis established that JQ1 effectively inhibited growth in both Y79 and WERI-Rb1. This inhibition also correlated with a marked reduction in *MYCN* mRNA levels in Y79 cells post-treatment. The combination of JQ1 and Nutlin-3a led to a dose-dependent increase in growth inhibition, suggesting a synergistic effect. Notably, pre-treatment with JQ1 enhanced the cells' sensitivity to subsequent Nutlin-3a treatment, indicating a potential priming effect of JQ1 on the p53 pathway.

However, this observation appeared to contradict earlier findings that increased *MYCN* expression sensitized Y79 cells to p53 activation. To reconcile these findings, the study examined the influence of JQ1 on other p53 targets, discovering that JQ1 treatment elevated *NOXA* expression, a direct p53 target involved in apoptosis, suggesting that JQ1's effects might be predominantly p53-dependent rather than solely through *MYCN* regulation.
Given the multifaceted roles of BRD proteins in cellular processes, including chromatin remodeling and transcriptional co-activation, the study posits that targeting these proteins, particularly Brd4, offers a strategic advantage in cancer therapy. This is underscored by the observed synergistic effect of combining JQ1 with Nutlin-3a, which not only suppresses *MYCN*-driven oncogenesis but also potentiates p53 pathway activation, providing a dual therapeutic approach for *MYCN*-driven retinoblastoma.

Significance

The findings of this study collectively emphasize the intricate interplay among *MYCN* expression, E2f activity, and the p53 regulatory axis in retinoblastoma. They offer a refined understanding of the disease's mechanism and suggest that targeted modulation of *MYCN* and E2F, alongside p53 activation, could be an effective approach in managing retinoblastoma.

Acknowledgements

I would like to express my gratitude to my supervisor Finn Hallböök for giving me the opportunity to conduct my PhD in his group, and on such an interesting project. Thank you for all the support and guidance on research ideas and on my development. I would also like to express my appreciation to my co-supervisor Malin Lagerström for her constructive suggestions and meaningful talks.

I would like to thank all the co-authors of the papers and former and present colleagues in the group. Without you, the work in this thesis would never be achieved. Thank you for your technical support, inspiration on research ideas and wonderful time we shared in the lab.

Lastly, a thank you from the bottom of my heart to my friends and family, my beloved ones. Thank you for being there for me no matter what. Thank you for backing me up when things were difficult. Thank you for being the light in the darkest of times.

This journey has been one of growth, challenge, and discovery, and each one of you has played an integral part in that. Your support has been a beacon of hope and a source of strength. I am profoundly grateful for the role each of you has played in bringing this work to fruition and in shaping the person I have become.

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