Exploring novel therapeutic strategies in neuroblastoma

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Cover illustration: Immunofluorescent staining of mitotic cells.

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”Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less”.

Maria Skłodowska-Curie

To my Family and Friends

Exploring novel therapeutic strategies in neuroblastoma

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ABSTRACT

Neuroblastoma (NB) is the most frequently diagnosed extracranial tumour in children, which arises from transient embryonal tissue of the neural crest that fails to complete terminal differentiation into neurons. Even after completion of successful therapy, high risk neuroblastoma patients typically suffer from post-treatment induced toxicity which impacts on their ability to lead a normal life. Traditional protocols including chemotherapeutic and radiation therapy treatments are associated with toxic side effects due to a lack of specificity for malignant cells. Therefore, a rapidly expanding panel of targeted therapy agents are actively being explored. One example of targeted therapy is the use of small-molecule tyrosine kinase inhibitors (TKIs), a number of which have been developed and FDA approved as cancer therapeutics. Anaplastic lymphoma kinase (ALK) is one such TKI target in NB, where genetic analysis has identified *ALK* mutations in both sporadic and inherited NB, and at a higher frequency in relapsed cases. ALK TKIs are currently employed in adult ALK-positive cancer patients where they elicit good responses, prior to development of resistance.

In this thesis, we have focused on improving our understanding of known and novel molecular pathways involved in NB progression for further targeting (study I and III). We also tested a recently developed novel ALK TKI (study II) in a preclinical setting as an alternative strategy to treat NB patients in the future.

Keywords: Neuroblastoma, Anaplastic Lymphoma Kinase, Targeted therapy, ATR inhibition

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SAMMANFATTNING PÅ SVENSKA

Neuroblastoma (NB) är den oftast diagnostiserade extrakraniella tumören hos barn, vilken härrör från tillfällig embryonal vävnad i neurallisten som inte nått slutgiltig differentiering till nervceller. Även efter framgångsrikt avslutad behandling drabbas patienter med hög risk av neuroblastom vanligtvis av toxicitet som påverkar deras förmåga att leva ett normalt liv. Traditionella behandlingsmetoder inklusive cellgifts- och strålbehandlingar är förknippade med toxiska biverkningar på grund av bristande specificitet för maligna celler. Därför utforskas en snabbt växande panel av medel för målinriktad terapi. Ett exempel på målinriktad terapi är användningen av småmolekylära tyrosinkinashämmare (TKI), av vilka ett antal har utvecklats och FDA-godkänts för cancerbehandling. Anaplastiskt lymfomkinas (ALK) är ett sådant TKI-mål i NB, där genetisk analys har identifierat ALK-mutationer i både sporadisk och ärftlig NB, och vid en högre frekvens i återfall. ALK-TKI används för närvarande hos vuxna ALK-positiva cancerpatienter där de framkallar goda resultat före resistensutveckling.

I denna avhandling har vi fokuserat på att förbättra vår förståelse för kända och nya molekylära signaleringsvägar delaktiga i NB-progression för vidare studier (studie I och III). Vi undersökte också en nyligen utvecklad ALK TKI (studie II) i prekliniska miljöer som alternativ strategi för att bota NB-patienter i framtiden.

LIST OF PAPERS

I. Van den Eynden J, Umapathy G, Ashouri A, Cervantes-Madrid D, **Szydzik J**, Ruuth K, Koster J, Larsson E, Guan J, Palmer RH, Hallberg B. Phosphoproteome and gene expression profiling of ALK inhibition in neuroblastoma cell lines reveals conserved oncogenic pathways. Sci Signal. 2018 Nov 20;11(557):eaar5680. doi: 10.1126/scisignal.aar5680

II. Cervantes-Madrid D, **Szydzik J**, Lind DE, Borenäs M, Bemark M, Cui J, Palmer RH, Hallberg B. Repotrectinib (TPX-0005), effectively reduces growth of ALK driven neuroblastoma cells. Sci Rep. 2019 Dec 18;9(1):19353. doi: 10.1038/s41598-019-55060-7

III. **Szydzik J**, Lind DE, Umapathy G, Hallberg B and Palmer RH, Modulation of SUN2 phosphorylation downstream of ALK pathway identifies a role for ATR in neuroblastoma cell survival. Manuscript 2020

CONTENTS

ABBREVIATIONS: v
1. INTRODUCTION: 1
1.1. Cancer 1
1.2. Hallmarks of cancer 2
1.3. Genome heterogeneity and instability 5
1.4. Adult versus childhood cancers 5
1.5. Neuroblastoma 6
1.5.1. Origin of neuroblastoma 6
1.6. Genetic aberrations in neuroblastoma 9
1.6.1. Chromosome 1p deletions 9
1.6.2. Chromosome 2p gain (*MYCN*, *ALK* and *ALKAL2*) 9
1.6.3. Chromosome 11q loss 10
1.6.4. Chromosome 17q gain 11
1.6.5. Aneuploidy 12
1.7. Diagnosis of NB 13
1.8. Risk stratification and current treatment options in NB 13
1.9. Treatment strategies in neuroblastoma 14
1.9.1. Surgery 14
1.9.2. Chemotherapy 14
1.9.3. Haemopoietic stem cell transplantation 15
1.9.4. Radiation 15
1.9.5. Retinoic acid treatment 15
1.9.6. Immunotherapy 15
1.10. Novel approaches for cancer treatment in neuroblastoma 16
1.10.1. Inhibition of ALK 17
1.10.2. Inhibition of ATR 17
1.10.3. Inhibition of Aurora A kinase 17
1.10.4. Inhibition of CDK4/6 18
1.10.5. Inhibition of WEE1 and Chk1 19
1.10.6. Alterations in *ATRX*, *TERT* and ALT 19
1.10.7. Inhibition of mTORC1/2 21
1.10.8. Targeting BIRC5 22
1.11. Targeting anaplastic lymphoma kinase in neuroblastoma 22

1.11.1. Receptor tyrosine kinases (RTK) 22
1.11.2. Anaplastic lymphoma kinase (ALK) 23
1.11.3. Activation of ALK 25
1.11.4. ALK in neuroblastoma 25
1.12. Oncogenic ALK signaling 26
1.13. ALK positive cancers 28
1.13.1. Anaplastic large cell lymphoma 28
1.13.2. Non-small cell lung cancer (NSCLC) 28
1.13.3. Inflammatory myofibroblastic tumours (IMT) 29
1.13.4. Diffuse large B-cell lymphoma (DLBCL) 30
1.14. ALK inhibitors 30
1.14.1. Crizotinib 31
1.14.2. Lorlatinib 32
1.14.3. Repotrectinib 33
1.15. Targeting ATR in neuroblastoma 34
1.15.1. Cell cycle and its regulation 34
1.15.2. Cell cycle checkpoints 35
1.15.3. An intrinsic S/G2 checkpoint enforced by ATR 37
1.15.4. The DNA damage response 38
1.15.5. Ataxia Telangiectasia Mutated and Rad3-related kinase 38
1.15.6. Mutations in DDR related genes 40
1.15.7. ATR inhibitors 40
1.15.7.1. AZD6738, ceralasertib 42
1.15.7.2. BAY1895344 42
1.15.7.3. M6620 (formerly VE-822, VX-970 berzosertib) 43
1.15.7.4. M4344 (VX-803) 44
2. AIMS: 45
3. MATERIALS AND METHODS: 46
3.1. Cell culture 46
3.2. Inhibition of ALK activity in neuroblastoma cell lines 46
3.3. Immunoblotting 46
3.4. Immunofluorescence 46
3.5. Apoptosis assay 47

3.6. Proliferation assay ... 47
3.7. Neurite outgrowth assay ... 47
3.8. ALK phosphorylation in PC-12 cells ... 47
3.9. RNA-seq sample preparation ... 47
3.10. RNA-seq data analysis ... 48
3.11. Proteomic/Phosphoproteomic sample preparation: ... 48
3.12. Proteomic/Phosphoproteomic sample analysis: ... 48
3.13. Xenograft neuroblastoma model ... 49
3.14. Software for data presentation ... 49
3.15. Statistical analysis ... 49
4. RESULTS AND DISSCUSION: ... 50
4.1. Paper I: ... 50
4.2. Paper II: ... 52
4.3. Paper III: ... 54
5. CONCLUSIONS: ... 56
5.1. Paper I: ... 56
5.2. Paper II: ... 56
5.3. Paper III: ... 57
6. ACKNOWLEGMENTS: ... 58
7. LIST OF FIGURES: ... 65
8. REFERENCES: ... 66

ABBREVIATIONS:

ALCL- anaplastic large cell lymphoma

ALK- anaplastic lymphoma kinase

ALKAL1- ALK and LTK Ligand 1

ALKAL2- ALK and LTK Ligand 2

ALO17- ALK lymphoma oligomerization partner on chromosome 17

ALT- alternative lengthening of telomeres

ATM- ataxia telangiectasia mutated

ATR- ataxia telangiectasia and Rad3 related

ATRA- all-transretinoic acid, tretinoin

ATRX- alpha thalassemia/mental retardation syndrome X-linked

BMP- bone morphogenetic protein

BIRC5- baculoviral inhibitor of apoptosis repeat-containing 5

CC- chromaffin cells

CDK- cyclin-dependent kinase

CHEK1- checkpoint kinase 1

CHEK2- checkpoint kinase 2

CKI- cyclin-dependent kinase inhibitor

CLTC- clathrin heavy chain

CNA- copy number alteration

CNS- central nervous system

CT- computerized tomography

CXCR4- C-X-C motif chemokine receptor

DA- dorsal aorta

DAXX- death-domain associated protein

DD- DNA damage

DDR- DNA damage response

DG- dorsal ganglia

DLBCL- diffuse large B-cell lymphoma

DLG2- disc large homolog 2

ECD- extracellular domain

EFS- event-free survival

EGFR- epidermal growth factor receptor

EML4- echinoderm microtubule associated protein-like 4

FDA- Food and Drug Administration

FGFR2- fibroblast growth factor receptor 2

FOXM1- forkhead Box M1

G1- gap 1 phase

G2- gap 2 phase

GD2- disialoganglioside 2

GOF- gain-of function

GR- glycine-rich region

GWAS- genome-wide association studies

HSC- hematopoietic stem cells

IGF-1R- insulin-like growth factor receptor 1

IMT- inflammatory myofibroblastic tumour

InR- insulin receptor

INSS- International Neuroblastoma Staging System

JAK- Janus kinase

KIF5B- kinesin family member 5B

KLC1- kinesin light chain 1

LDLa- low density lipoprotein class A

LOF- loss-of function

LTK- leukocyte tyrosine kinase

M- mitosis

MAM- meprin A5 protein and receptor protein tyrosine phosphatse mu

MHY9- non-muscle myosin heavy chain 9

MIBG- metaiodobenzylguanidine

MRI- magnetic resonance imaging

MSN- moesin

mTOR- mammalian target of rapamycin

N- notochord

NB- neuroblastoma

NCC- neural crest cells

NPM- nucleophosmin

NRG1- neuregulin 1

NRTK- non-receptor tyrosine kinase

NSCLC- non- small cell lung cancer

NT- neural tube
PC12- pheochromocytoma 12 cells
PI3K- phosphoinositide 3-kinase
PK- protein kinase
PLCγ- phospholipase Cγ
PTK- protein tyrosine kinase domain
PTPN3- protein tyrosine phosphate non-receptor type 3
RA- retinoic acid
RB- retinoblastoma
RS- replication stress
RTK- receptor tyrosine kinase
S- synthesis
SAP- sympathoadrenal precursor cells
SBRCT- small blue round cell tumour
SCA- segmental chromosomal alterations
SCP- Schwann Cell Precursors
SDF1- stromal cell-derived factor 1
SG- sympathetic ganglion
SHANK2- SH3 and Multiple Ankyrin Repeat Domains 2
SN- sympathetic neurons
SNV- single nucleotide variant
SRG- suparenal symphatetoc ganglion
STAT- signal transducer and activator of transcription
STRN- striatin
SUN2- Sad1 and UNC84 Domain Containing 2
SV- structural variation
TAT- targeted alpha therapy
TERT- telomerase reverse transcriptase
TFG- TRK fused gene
TMD- transmembrane domain
TPM3/4- tropomyosin 3 and 4
TSG- tumor suppressor gene
TT- targeted therapy

1. INTRODUCTION:

1.1. Cancer

Cancer is one of the major causes of morbidity and mortality worldwide. Cancer refers to a heterogeneous group of diseases that share biological properties such as uncontrolled growth and abnormal cellular phenotypes as well as the potential to spread to other parts of the body. The transformation of normal cells into tumour cells is usually caused by series of events, including somatic mutation, and/or hereditary predisposition, as well as involving activation of oncogenes and inactivation of tumour suppressor genes or altering DNA repair genes. Therefore, each patient's tumour is characterised by a unique combination of genetic and epigenetic changes (Vogelstein et al., 2013). The transformation process of normal cell into a cancer cell is called carcinogenesis and typically progresses from a pre-cancerous lesion (neoplastic transformation) to a malignant tumour. Increased incidence of developing cancer is also associated with exposition to carcinogenic agents (physical, chemical, biological) as well genetic predispositions (Fig. 1). Untreated tumours eventually affect surrounding tissues, penetrating the organs and cause tremendous damage to patients' health.

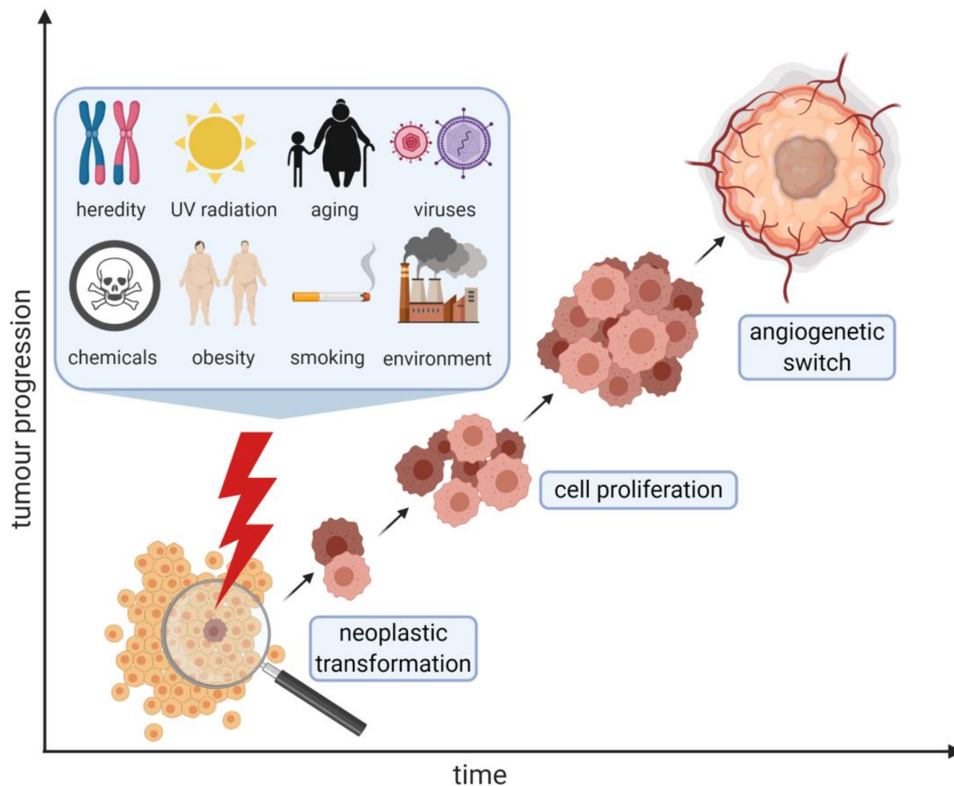

Fig. 1 Cancer initiation and progression.

The neoplastic transformation of adult cancer is usually an effect of life-lengthening accumulative exposure to environmental causes and inherited genetic predispositions.

The carcinogenic cascade is usually irreversible, often resulting in chronic inflammation (which causes immune system malfunction due to persistent activation) or pain due to increased pressure on nerves. Early diagnosis of cancer is a key factor in patient outcome. In many cases, delayed cancer diagnosis reduces patient survival, however progression varies greatly between types of cancer and between individual patients (Vogelstein et al., 2013).

1.2. Hallmarks of cancer

In 2000, Hanahan and Weinberg defined six characteristics associated within processes that transforms normal cells into cancer including self-sufficiency for growth signals, insensitivity to growth inhibition signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, which are briefly described below:

(1) Self-sufficiency for growth signals

Normal cells are dependent on growth-promoting signaling, both autocrine and paracrine, that controls the tightly-regulated cell cycle machinery to actively proliferate and maintain tissue homeostasis. In contrast, cancer cells reduce their dependence on paracrine signals, therefore staying independent of the cellular microenvironment. The autocrine signaling includes growth ligands, their receptors as well as cytosolic signaling molecules which play important role in cancer self-sufficient maintenance.

(2) Insensitivity to inhibitory growth signals

Maintaining a high level of cellular homeostasis is a main goal for healthy cells. Tumour suppressor genes (TSG) induce quiescence or differentiation in cells and ensure that they are ready to divide or to halt division when DNA damage occurs. In contrast, cancer cells perturb cellular homeostasis and alter tumour suppressor functions, which lead to inefficient prevention of abnormal cell division. Cancer cells are also insensitive to contact inhibition and are able to continue growth and division, regardless of their surroundings.

(3) Evasion of apoptosis

Apoptosis is a naturally occurring cellular mode of cell death that organisms have evolved to eliminate unnecessary or unhealthy cells. This process is highly regulated upon extra- or intracellular signals, however cancer cells acquire resistance to escape apoptotic programs.

(4) Limitless replicative potential

The replicative potential of most mammalian cells is limited by the length of the telomeres. Telomeres are specialized structures of repetitive DNA at the end of chromosome that are involved in the cell replication. They are also extremely important to maintain chromosomal stability and protect the ends of the chromosomes against degradation. Each replication event leads to degradation between 50-200 base pairs of telomeric DNA (Zhao et al., 2009). Shortening of telomeres is associated with aging and when telomeres are critically short, a process called ‘crisis’ takes place and this eventually leads to cell death (Shay, 2016). Cancer cells are able to disturb this dynamic equilibrium and maintain high levels of telomerase activity to achieve limitless replication potential and avoid telomere shortening.

(5) Sustained angiogenesis

Growth of blood vessels, in a process called angiogenesis, helps cancer cells to build a potent network that provides better access to nutrients and facilitate metastasis.

(6) Tissue invasion and metastasis

In order (for tumours) to acquire metastatic properties, an ability to spread throughout the body, two crucial mechanisms are involved: invasion and metastasis. Invasive cancers are those which directly expand and penetrate the neighbouring tissues, whereas the process of metastasis is highly complex, involving migration of the malignant cells from the origin site which are able to invade blood and/or lymph vessels in order to spread at distal sites.

Weinberg and Hanahan (Hanahan and Weinberg, 2011) later proposed two additional hallmarks: (7) abnormal metabolic pathways and (8) evasion of the immune system, and two supportive characteristics: genome instability, and tumour-promoting inflammation that facilitates neoplasia (Fig. 2) (Hanahan and Weinberg, 2000).

(7) Abnormal metabolic pathways

In contrast to normal cells, which produce energy by glycolysis followed by oxidation of pyruvate in mitochondria, tumour cells have a much higher rate of glucose consumption and largely relay on glycolysis followed by lactic acid fermentation in the cytosol (the Warburg effect).

(8) Evasion of the immune system

The immune system possesses a capacity to identify abnormal and damaged cells and destroy them before they can develop into malignancy. Cancer cells acquire the ability to evade destruction by the body’s immune system. Tumour cells produce several

immune suppressing cytokines that interfere with checkpoint pathways of the immune system so that they cannot be recognised and eliminated (Hanahan and Weinberg, 2011).

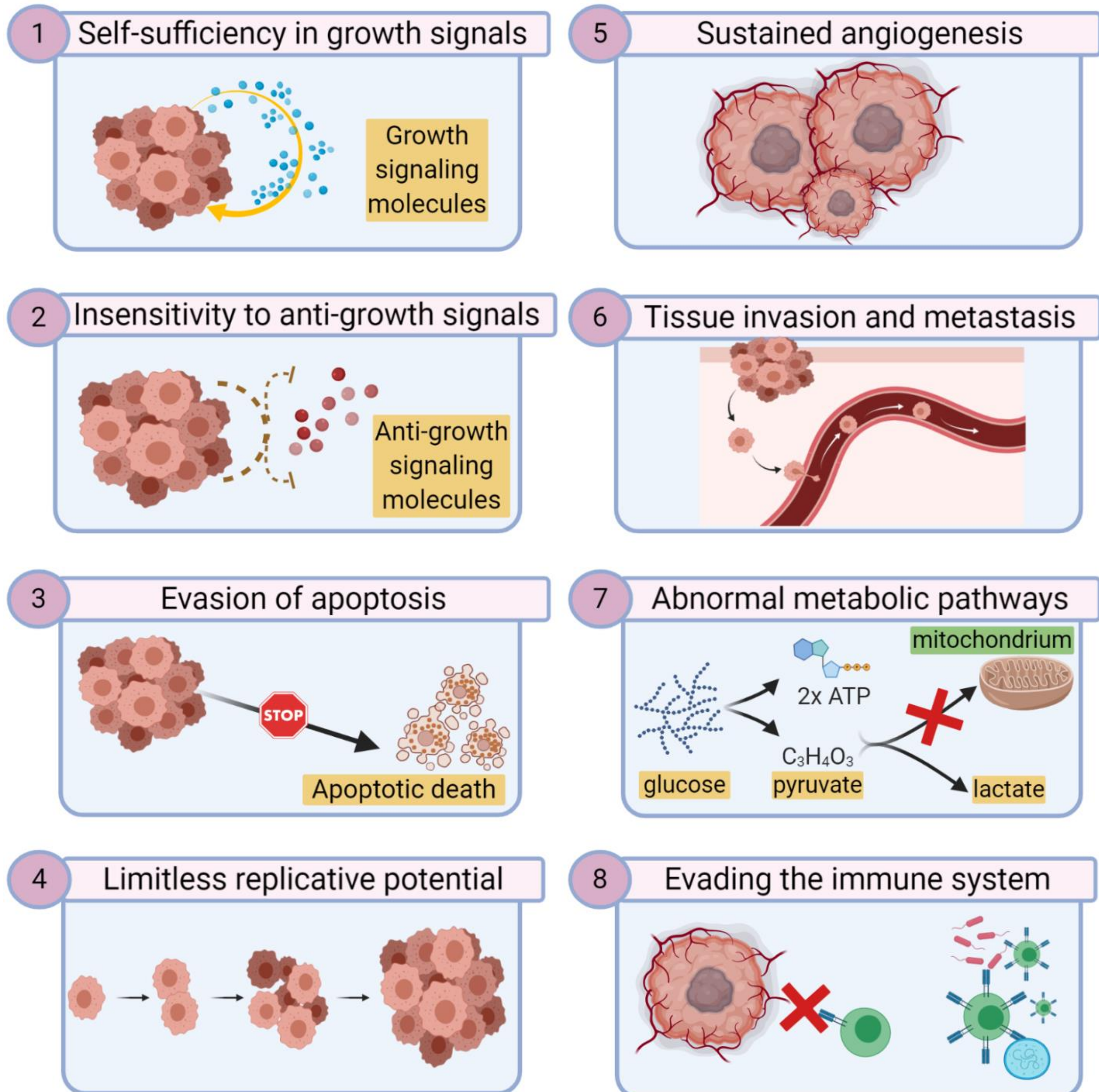

Fig. 2 Hallmarks of cancer.

Eight biological capabilities of cancer cells: 1. Self-sufficiency in growth signals, 2. Insensitivity to anti-growth signals, 3. Evasion of apoptosis, 4. Limitless replicative potential, 5. Sustained angiogenesis, 6. Tissue invasion and metastasis, 7. Abnormal metabolic pathways, 8. Evading the immune system. Based on (Hanahan and Weinberg, 2011).

1.3. Genome heterogeneity and instability

The complexity of heterogeneous interactions of different cell types in tumour can create a favourable microenvironment for neoplasia. Tumour DNA is characteristically more fragile than DNA in normal cells, leading to an elevated replication stress (Dillon et al., 2010). The majority of cancers accumulate somatic mutations over time. Among these mutations we can distinguish so called “driver gene” mutations, which give a selective advantage to a clone in its microenvironment and “passenger” mutations that confer no selective growth advantage (Vogelstein et al., 2013). Those changes influence the signaling pathways which determine cell fate by dysregulating proliferation through loss of checkpoint control or elevated level of oxidative damage (Wiseman and Halliwell, 1996). Intra-tumour heterogeneity can lead to expansion of particular subpopulation. Such tumour cells can be insensitive to the treatment regimen, and able to infiltrate surrounding tissue and spread to nearby as well as distal organs and glands causing metastasis (Negrini et al., 2010). Genomic instability has been described as a “supportive feature” of cancer cells and is caused by errors in DNA replication and repair machinery (Hanahan and Weinberg, 2011).

1.4. Adult versus childhood cancers

One difference between childhood and adult solid tumours is that adult tumours arise from differentiated adult tissues (such as epithelia of gastrointestinal tract and skin) after accumulation of multiple sequential mutations due to increasing life span and exposure to dietary, lifestyle, hereditary predisposition and others previously mentioned in section 1.2. In contrast, paediatric malignancies often originate in precursor cells of non-self-renewing tissues and have less single nucleotide variant (SNV) and nucleotide insertions/deletions than most adult malignancies (Rahal et al., 2018). Comprehensive analysis of various paediatric tumours identified at least one significantly mutated gene in 47%, with most tumours having only one. In contrast, the majority of adult cancer (76%) harbour recurrent mutations in multiple genes. In addition, TP53 was identified as the most frequently somatically mutated gene in both childhood and adulthood malignancies (Gröbner et al., 2018). Development of cancer early in life is associated with specific (rare) birth defects, that are consequences of perturbations in certain cellular signaling processes during development and neuroblastoma is one such example.

1.5. Neuroblastoma

Neuroblastoma (NB) is the most common extracranial solid tumour in children. After leukaemia and cranial tumours, it is the third most common paediatric malignancy worldwide (Park et al., 2013). This relatively rare disease affects 1 in 8,000 live births (20-25 individual per 1 million) and accounts for 6-10% of all childhood tumours. Sadly, that makes NB the most deadly tumour of childhood, which accounts for 12-15% of all paediatric cancer related deaths (Brodeur, 2003; Park et al., 2010). The majority (90%) of NB tumours arise in children younger than 10 years (including 40% of children younger than a year) with a median age at diagnosis of 17–18 months (London et al., 2005; Maris, 2010; Stiller and Parkin, 1992). Almost all NB arises sporadically, with the familial form of neuroblastoma being rare and accounting for only 1% of cases.

Several genetic alterations are often observed in NB, including gains and losses of chromosomal parts as well as whole karyotype near diplo- and tetraploid aberrations which are correlated with poor patient survival (discussed in section 1.6). Histologically, this solid tumour of infancy has been described as a small blue round cell tumour (SBRCT) characterised by high heterogeneity and poorly differentiated cells. NBs are localised along the sympathetic chain with the mass originating in the adrenal medulla of the adrenal gland (47%), nerve tissues of abdomen (24%), thoracic (15%) or in the pelvis (3%) or neck (2.7%), or other sympathetic ganglia near the spine in the chest (7.9%), (Maris, 2010; Tolbert and Matthay, 2018; Vo et al., 2014). Genome-wide association studies (GWAS) describe NB as a complex genetic disease, characterised by presence of common polymorphic alleles that can influence tumour formation and patient status at diagnosis (Manolio et al., 2009; Ritenour et al., 2018).

1.5.1. Origin of neuroblastoma

Neuroendocrine NB tumours arise from sympathoadrenal cells during foetal development of sympathetic nervous system. The sympathoadrenal lineage originates from multipotent migratory neural crest cells (NCCs) that are localised in the dorsal part of the neural tube (NT) (Takahashi et al., 2013). Early migration of undifferentiated NCCs (Fig. 3, left panel) depends on chemoattraction, and is followed by a later migration that relies on sympathetic neurons (Baker et al., 1997). In the initial early migratory pathway, the dorsal aorta provides chemoattractant signals to the SOX10 positive early NCC via aortic bone morphogenetic proteins (such as BMP4 and BMP7) which induce the expression of the chemokine stromal cell-derived factor 1 (SDF1) and

neuregulin 1 (NRG1) (Saito et al., 2012). Both receptors, C-X-C motif chemokine receptor 4 (CXCR4) and epidermal growth factor receptor (EGFR) can be stimulated by their ligands SDF1 and NRG1. The active secretion of SDF1 and NRG1 ligands by the para-aortic mesenchyme direct the SOX10 positive early NCCs expressing CXCR4 and EGFR to start their migration towards the dorsal aorta. After reaching the vicinity of the dorsal aorta, migrating neural crest cells are no longer called SOX10 positive early NCCs, but are instead known as sympathoadrenal precursor cells (SAPs). In the so called dorsoventral split, SAPs commit to further differentiation in distinct regions of the embryo.

The late migratory event of NCCs (Fig. 3, right panel) starts when the neural crest cells start to migrate on the sympathetic neurons, from which moment they are known as Schwann cell precursors (SCPs). To reach the cortex and invade the developing adrenal medulla, SCPs migrate on the sympathoadrenal neurites which distinguish them from free migrating NCC. After reaching the medulla, SCPs differentiate to become catecholamine-secreting cells of the adrenal gland called chromaffin cells.

Lineage tracing experiments in mice have estimated that 80% of chromaffin cells of the adrenal medulla originate from late migratory NCC Schwann cell precursors, while 20% are due to migration and differentiation of sympathoadrenal precursor cell. (Furlan et al., 2017).

The exact origin of NB is still enigmatic, however a better understanding of the sympathoadrenal differentiation is crucial as NB are considered to arise due to failure of differentiation, growth and migration of the emerging sympathetic lineage. This failure to complete terminal differentiation into neurons or chromaffin cells in the adrenal medulla and instead transform to become malignant is thought to involve abnormal maintenance of stemness signals, which arise from genetic and epigenetic lesions. Due to the observed lack of differentiation features, NB has been called a malignancy with differentiation block (Huber et al., 2009; Maris, 2010).

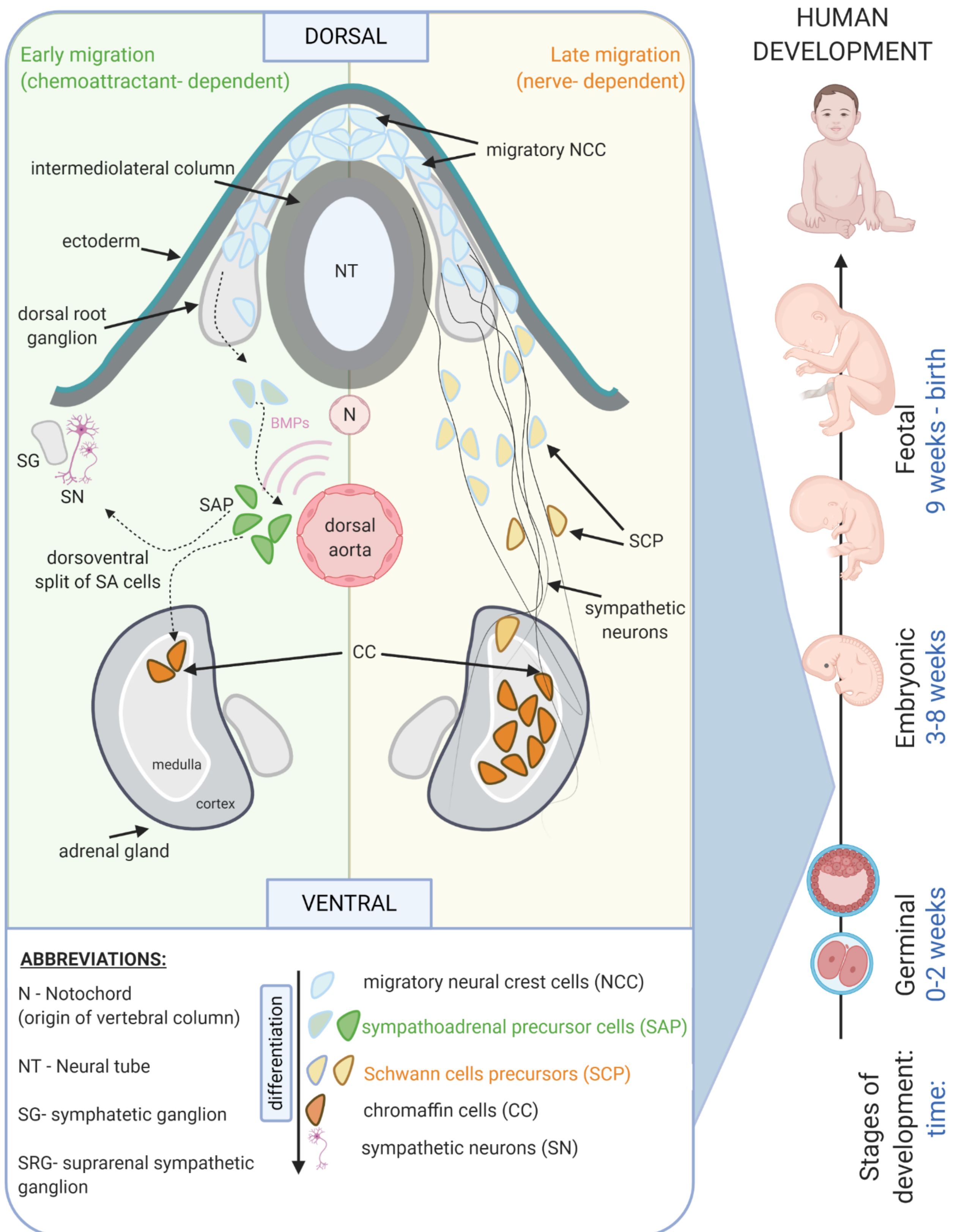

Fig. 3 Neural crest cells migration

NCC migration in humans takes place in week 4. Left panel: Early migration of chemoattractant-dependent NCCs. Right panel: Late migration of nerve dependent migratory neural crest cells. Adapted with permission from (Furlan et al., 2017; Tsubota and Kadomatsu, 2018).

1.6. Genetic aberrations in neuroblastoma

While mutation poor, high-risk NB characteristically exhibits a high number of somatic chromosomal lesions at the genomic level, including structural variations (SVs) and copy number alterations (CNAs) (De Brouwer et al., 2010; Gröbner et al., 2018; Pugh et al., 2013). Besides *MYCN* amplification which is found in 20% of NB overall, and about 50% in high-risk patients, the most frequently mutated gene in NB is *ALK* followed by *PTPN11*, *ATRX, PHOX2B, NRAS, TERT, CHEK2, PINK1,* and *BARD1* (De Brouwer et al., 2010; Molenaar et al., 2012; Pugh et al., 2013). Below, some of the most important segmental chromosomal alterations (SCA) in NB are described:

1.6.1. Chromosome 1p deletions

Segmental chromosomal loss of the distal short arm of chromosome 1p is reported in one-third of all NB cases (approximately 35%) and with even higher frequency in high risk NB where it correlates with *MYCN* amplification and poor patient survival (Ejeskär et al., 2001; Fong et al., 1989; Mora et al., 2000). Investigation of the importance of chromosome 1 in NB by transferring 1p arm of chromosome into the NB cell line (with deletion of distal arm 1p) showed that cells induced neuronal differentiation, suppressed proliferation and caused major cell death (Bader et al., 1991). This observation, and many others in a range of human malignancies (Bagchi and Mills, 2008; Schwab et al., 1996) indicated the existence of tumour suppressors encoded in this region. Several tumour suppressor genes are localised in the 1p36 region including: *CAMTA1, CHD5, KIF1B, CASZ1* and *ARID1A*. Those genes are associated with reduction of cell proliferation and activation of apoptosis (Bagchi et al., 2007; Fransson et al., 2007; García-López et al., 2020; Henrich et al., 2012; Katoh and Katoh, 2003; Liu et al., 2011; Yang et al., 2001).

1.6.2. Chromosome 2p gain (*MYCN*, *ALK* and *ALKAL2*)

The p arm of chromosome 2 is a location of three key players of the ALK signaling pathway: *ALK* itself, *MYCN*, and one of the ALK ligand - *ALKAL2.* Moreover, this SCA is associated with unfavourable outcome in NB patients (Javanmardi et al., 2019; Jeison et al., 2010). Located at 2p23.2-2p23.3, the *ALK* locus spans 728 kb, and with *MYCN* (6.4kb at 24.3), coding for a downstream transcriptional target and *ALKAL2* (8.7kb at 25.3) all are found in the distal 2p arm (Fig. 4). Oncogenic *ALK* mutations have been described in both familial and sporadic NB and are observed in 7-10% of

patients (Carén et al., 2008; Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mossé et al., 2008). The amplification of *MYCN*, which is localised at 2p24.3 remains a valid and critical prognostic marker for NB diagnosis. Approximately 20-30% of all primary NB express high levels of *MYCN*, and this percentage increases in high risk NB cases to around 50%, where it is associated with advanced disease stage and poor prognosis (Maris and Matthay, 1999). Amplification of *MYCN* greater than 10 copies per haploid genome is associated with poor prognosis regardless of other strategic factors (Maris and Matthay, 1999). The *MYCN* gene encodes for its product, MYCN, which is transcription factor with a short half-life that is involved in a range of cellular process like cell proliferation and differentiation (Brodeur, 2003; Eilers and Eisenman, 2008; Maris et al., 2007). MYCN activates targets such as ODC and MCM7, which leads to cell cycle progression (Hogarty et al., 2008; Shohet et al., 2002). The oncogenic association of *ALK* and *MYCN* together with ALKAL2 represent a growth control loop that could potentially be involved in the development and progression of NB (Javanmardi et al., 2019).

1.6.3. Chromosome 11q loss

Loss of parts of the long arm of chromosome 11 (11q) are observed in about 43% of tumours, making this one of the most common chromosomal aberration in NB. 11q deletion is generally mutually exclusive to *MYCN* amplification and is a marker of an unfavourable phenotype for patients (Carén et al., 2008; Carén et al., 2010). The loss of part of chromosomes in cancer cells often indicates the sites where important tumour suppressor genes reside. Deletion of part of a chromosome or gene or even point mutations in tumour suppressor genes can lead to carcinogenesis (Maris and Matthay, 1999). Recently, a strong tumour suppressor gene candidate Disc Large Homolog 2 (DLG2), localised on this high-risk deletion region was identified by Siaw et al. DLG2 is located at chromosome 11q14.1 and is a part of the 'bridge signature' which characterises the transcriptional transition of SCPs towards adrenal chromaffin cell differentiation (Furlan et al., 2017). Overexpression of DLG2 induces differentiation of NB cells and inhibits tumour growth in xenograft models. DLG2 was also found as downregulated target of oncogenic ALK signaling (Siaw et al., 2020). Also at 11q, Lopez et al., identified the postsynaptic adaptor protein-coding gene *SHANK2* (located at 11q13.3-13.4) as associated with high-risk NB. Overexpression of SHANK2 results in significant reduction of cellular proliferation and stimulates differentiation of NB cells

upon RA treatment (Lopez et al., 2020). The inactivation of tumour suppressor genes such as *DLG2* and *SHANK2* could lead to disturbance in neurodevelopmental processes and enhance tumorigenesis in NB (Keane et al., 2020; Lopez et al., 2020; Siaw et al., 2020). In keeping with the complexity of tumour suppressor regions in NB, deletion of parts of chromosome arm 11q, result in loss of DNA damage response (DDR) genes that are known drivers of NB, such as *ATM*, *CHK1*, *MRE11* and *H2AFX* representing an additional important chromosomal aberrations in NB for diagnostic approach to separate high- and low-risk cases (Brodeur, 2003; Carén et al., 2008; Carén et al., 2010).

1.6.4. Chromosome 17q gain

The gain of a fragment of the long arm of chromosome 17q (17q25) is common genetic alteration in primary NB, detected in about 50% of tumours and correlated with adverse outcome (Abel et al., 1999). Unbalanced translocation of chromosome 17q involves many different chromosomes and in particular short arm of chromosome 1p (Bown et al., 1999). This translocation leads to loss of distal 1p arm with simultaneous gain in chromosome 17q and is found often in primary NB (Savelyeva et al., 1994; Van Roy et al., 1994). The genomic region of unbalanced chromosome 17q contains genes such as: *BIRC5* (at 17q25.3), *NM23A* (at 17q21.33) and *PPM1D* (at 17q23.2) that contribute to the growth advantage of tumour cells (Godfried et al., 2002; Islam et al., 2000; Saito-Ohara et al., 2003). Baculoviral inhibitor of apoptosis repeat-containing 5, *BIRC5* encodes survivin protein, that is an inhibitor of apoptosis associated with poor patient outcome and therefore is useful for patient stratification (Caron, 1995; Islam et al., 2000).

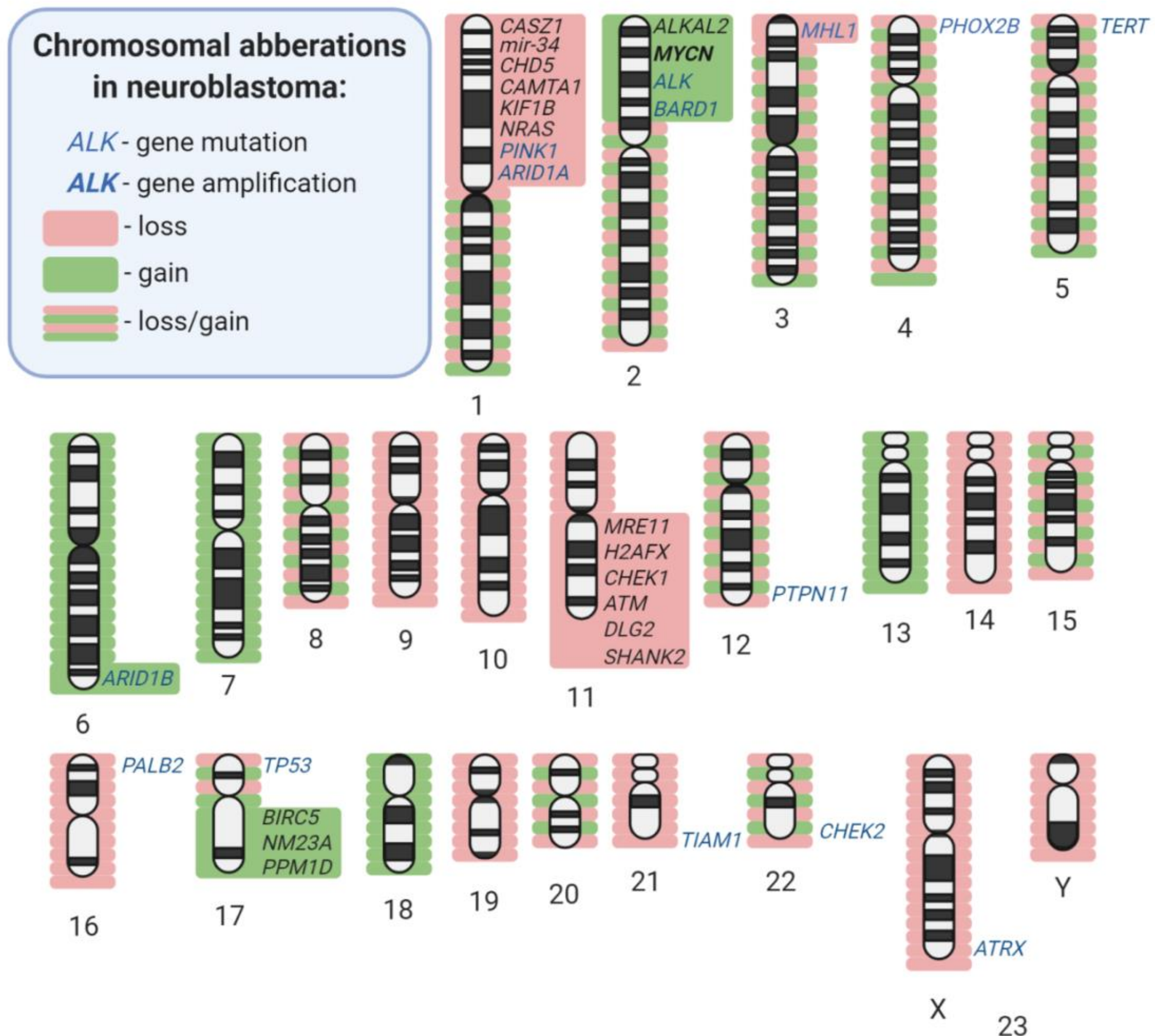

Fig. 4 Schematic representation of SCV in NB patients.

Segmental chromosomal gains (indicated in green) and losses (in red). *Italics* represent of genes which are reported to be mutated in NB, and ***bold italics*** symbolise genes that have been identified as amplified in NB. Adapted with permission from (Maris and Matthay, 1999) based on (De Brouwer et al., 2010; Pugh et al., 2013).

1.6.5. Aneuploidy

Ploidy of genetic materials represents the number of complete sets of chromosomes, and in NB, genome ploidy is an important prognostic marker. Near diploid or near-tetraploid DNA content correlates with more aggressive primary tumours and correlates with chromosomal aberrations including: segmental amplification, deletion and unbalanced translocations. In contrast, less aggressive tumours and more favourable prognosis is associated with hyperploid and near-triploid DNA state, where whole chromosome gains and very few structural rearrangements are present (Brodeur, 2003; Maris and Matthay, 1999).

1.7. Diagnosis of NB

The majority of neuroblastoma patients present diseases at an advanced stage, due to symptoms including: lumps or swellings in the abdomen, neck, pelvis or chest region (main primary tumor location), enlarged belly, weight loss and problems with breathing or swallowing. After physical examination, urine and blood tests are performed. If increased levels of catecholamines (hormones released by chromaffin cells of adrenal medulla, such as epinephrine/adrenaline and norepinephrine) are found, then a positive identification of tumor cells in a bone marrow sample is enough to diagnose NB. Imaging tests may include an ultrasound, magnetic resonance imaging (MRI), X-ray, computerized tomography (CT) scan, followed by metaiodobenzylguanidine (MIBG) if NB was diagnosed. MIBG contains a minimum required amount of radioactive iodine, which is intravenously given to patients and is the most sensitive metastatic investigation to detect NB cells in skeletal/soft tissue. If imaging identifies a suspicious mass, biopsy of the tumour tissue and/or bone marrow is performed to confirm the diagnosis.

1.8. Risk stratification and current treatment options in NB

Precise NB staging is critical to choose the most effective treatment protocol. NB staging has evolved dramatically and has been extensively reviewed by Sokol and Deasi (Sokol and Desai, 2019). According to the International Neuroblastoma Staging System (INSS) neuroblastoma is classified into 5 stages (1-4 and 4S) to evaluate the risk assessment. Stage 1 and 2 are usually characterized as a mild form of NB without metastasis into the bone marrow whereas stage 3 and 4 mainly metastasize and display resistance to chemo- and radiotherapies. The first line of treatment for patients with stage 1 NB is surgery following chemotherapy and/or radiotherapy. For patients with stage 2-4 NB treatment is more complex.

Patients diagnosed with stage 4S NB (mostly children under 1 year old) have a better prognosis, and in these cases NB can spontaneously regress without any treatment (Uemura et al., 2019). Risk of relapse in low/intermediate NB patients is between 5-15%, whereas in high-risk neuroblastoma this risk is around 50-60%. Currently, despite highly aggressive therapy, only approximately half of patients with high-risk neuroblastoma survive 5 years. Therefore, there is an unmet need for new therapeutic strategies in this NB patient subgroup (Brisse et al., 2011; Cohn et al., 2009).

1.9. Treatment strategies in neuroblastoma

1.9.1. Surgery

In solid tumors such as NB, surgery is an efficient way to eliminate carcinogenic tissue. However, complete resection is problematic in NB due to high vascularization of primary tumors and encasement of surrounding nerves by the tumor. Surgery is usually followed by chemo- or/and radiation therapy targeting the remaining cancer cells. In cases where surgical restriction is not recommended based on size, localization and observed metastasis, surgery is limited to collect enough tumor tissue for diagnostic analyses. Presurgical chemotherapy is often administrated to debulk the tumour and allow safe tumour resection (Günther et al., 2011; Lim et al., 2016; Monclair et al., 2015). A small group of patients, usually very young (especially in infants below 18 months of age) categorized as 4S group, are exempt from surgery, as tumors spontaneously regress and patients require no further treatment (Maris, 2010; Maris et al., 2007).

1.9.2. Chemotherapy

One of the most common treatment options for many cancers is chemotherapy. Chemotherapeutics can be a single compound or combination of drugs which are highly toxic to dividing cells, leading to cell death or growth inhibition. While effective in killing uncontrollably dividing cancer cells, this toxicity also affects the normal cells of the body, which typically include cells of the immune system, gut, and hair follicles with common side effects of neutropenia, anemia, diarrhea, hair loss and vomiting. Chemotherapy can be given to downsize the tumour (so called pre surgical) or after surgery to eliminate remaining cancer cells. The chemotherapy treatment protocol is based on size and location of the tumour, whether it has spread, the age of the child and biopsy results. Cytotoxic chemotherapy includes DNA-binding drugs such as carboplatin/cisplatin, which can be combined with etoposide (a topoisomerase type II inhibitor) or vinorelbine (an anti-mitotic). Children in intermediate risk groups often receive carboplatin, cyclophosphamide, doxorubicin or etoposide whereas high-risk NB patients often receive cisplatin, cyclophosphamide, etoposide, topotecan, vincristine or melphalan (Matthay, 2008; Pearson et al., 1992).

1.9.3. Haemopoietic stem cell transplantation

Heavy chemotherapy and/or radiotherapy destroys not only cancer cells but causes extensive damage to the patient's body. The bone marrow harbours hematopoietic stem cells (HSC), which give rise to specialized blood cells as a result of haematopoiesis. High-risk NB patients commonly undergo either an autologous (self-transplantation), (or sometimes allogeneic- from a donor) bone marrow transplantation after chemotherapy. (Fish and Grupp, 2008).

1.9.4. Radiation

131I-metaiodobenzylguanidine (131I-MIBG), which is used in the diagnosis of NB, is a radionuclide that is also employed as a therapeutic agent in NB. 131I-MIBG, similar to norepinephrine, is taken up by sympathomedullary tissues (mainly by a norepinephrine transporter system) and into intracytoplasmic vesicles (through a vesicular transporter system). Accumulation of MIBG takes place within the adrenergic tissues and is associated with severe side effects (Garaventa et al., 1999; Ilias et al., 2011).

1.9.5. Retinoic acid treatment

NB is a disease characterized by poorly differentiated cells. Introducing retinoid derivatives of vitamin A is known to be impact to process of differentiation. Retinoid therapy is widely employed in clinical oncology as a treatment option in NB to differentiate cells into postmitotic neuroendocrine cells. *In vitro* induction of cell differentiation with 13-cis retinoic acid (13-cis-RA, isotretinoin) and all-transretinoic acid (ATRA, tretinoin) has been reported in several studies (Matthay et al., 1999; Reynolds et al., 2003; Sidell et al., 1983). Patients with less severe disease follow a retinoid-induced differentiation protocol, that is also employed as maintenance therapy in high risk patients, for treatments of minimal residual disease (Smith and Foster, 2018). For NB patients, 13-cis-retinoic acid is more preferential (clinically effective) than ATRA, firstly because of its higher half-life time (more than 5 times in comparison to ATRA) and higher level of plasma peak 13-cis-RA (ATRA peak level= 0.62-1 μM and 13-cis-RA is 7.4 μM) (Reynolds et al., 1994).

1.9.6. Immunotherapy

Early investigation of NB cells revealed the enrichment of sialic acid and gangliosides on their surface (Shochat et al., 1977). Disialoganglioside (GD2) is a sialic acid-

containing glycosphingolipid whose high expression level is mainly limited to the surface membrane of neuroectodermal origin cell with examples of neurons, astrocytes, skin melanocytes, and peripheral pain fibers of normal human tissues (Graus et al., 1984). Functionally, they are important for attachment capacity of neuroblastic cells and are required for migration, metastasis and adhesion (Hakomori and Igarashi, 1995). NB patients have elevated free GD2 levels in serum compared with normal children and children with other tumour types however expression does not correlate with patient prognosis (Shochat et al., 1977). In 1985, Cheung and colleagues produced for the first time four monoclonal antibodies (three immunoglobulin M and one immunoglobulin G3) against a human NB cell surface glycolipid antigen (Cheung et al., 1985). Currently, immunotherapy using anti-GD2 monoclonal antibodies has been integrated as a frontline treatment of patient with high risk NB starting with the first drug Dinutuximab (Unituxin) approved by FDA in 2015 (2015; Dhillon, 2015; Yang and Sondel, 2010).

1.10. Novel approaches for cancer treatment in neuroblastoma

High-risk NB patients that complete therapy successfully typically suffer from post-treatment induced toxicity which leads to growth and mental retardation and affects their ability to lead a normal life. Traditional protocols containing chemotherapeutics and radiation therapy demonstrate additional harmful effects due to a lack of specificity for malignant cells. Therefore an important aim of targeted therapy (TT) is to identify less toxic compounds in comparison to conventional chemotherapeutics and highly potent and specific therapeutic molecularly targeted drugs (Tsubota and Kadomatsu, 2018). The ideal example of TT is a compound (or combination of compounds) that eliminates only the cancerous cells while leaving normal cells intact before drug-resistance occurs (Amoroso et al., 2018; Ladenstein et al., 2017).

The development of novel therapeutic strategies in paediatric cancer remains limited, especially in childhood neuroblastoma, which is relatively uncommon. Despite increasing preclinical research efforts with a wide spectrum of inhibitors, phase III clinical trials in NB are still largely limited due to rarity of this malignancy (https://clinicaltrials.gov/).

1.10.1. Inhibition of ALK

Targeting tyrosine kinases, such as ALK, by using a small inhibitors has been shown to inhibit the growth of NB cell *in vitro* and *in vivo* models in preclinical studies (Alam et al., 2019; Cervantes-Madrid et al., 2019; Guan et al., 2016; Infarinato et al., 2016; Schönherr et al., 2011; Siaw et al., 2016; Trigg et al., 2019). Targeting *ALK*, the most frequently mutated gene in NB is only one, well advanced example of targeted therapy in NB. ALK and its inhibition is described in more detail in section 1.11.

1.10.2. Inhibition of ATR

Clinical studies targeting Ataxia telangiectasia and Rad3 related (ATR) in adult patients are ongoing with four different compounds study as monotherapeutics, as well in combination with other drugs (NCT03188965, NCT02264678, NCT02157792, NCT02278250). ATR has been identified in 'omics' analysis as a target of ALK signaling and is one interest of this thesis described in section 1.15. (Van den Eynden et al., 2018).

1.10.3. Inhibition of Aurora A kinase

Despite its key role, the *MYCN* oncogene is currently considered clinically undruggable, prompting approaches that target *MYCN* indirectly (Maris and Matthay, 1999). Otto and colleagues used a synthetic-lethal screening strategy in NB cell to identify genes overexpressed in *MYCN*-amplified tumours and/or genes with direct evidence for being a MYCN target. One out of 17 genes was *AURKA* which showed selective growth-halted effects in the knockdown of MYCN in *MYCN*-amplified cells (Otto et al., 2009). They also demonstrated that Aurora A protects MYCN from ubiquitin-mediated proteolytic degradation (Otto et al., 2009). On the other hand, to initiate transcription, MYCN interacts with MAX, uA4, BPTF, p400 and PAF1 to assemble the specific effector complex with its specific interaction partners: TFIIIC, TOP2A and RAD21. During S phase oncogenic Aurora A kinase (AURKA) displaces specific interaction partners of MYCN to bind to the amino-terminus domain of MYCN, which as a consequence, avoids the release of POL II and stabilizes MYCN transcription. Inhibition of the MYCN dependent pause release of POL II prevents activation of the ATR checkpoint kinase (Büchel et al., 2017). The stabilisation of MYCN by AURKA contributes to development of NB malignancies. Increased expression of AURKA is correlated with unfavourable outcome for NB patients (Shang

et al., 2009). Otto *et al.* postulated that stabilisation of MYCN is independent of AURKA kinase activity therefore the application of small molecules such as kinase inhibitors could not be the ideal therapeutic strategy (Otto et al., 2009). Several AURKA inhibitors have been developed such as: LY3295668, alisertib (MLN8237), ZM447439 (Ditchfield et al., 2003; Gong et al., 2019; Sloane et al., 2010). Nonetheless, the Paediatric Preclinical Testing Program demonstrated that the AURKA inhibitor MLN8237 abrogated proliferation in NB cell lines in a *MYCN*-independent manner. Moreover, studies with MLN8237 showed promise in cell line and *in vivo* xenograft experiments (Maris et al., 2010). Inhibition of Aurora A with alisertib has been currently enrolled in phase II of clinical trials in combination with irinotecan and temozolomide for patients with recurrent NB (DuBois et al., 2018). Erbumine (LY3295668) clinical phase I (NCT04106219) has just opened. Based on mechanistic evidence, Aurora A kinase inhibitors may synergise with ATR inhibitors (Büchel et al., 2017; Moreno et al., 2017).

1.10.4. Inhibition of CDK4/6

The cyclin-dependent kinases 4 and 6 (*CDK4/6*) encode cyclin-dependent serine-threonine kinases which tightly regulate cell cycle progression. Upon mitogenic or pro-proliferative stimuli, once cells commit their re-entry into cell cycle and by exiting G0 phase, followed by replication and resulted in cell division, the elevated Cyclin D1-cyclins form catalytic heterodimers with CDK4 and CDK6. The assembled complex mediates phosphorylation of retinoblastoma (RB) tumour suppressor via phosphorylation of threonine 821 by CDK4 and threonine 826 by CDK6 (Takaki et al., 2005). Phosphorylation of RB leads to its inactivation and this event triggers cell proliferation. Phosphorylated RB releases the E2F transcription factor and this event leads to transcription of genes involved in the G1/S cell cycle progression to continue cell proliferation. Preventing the phosphorylation of RB (RB is active when bound to E2F) helps to keep the cell cycle under control (Narasimha et al., 2014). Therefore, CDK4/6 inhibitors have been identified as potential candidates for targeted therapy in NB with mechanism of action targeting the activity of retinoblastoma tumour suppressor in cancer (Harbour et al., 1999). Additionally, homozygous deletion of *CDKN2A* and amplification of *CCND1* (Cyclin D1) and *CDK4* have been identified in a subset of NB (Mosse et al., 2005). *CDK4* and *CCND1* are major oncogenic drivers among members of the CDK superfamily with the *CCND1* genomic locus most frequently amplified among all tumour types. The cyclin D1 – CDK4/6 complex is strictly

reliant on MAPK/ERK signaling to mediate G1 phase progression to a stage which does not need mitogen induction. (Choi and Anders, 2014). Rader and colleagues performed a successful study using ribociclib (LEE011), a small molecule inhibitor of both CDK4 and CDK6, in a NB setting. Upon treatment with LEE011, reduction in cell proliferation was observed in 12 out of 17 human NB cell lines and tumour growth was delayed in *in vivo* xenografts (Rader et al., 2013). Ribociclib efficiently reduced the phosphorylation of RB and Forkhead Box M1 transcription factor (FOXM1) and this event lead to cell cycle arrest followed by cellular senescence (Anders et al., 2011). Ribociclib is currently in two clinical trials: ESMART (NCT02813135) in combination with topotecan and temozolomide for mainly children and young adults with refractory or recurrent malignancies and NEPENTHE (NCT02780128) where it is tested in combination with ceretinib in children with ALK positive relapsed NB.

1.10.5. Inhibition of WEE1 and Chk1

WEE1 and CHK1 are kinases which are vital in regulation of cell cycle checkpoints and mediate cell cycle arrest when DNA damage occurs (Otto and Sicinski, 2017). The main function of the serine/threonine specific protein kinase Chek1 is to coordinate the cell cycle arrest due to DNA damage or unreplicated DNA. Wee1 kinase controls cell size by restricting mitotic entry via CDK1 inhibition. Loss of Wee1 function results in smaller than normal progeny, because cell division occurs prematurely. Wee1 inhibits Cdk1 by phosphorylating it on two different sites, Tyr15 and Thr14. Silencing of CHK1 or WEE1 blocks cell cycle arrest during S or G2 phase thereby allowing cell cycle progression in spite of DNA damage accumulation, which leads to mitotic cell death catastrophe (Castedo et al., 2004). The CHK1 inhibitor CCT244747 displayed antitumor activity in NB cell lines and in a MYCN-driven NB transgenic mice model (Walton et al., 2012). Currently, a phase I trial of prexasertib (CHK1/2 inhibitor) is ongoing (NCT02808650). Clinically, AZD1755, a WEE1 inhibitor is combined with irinotecan and carboplatin in paediatric phase I trials by COG (NCT02095132) and ITCC (ESMART) and in phase II for other malignancies in adult patients.

1.10.6. Alterations in *ATRX*, *TERT* and ALT

Cancer cells are able to disturb cellular equilibrium by increasing telomerase activity to achieve limitless replication potential (Fig. 5) (Hanahan and Weinberg, 2011). The whole-genome sequencing analysis of NB patients specimen identified a loss-of

function (LOF) genetic alterations in the alpha thalassemia/mental retardation syndrome X-linked RNA helicase (*ATRX*), in approximately 10% of NB patients and rearrangements of the reverse transcriptase telomerase (*TERT*) leading to increased telomerase activity in approximately 25% of NB patients (Peifer et al., 2015; Valentijn et al., 2015). Both genetic aberrations are mutually exclusive to *MYCN* amplification (Cheung et al., 2012; Pugh et al., 2013). Additionally, *TERT* rearrangements are associated with the alternative lengthening of telomeres (ALT), a telomerase-independent mechanism used by many cancers to elongate the telomere via homologous recombination (Cesare and Reddel, 2010). Rearrangement in *TERT*, a target of MYCN has been correlated with high-risk NB and used as a marker for poor outcome NB (Ackermann et al., 2018; Lundberg et al., 2011; Ohali et al., 2006). Zeineldin et al., showed that inactivation of tumour suppressor *ATRX* and activation of oncogenic *MYCN* are incompatible and lead to synthetic lethality (Zeineldin et al., 2020). An important function of ATRX is complex formation with death-domain associated protein (DAXX) which recruits histone H3.3 within telomeric DNA of PML nuclear bodies to maintain proper replication. In the absence of ATRX, the MRN complex co-localise with PML nuclear bodies and a failure of telomeric histone H3.3 deposition results in guanine (G)-rich stretches of DNA called quadruplex, which can block DNA replication by formation of DNA-RNA hybrids (called R-loops) leading to replication dysfunction, telomeric DDR and ALT (George et al., 2020; Zeineldin et al., 2020). Both alterations: *MYCN* amplification and *TERT* rearrangements, lead to telomere maintenance, therefore targeting telomerase activity and ALT can serve as a novel therapeutic strategy for treating a high-risk NB patient, however no clinical trial is currently ongoing (George et al., 2020; Matthay et al., 2016; Peifer et al., 2015). So far, only imetelstat (GRN163L), a telomerase enzymatic activity inhibitor has entered paediatric clinical trials, but due to excessive toxicity the trial was suspended (Salloum et al., 2016; Thompson et al., 2013). 6- thio-20-deoxyguanosine is a promising agent to target telomerase activity in cells expressing telomerase (Moreno et al., 2020). On the other hand, Tetra-Pt (bpy), structurally similar to cisplatin, inhibits strand invasion/annealing step during ALT and specifically halts proliferation of ALT dependent cell. However, production of this compound has been discontinued (Zheng et al., 2017).

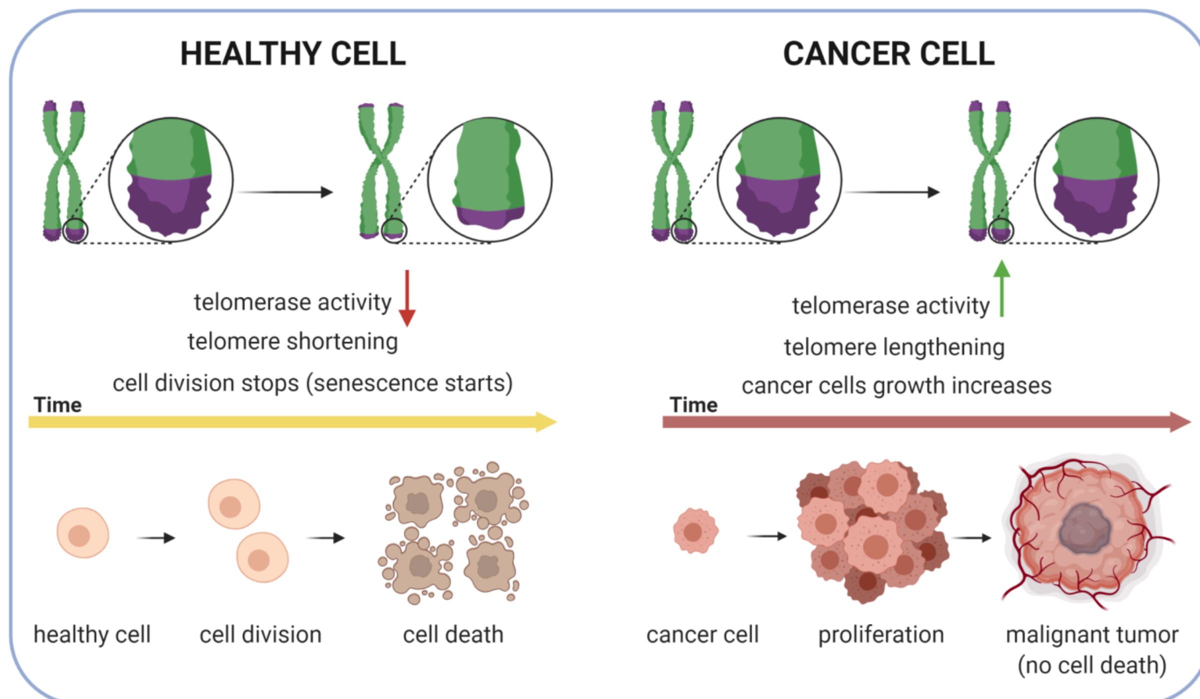

Fig. 5 Telomere biology in cells.

In normal cells every division decreases the length of the distal chromosomal fragments called telomeres. Cancer cells can hijack this by increasing telomerase activity and avoiding telomere shortening.

1.10.7. Inhibition of mTORC1/2

MYCN amplification is associated with poor outcome of NB patients (Maris and Matthay, 1999). Vaughan et al., aimed to find a compound which can destabilise and kill MYCN expressing cells. They showed that PI3K/mTOR inhibitors selectively eliminated MYCN-expressing tumour cells by apoptosis induction. They highlighted the NVP-BEZ235 as a compound which degraded MYCN via inhibiton of mTOR signaling but not PI3 kinase activity (Vaughan et al., 2016). Also, Xu et al. showed that acquired resistance against AZD8055 in NB cell lines correlated with activation of MEK/ERK signaling pathway, leads them to combine AZD8055 with the MEK inhibitor U0126, observing cellular growth inhibition in both *in vitro* and *in vivo* models. Further optimization of AZD8055 (Pike et al., 2013), resulted in the discovery of the more potent candidate AZD2014 which was accessed in the clinical trial ESMART (NCT02813135), but has since been discontinued (Moreno et al., 2020).

1.10.8. Targeting BIRC5

An interesting candidate for targeted therapy in NB treatment is BIRC5, an inhibitor of apoptosis, which is associated with poor patient outcome. The therapeutic agent YM155 (sepantronium bromide) is a potent suppressant which inhibits survivin promoter activity. YM155 suppressed expression of survivin leading to apoptotic cell death in human retinal progenitor cell (HRPC) lines (Nakahara et al., 2007). YM155 was also shown to sensitize NSCLC cells to radiation both *in vitro* and *in vivo*, followed by induction of apoptosis and resulting in downregulation of survivin expression (Iwasa et al., 2008).

1.11. Targeting anaplastic lymphoma kinase in neuroblastoma

The protein kinase (PK) family is a large family of enzymes that facilitate the transfer of the γ phosphate of ATP to specific amino acids such as: tyrosine or serine/threonine residues on protein substrates (Hubbard and Till, 2000; Hunter, 2014). Among the tyrosine protein kinases there are two subclasses: the receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (NTRKs).

1.11.1. Receptor tyrosine kinases (RTK)

Receptor tyrosine kinases (RTKs) are crucial for cell proliferation and differentiation as well as share a common structure architecture consisting of an extracellular ligand-binding domain (ECD), a protein tyrosine kinase domain (PTK), and a transmembrane domain (TMD) (Schlessinger, 2000). Binding of the ligand to the ECD induces receptor dimerization and leads to tyrosine trans-auto-phosphorylation and activation of signaling (Brognard and Hunter, 2011; Heldin, 1995). Humans have 58 known RTKs, which fall into 20 subfamilies (Manning et al., 2002; Robinson et al., 2000). Additionally, RTKs exhibit oncogenic properties when their kinase activity is permanently enhanced and uncontrollably potentiated due to point mutations, amplification or rearrangements. Constant activation of RTKs can lead to uncontrolled cell proliferation, influence cell motility, migration or invasion, as well cause angiogenesis and inhibit apoptosis (Robertson et al., 2000).

1.11.2. Anaplastic lymphoma kinase (ALK)

Anaplastic Lymphoma Kinase (ALK) was originally reported in 1994 when it was first identified as truncated and fused to nucleophosmin (NPM) in the t(2;5) chromosomal rearrangement (t(2;5)(p23:q35)23,24) associated with non-Hodgkin's lymphoma (Fujimoto et al., 1996; Morris et al., 1994; Shiota et al., 1995). The ALK protein is encoded by the *ALK* gene located at 2p23. Further characterisation of the full-length ALK receptor was first described in 1997 by two independent groups (Iwahara et al., 1997; Morris et al., 1997) and ALK expression was detected in the developing central and peripheral nervous system. Full-length human ALK consist of 1620 amino acids and the unmodified protein has molecular weight of 180 kDa. Upon post-translation modification such as N-linked glycosylation, can increase up to 220 kDa (Iwahara et al., 1997; Morris et al., 1997). The homology of the ALK kinase domain to insulin-like growth factor receptor 1 (IGF-1R) and insulin receptor (InR) is 47%, placing ALK within the family of insulin RTKs (Morris et al., 1994). *ALK* expression was identified in the define area of the developing brain, with highest expression observed in regions such as: thalamus, mid-brain, olfactory bulb and selected cranial, peripheral ganglia of mice (Iwahara et al., 1997; Morris et al., 1997; Vernersson et al., 2006). Knockdown of ALK strongly reduced sympathetic neuron proliferation (Reiff et al., 2011). The *ALK* loss of function mice are viable and do not show any gross phenotypes however defects in neurogenesis (the number of neurons, regeneration of myelinated axons) and delayed testosterone production, as well as the behavioural responses to ethanol have been reported (Bilsland et al., 2008; Lasek et al., 2011; Weiss et al., 2012; Witek et al., 2015). A lesson learnt from gain-of-function ALK mice highlights its role in neurogenesis and neuroblastoma progression in combination with oncogenic MYCN (Berry et al., 2012; Cazes et al., 2014; Ueda et al., 2016). Taken together, those data suggest the importance of *ALK* in behaviour, fertility and development of both brain and testis. To date, many chromosomal rearrangements resulting in ALK activation have been reported, and are involved in a variety of cancer types (Hallberg and Palmer, 2016; Li et al., 2007; Turner and Alexander, 2005). The discovery in 2007 of the EML4-ALK oncoprotein in non-small cell lung cancer (NSCLC) (Rikova et al., 2007; Soda et al., 2007), and in late 2008 of ALK gain-of-function mutations in paediatric NB (Carén et al., 2008; Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mossé et al., 2008) has focused attention on ALK as a prominent target for drug development (Chiarle et al., 2008; Hallberg and Palmer, 2010; Palmer et al., 2009).

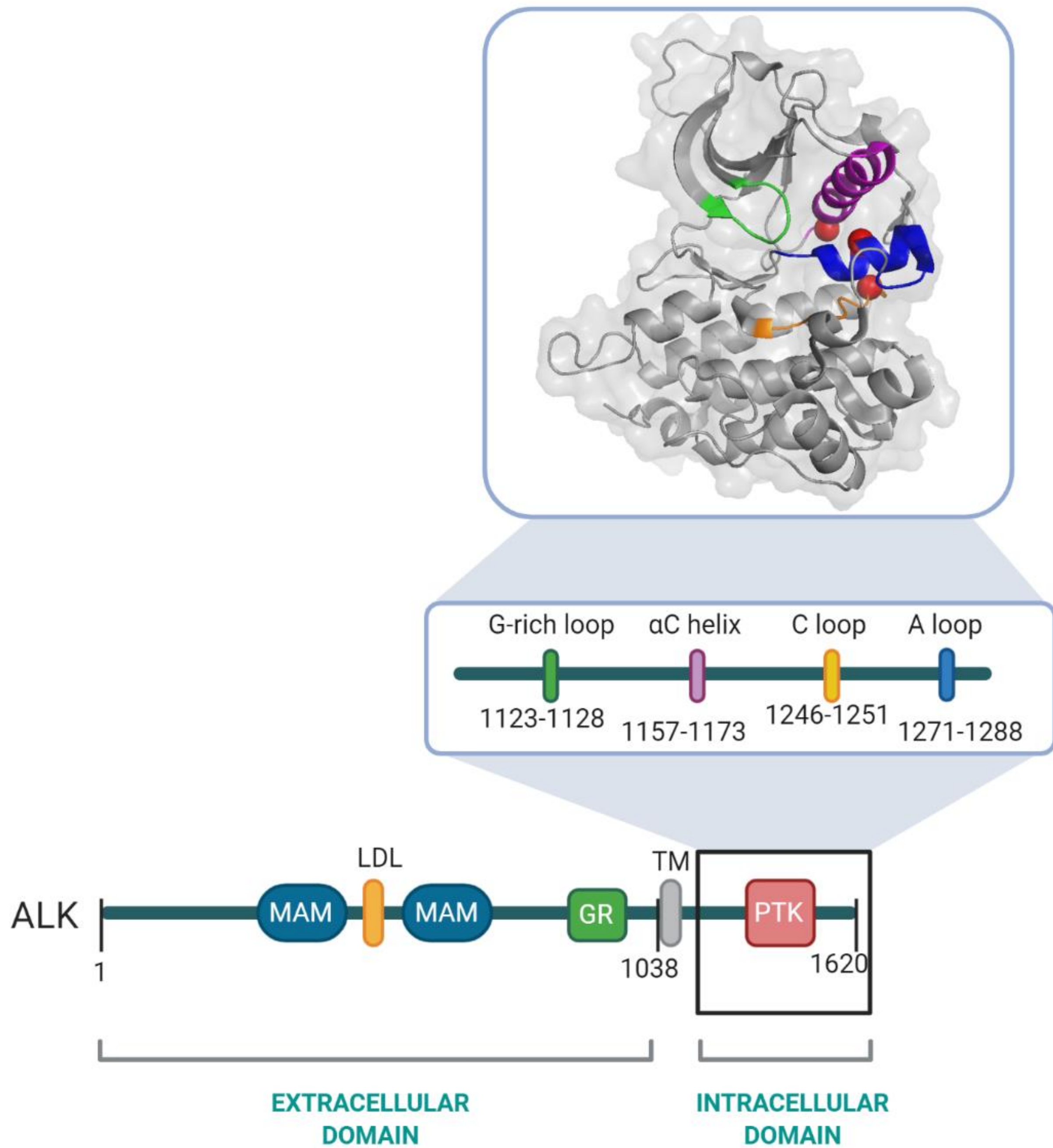

Fig. 6 Architecture of ALK receptor.

The RTK ALK consist of 1620 aa. The ECD of ALK carry a two MAM (meprins, A-5 protein and receptor protein tyrosine phosphatase mu) domains separated by LDLa (Low density lipoprotein class A) domain and those are followed by a glycine-rich region (GR). ALK kinase domain include a conserved small N-terminal lobe and a large C-terminal lobe. In the top box, crystal structure of ALK kinase domain (PDB: 3LCT) consists of: glycine-rich loop (green), alfa C helix (magenta), catalytic loop (yellow) and activation loop (blue). Hot spot mutations: Phe1174, Arg1275, Phe1245 (red balls). Adapted with permission from (Hallberg and Palmer, 2013).

1.11.3. Activation of ALK

Similar to all other RTKs, ALK possesses a ligand binding extracellular region, transmembrane domain and intracellular region which harbours the protein tyrosine kinase domain (PTK) (Fig. 6) (Iwahara et al., 1997; Morris et al., 1997). In ALK, the extracellular domain consists of 1020 aa, followed by a transmembrane-spanning region of 21 aa and an intracellular domain of 561 amino acids (Hallberg and Palmer, 2013). Ligand interaction with the extracellular domain of the receptor is thought to effectively crosslink them in a dimeric complex. Ligand induced dimerization results in trans-autophosphorylation and activation. In both familial and sporadic NB, full-length ALK is activated by point mutations, almost exclusively in the kinase domain (Carén et al., 2008; Chen et al., 2008; George et al., 2008; Hallberg and Palmer, 2013; Janoueix-Lerosey et al., 2008; Matthay et al., 2016; Mossé et al., 2008). Recently described ligands of ALK, named ALKALs, can also potently lead to the activation of wildtype ALK in NB cell (Guan et al., 2015; Reshetnyak et al., 2015), as well as in vertebrate neural crest tissues (Fadeev et al., 2018; Mo et al., 2017).

1.11.4. ALK in neuroblastoma

Oncogenic *ALK* mutations has been identified in primary and relapsed NB tumours. (Martinsson et al., 2011; Schleiermacher et al., 2014). Sporadic NB is more common than familial NB with heritable mutations (Knudson and Strong, 1972). Patients with a family history of NB inherit the disease in an autosomal dominant Mendelian fashion and these patients represent 1-2% of all NB cases with their tumours mostly harbouring mutations in *ALK*. Overall, *ALK* point mutations are observed in 7-10% of NB patients (Bresler et al., 2014; De Brouwer et al., 2010) and a higher percentage, 26%, in relapsed NB cases (Martinsson et al., 2011; Schleiermacher et al., 2014).The “hotspot” residues- Phe1174, Arg1275, or Phe1245 in the ALK kinase domain account for of 85% of all ALK point mutations (Eleveld et al., 2015; Martinsson et al., 2011; Mossé, 2016; Schleiermacher et al., 2014). There is now ample mechanistic evidence of oncogenic cooperation between ALK and MYCN to promote NB pathogenesis and the combined occurrence of *ALK* mutations and *MYCN* amplification is associated with poor prognosis (Berry et al., 2012; Cazes et al., 2014; De Brouwer et al., 2010; Heukamp et al., 2012; Schönherr et al., 2012). This implies that targeting of ALK with tyrosine kinase inhibitors (TKIs) may provide therapeutic benefits in NB. Studies in cell lines and transgenic mouse models have shown that multiple intracellular signal

cascades are triggered by ALK and mutated forms of the receptor (Emdal et al., 2018; Gouzi et al., 2005; Sattu et al., 2013; Turner and Alexander, 2005; Van den Eynden et al., 2018).

1.12. Oncogenic ALK signaling

Oncogenic ALK signaling mediates downstream signaling cascades via the complex interactions of various protein molecules. A vast majority of the knowledge about ALK signaling is a result of genetic abnormalities leading to constitutive activation of this oncogenic kinase. There are three main ways to activate ALK: (1) via translocation and dimerization with the fusion partner, (2) gain of function mutation in the ALK kinase domain, or (3) ALK receptor amplification identified in many malignancies. It is important to know that the network of interacting proteins and downstream signaling of any receptor is complex and may involve multiple feedback loops and association with other oncogenes.

Phosphorylated ALK leads to activation of multiple downstream pathways such as: Janus kinase (JAK)–signal transducer and activator of transcription (STAT), sonic hedgehog (SHH), JunB Proto-Oncogene signaling (JUNB), Mitogen Activated Protein Kinase (MAPK) signaling cascades, mTOR PI3K–AKT, CRKL-C3G-RAP1 and phospholipase Cγ (PLCγ) among others. Molecular events in these signaling pathways lead to activation of transcription factors such as MYCN, HIF1α, ETV's and FOXO's resulting in stimulation of a range of cell-specific responses such as cell growth, differentiation or anti-apoptotic signaling (Fig. 7) (Barreca et al., 2011; Chiarle et al., 2008; Mossé et al., 2009; Palmer et al., 2009). Understanding of oncogenic ALK signaling comes mainly from study of the ALK fusions: NPM-ALK and EML4-ALK in ALCL and NSCLC as well as from the mutated full length receptor (Hallberg and Palmer, 2016; Mazot et al., 2011; Mossé, 2016).

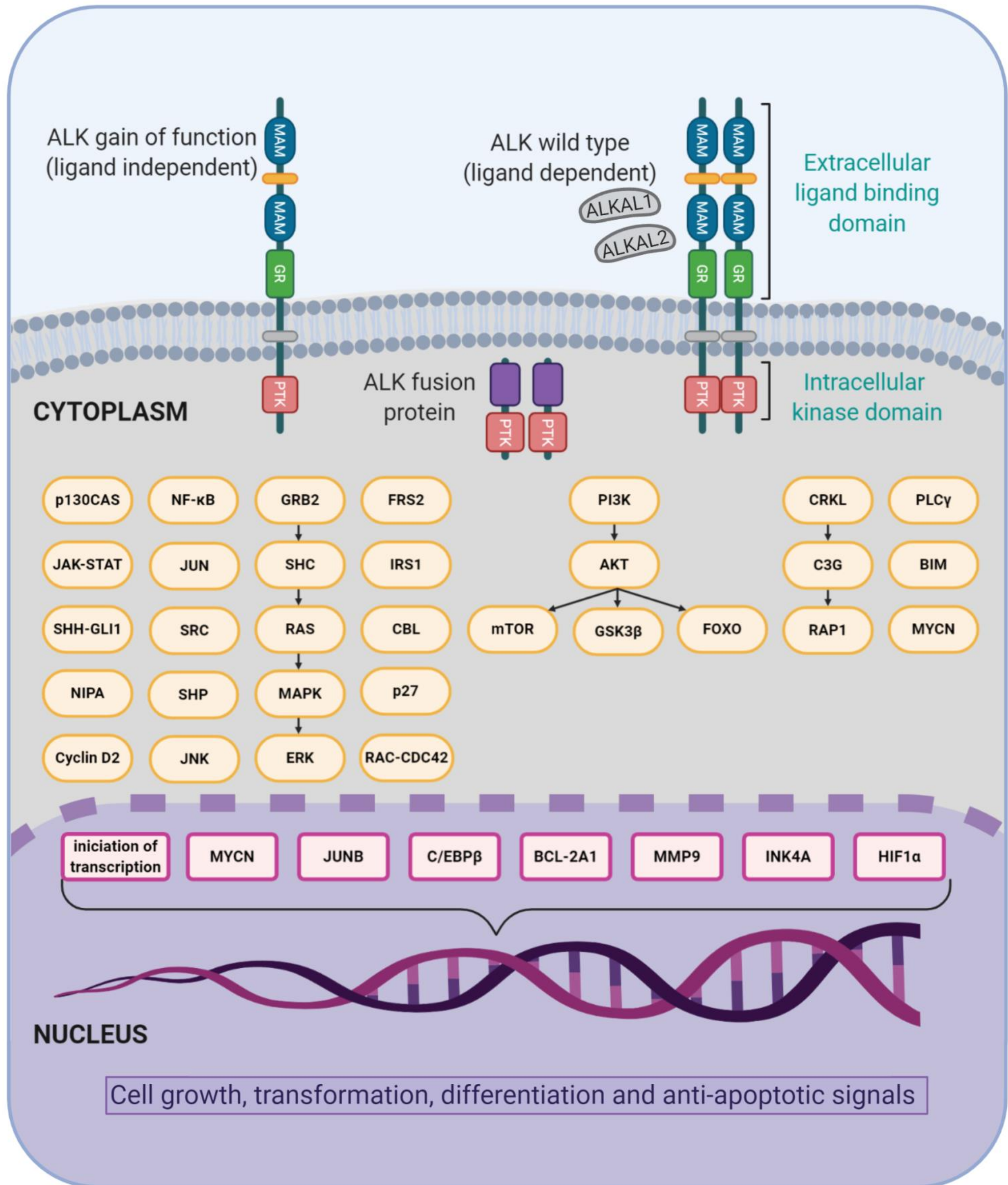

Fig. 7 Signaling downstream of ALK.

Anaplastic lymphoma kinase (ALK) mediates signaling via many pathways including the RAS–MAPK, PI3K–mTOR, phospholipase Cγ (PLCγ), RAP1, Janus kinase (JAK)–signal transducer and activator of transcription (STAT) and JUN pathways activated during cell growth, transformation, differentiation and anti-apoptotic signals. Adapted with permission from (Hallberg and Palmer, 2013).

1.13. ALK positive cancers

Activation of ALK in NB generally occurs due to mutation in the kinase domain of receptor, however ALK overexpression has also been described. Oncogenic ALK aberrations are also associated with many types of cancers. Apart from mutation and overexpression, ALK also exists as a fusion partner in many malignancies such as: anaplastic large cell lymphoma, non-small cell lung cancer, inflammatory myofibroblastic tumours, diffuse large B-cell lymphomas and squamous cell carcinoma of the esophagus. Here the four first malignancies are described:

1.13.1. Anaplastic large cell lymphoma

Anaplastic large cell lymphoma (ALCL), a subtype of human non-Hodgkin lymphoma, was originally described as a neoplasm with predisposition to invade lymph node sinuses that expresses Ki-1 (CD30) antigen (Stein et al., 1985). ALCL is the malignancy in which *ALK* translocation was first described and to which also owes its name. In ALCL, activation of oncogenic ALK signaling is a direct result of its fusion to nucleophosmin (NPM) as a result of NPM-ALK t(2;5)(p23;q35) translocation (Fujimoto et al., 1996; Morris et al., 1994; Shiota et al., 1995). This genetic rearrangement is extremely common in ALCL and occurs almost in 80% of cases (Amin and Lai, 2007). The comprehensively studied NPM-ALK oligomeric fusion consists of the first 117 aa from NPM followed by 563 aa residues including the kinase domain of ALK. The translocations of the amino-terminal part of the fusion partner lead to dimer formation that facilitate autophosphorylation of ALK kinase domain and result in tumorigenic potential of fusion oncogene. The common characteristic among the fusion partners of ALK is that they determine the cellular localisation of their complex and drive the transcription of fusion genes via own promotor. Besides NPM, several other fusion partner genes for *ALK* have been identified in ALCL: moesin (MSN), ALK lymphoma oligomerization partner on chromosome 17 (ALO17), TRK fused gene (TFG), Tropomyosin 3 and 4 (TPM3, TPM4), non-muscle myosin heavy chain 9 (MHY9), 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC), clathrin heavy chain (CLTC), TRAF1 (Boi et al., 2015).

1.13.2. Non-small cell lung cancer (NSCLC)

Lung cancer is associated with the highest mortality among both men and women worldwide, accounting for approximately 1.7 million (in 2019) deaths per year, and can

be classified into two histologically distinguishable subgroups: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), with 85% and 15% occurrence, respectively. The main risk factor in relation to developing NSCLC is cigarette smoking (Collins et al., 2007; Zappa and Mousa, 2016). Approximately 4-6% of NSCLCs carry *ALK* rearrangements and given the high incidence of lung cancer cases worldwide this accounts for 40,000 ALK dependent NSCLC new cases every year (Bayliss et al., 2016; Shaw and Engelman, 2013). ALK-positive NSCLC patients are mainly younger and light or non-smokers (Rodig et al., 2009; Sasaki et al., 2010). The most frequent ALK fusion in NSCLC is the translocation with a cytoplasmic protein echinoderm microtubule associated protein-like 4 (EML4). Like *ALK*, *EML4* is located on chromosome 2, and to date, at least 15 different EML4-ALK chimeric variants have been identified (Bayliss et al., 2016; Soda et al., 2007). Other ALK fusion partners in NSCLC include are TFG (Rikova et al., 2007), kinesin light chain 1 (KLC1) (Togashi et al., 2012), kinesin family member 5B (KIF5B) (Takeuchi et al., 2009; Wong et al., 2011), protein tyrosine phosphate non-receptor type 3 (PTPN3) (Jung et al., 2012) and striatin (STRN) (Yang et al., 2017).

1.13.3. Inflammatory myofibroblastic tumours (IMT)

IMT is a rare myofibroblastic soft tumour composed of malignant myofibroblasts with prominent inflammatory components, such as lymphocytes, eosinophils, and plasma cells that commonly originate in soft tissue of lung, abdomen, pelvis and retroperitoneal region and mostly found in children and young adults (Gleason and Hornick, 2008; Meis and Enzinger, 1991). IMTs were the first reported non-haematological tumours with *ALK* aberration. *ALK* rearrangement has been identified in 50% of IMT cases with the *TPM3* gene localised on chromosome 1 that encodes for a non-muscle tropomyosin being the most common fusion (Griffin et al., 1999). Several other fusion partners of ALK identified in IMT are: TPM4-ALK (Griffin et al., 1999), ATIC-ALK (Debiec-Rychter et al., 2003), CLTC1-ALK (Bridge et al., 2001; Patel et al., 2007), cysteinyl-tRNA synthetase, CARS-ALK (Debelenko et al., 2003), Ras-related nuclear protein-binding protein 2 (RANBP2), RANBP2-ALK (Ma et al., 2003; Patel et al., 2007), protein-tyrosine phosphatase receptor-type F polypeptide-interacting protein-binding protein 1, PPFIBP1-ALK (Takeuchi et al., 2011b), SEC31 homologue A, SEC31L1-ALK (Panagopoulos et al., 2006).

1.13.4. Diffuse large B-cell lymphoma (DLBCL)

DLBCL is a B-cell type neoplasm which is the most common among all lymphomas and accounts for 30-40% of all lymphoma cases with primary tumours localised among lymph nodes and infrequently at sites such as tongue, nasopharynx, and stomach (Laurent et al., 2009; Lenz and Staudt, 2010). However aberrations in ALK are very rare, representing less than 1% of all DLBCL, and are associated with unfavourable prognosis and ineffective response to chemotherapeutics (Beltran et al., 2009; Laurent et al., 2009). Therefore, targeted therapy against ALK would be a good strategy for ALK positive DLBCL patients (Tanaka et al., 2020). The most common ALK rearrangement is the t(2; 17) (p23; q23) translocation leading to a fusion *CLTC* gene localised on the chromosome 17q23 (Gascoyne et al., 2003). Other ALK fusions in DLBCL include NPM-ALK, SEC31A-ALK (Van Roosbroeck et al., 2010), sequestosome -1 SQSTM1-ALK (Adam et al., 2003; Onciu et al., 2003; Takeuchi et al., 2011a)

1.14. ALK inhibitors

Since the first report of ALK in the pathogenesis of ALCL in 1994 (Fujimoto et al., 1996; Morris et al., 1994; Shiota et al., 1995), oncogenic ALK signaling has been implicated in a number of cancer forms (Hallberg and Palmer, 2016; Prokoph et al., 2018; Shaw and Engelman, 2013; Trigg and Turner, 2018). ALK positive cells are strongly dependent on the ALK kinase activity, therefore targeting ALK is a good strategy to inhibit tumour cell proliferation. Additionally, in non-cancerous tissue expression of ALK is restricted to the early stages of development and then retained at low levels in few specific tissues. This makes ALK an ideal target to be used in clinic because its inhibition is catastrophic for cancer cells.

A spectrum of small-molecule TKI inhibitors (TKIs) selectively inhibiting ALK have been designed and investigated in ALK positive cancers. NVP-TAE684 was one of the first ALK inhibitors shown to reduce cell proliferation in ALCL and NSCLC (Galkin et al., 2007; McDermott et al., 2008). To date, several ALK inhibitors have been through clinical trials and are clinically employed for ALK-positive cancer. Crizotinib was the first generation ALK specific inhibitor approved by FDA (FDA, 26 August 2011, date last accessed). After crizotinib, a second generation of inhibitors was developed including: ceritinib, alectinib and brigatinib and entrecitinib. Here highlighted are a few that have been used in my studies:

1.14.1. Crizotinib

The first generation ALK TKI to enter clinical trials that was approved for treatment in ALK-positive NSCLC in 2011 was crizotinib (Fig. 8). Crizotinib (PF-2342066 or XalkoriTM), is an orally bioavailable, ATP competitive TKI (Christensen et al., 2007). Initially, this drug was developed as a potent c-Met kinase inhibitor, but later studies showed that crizotinib was an effective inhibitor of other RTKs, such as ALK and ROS-1, at pharmacologically relevant concentrations. Based on the remarkable success in Phase I and II of clinical trials in ALK-positive NSCLC and since ALK was implicated in other malignancies (ALCL and IMT) crizotinib was proposed as a candidate treatment option for NB patients (Kwak et al., 2010; Mossé et al., 2013). Currently, crizotinib has been evaluated in an ongoing COG phase III trial (COG ANBL1531, NCT number NCT03126916) for paediatric patients. However the initial phase I of clinical trial shows that only 1 out of 11 NB patient with mutated or amplified ALK had objective response (9%) (Mossé et al., 2013). In contrast to NB, treatment with crizotinib resulted in robust responses in other paediatric cancers such as IMT, NSCLC and ALCL (Mossé et al., 2017). The phase II study with crizotinib as a single agent and in combination with chemotherapy are yet to be reported (NCT00939770 and NCT01606878) (Moreno et al., 2017).

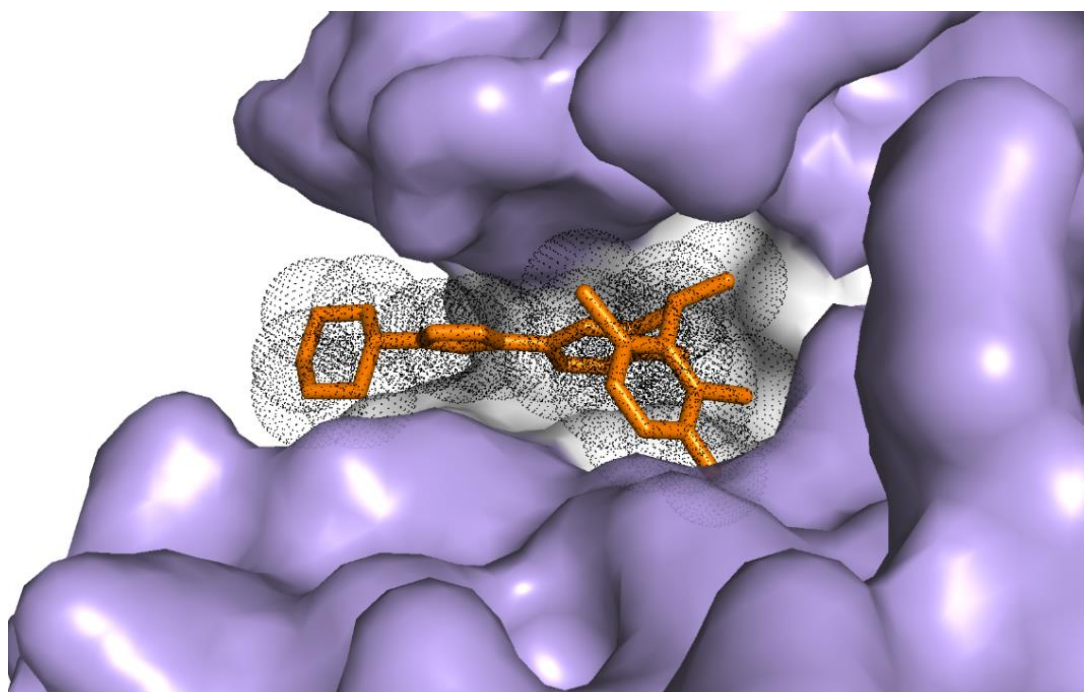

Fig. 8 Crystal structure of crizotinib binding to the KD of ALK.

The figure represents the tyrosine kinase inhibitor crizotinib (orange) in the ATP-binding site of the ALK kinase domain. Generated with PyMol using published coordinates (Protein Data Bank code: 2XP2).

1.14.2. Lorlatinib

The third generation inhibitor, PF-06463922 with the molecular formula $C_{21}H_{19}FN_6O_2$ known as lorlatinib (Fig. 9), is a third generation inhibitor and one of the most recent additions to the list of ALK TKIs. This ATP-competitive molecule is a highly selective and highly potent ALK/ROS1 inhibitor capable of CNS penetration. Lorlatinib, is rather unique as it possesses a macrocyclic structure developed mainly to overcome ALK-TKI resistant mutations, in addition with improved CNS activity (Johnson et al., 2014). In biochemical assays, lorlatinib inhibited wild-type ALK, represented by Ki value (mean inhibitory constant) of less than 0.07 nM. In addition, lorlatinib potently inhibits crizotinib-resistant ALK mutants such as L1196M, G1269A, 1151Tins, and F1174L with Ki value in a range between 0.1-0.9 nM (Zou et al., 2015). Preclinical investigation of lorlatinib in NB shows that this inhibitor is capable of inhabiting almost all ALK mutants including those previously described to be resistant to other ALK TKIs. Furthermore, lorlatinib was superior to crizotinib and alectinib in ALK- dependent *in vitro* and *in vivo* xenograft mice models (Guan et al., 2016; Infarinato et al., 2016; Lin et al., 2017). Lorlatinib is currently in paediatric phase I/II clinical trials for NB (NANT, NCT03107988) (Moreno et al., 2017).

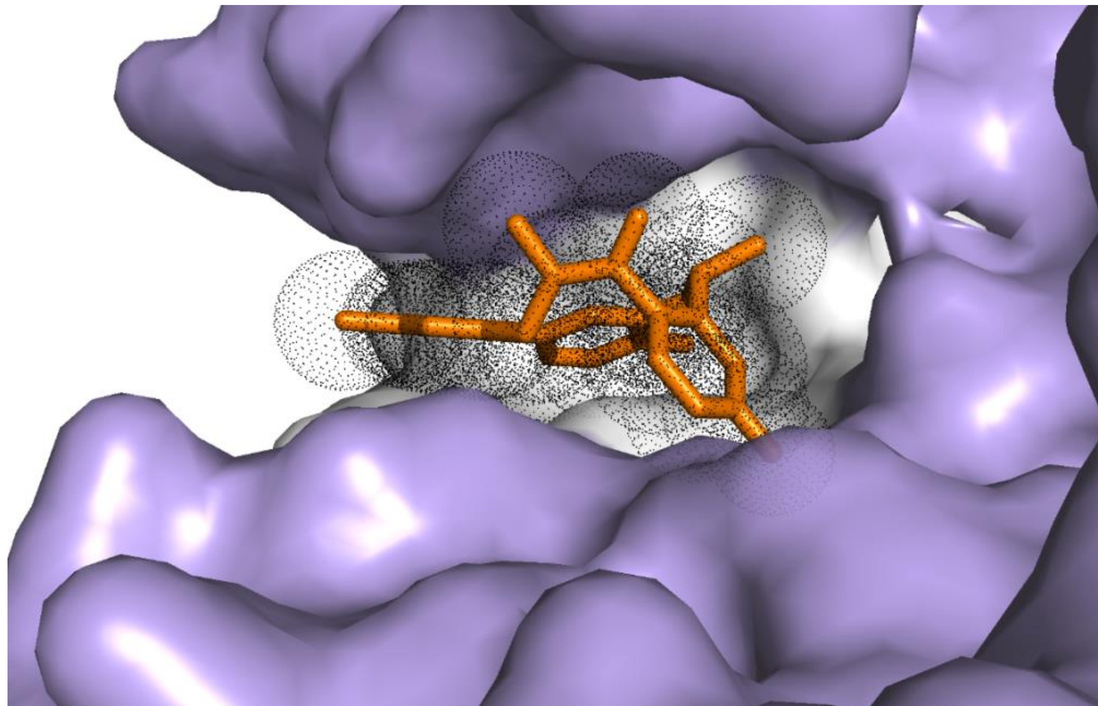

Fig. 9 Crystal structure of lorlatinib binding to the KD of ALK.

The figure represents the tyrosine kinase inhibitor lorlatinib (orange) in the ATP-binding site of the ALK kinase domain. Generated with PyMol using published coordinates (Protein Data Bank code: 4CLI).

1.14.3. Repotrectinib

Repotrectinib (TPX-0005) is a novel rationally designed ATP-competitive TKI developed by TP Therapeutics. This inhibitor was designed based on the structure of lorlatinib (Fig. 10). Repotrectinib possesses a low-molecular weight and three-dimensional macrocyclic structure that precisely anchors in the adenine-binding site. It is selective and highly potent against ROS1, TRKA-C, and ALK. Repotrectinib can overcome resistance acquired due to solvent-front mutation (G1202R rearrangement), thanks to its small structure which helps to avoid steric hindrance**.** Repotrectinib is currently in phase I and phase II clinical trials for adult cancer patients (*NCT03093116).* Preliminary data shows that is well tolerated, and has low side effects in patients with TRK and ROS1 mutations *(Adult patients: ETV6–NTRK3*-rearranged MASC with *NTRK3*^{G623E}-mediated resistance to entrectinib and *CD74–ROS1*-rearranged NSCLC with *ROS1*^{G2032R}-mediated resistance to crizotinib. It has been shown that this inhibitor can overcome multiple resistance mechanisms by targeting SRC/FAK signaling (Drilon et al., 2018).

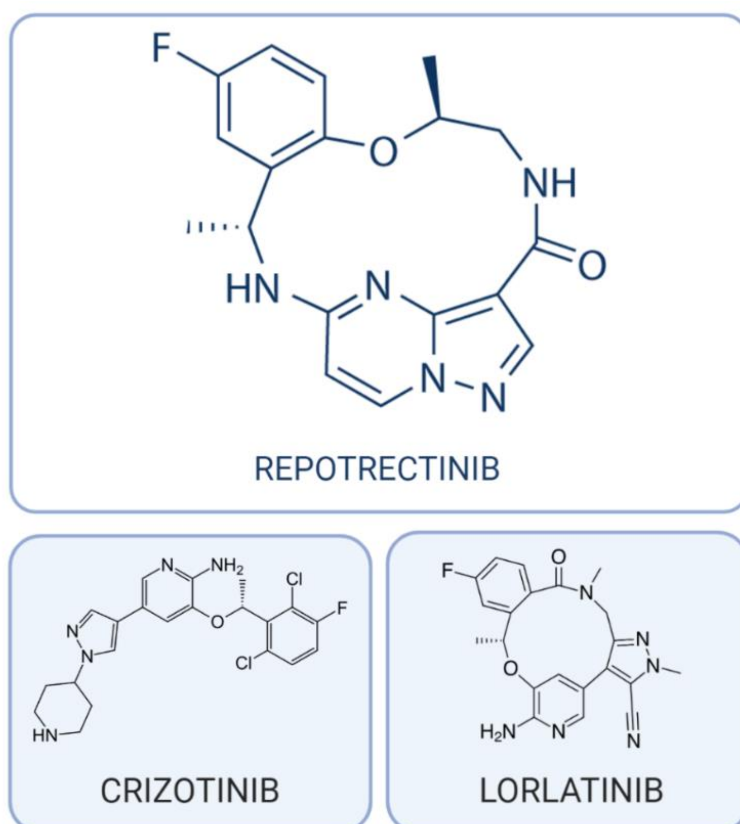

Fig. 10 Comparison of the structures of crizotinib, lorlatinib and repotrectinib.
Repotrectinib has been designed based on macrocyclic structure of lorlatinib. The crystal structure of repotrectinib binding to the KD of ALK is not available yet.

1.15. Targeting ATR in neuroblastoma

1.15.1. Cell cycle and its regulation

The eukaryotic cell cycle is a highly evolutionary conserved chain of events by which a mother cell replicates its genome, grows and segregates into two daughter cells. It is therefore very important that this process is performed with great precision to avoid cellular damage and to prevent potential errors to be transferred to the progeny. The majority of the cells in adult tissues are in G0 phase and cells in this phase consist of two different cellular states: transient (quiescence) or permanent (upon terminal differentiation or senescence). However, to proliferate they need to commit to re-enter the cell cycle in response to mitogenic factors (Otto and Sicinski, 2017). The mammalian cell cycle comprises of interphase which consists of three active phases: gap 1 phase (G1), DNA synthesis phase (S) and gap 2 phase (G2) and is followed by mitosis (M) phase (Blagosklonny and Pardee, 2002). Cellular reprograming relies on the complex communication between three groups of signaling proteins that ensure that the cell cycle is performing properly. Cyclin-dependent kinases (CDKs) are members of the serine/threonine kinase family and their catalytic activities are modulated by interactions with cyclins and cyclin-dependent kinase inhibitors (CKIs) which control kinase activity and substrate specificity during cell cycle progression. CDKs are recognised as the engine that drives cell cycle progression, while CKIs and cyclins are considered to be the gears and their presence or absence strictly controls the smooth transition between cell cycle phases (Lim and Kaldis, 2013). Any alteration in the genes controlling the cell cycle progression can lead to reproduction of progeny with defective genetic material and therefore increased genetic instability. In malignant cells, the restriction points that control cell cycle progression often become non-functional for various reasons, leading to uncontrolled proliferation (Wenzel and Singh, 2018). Therefore, it is critical that cells possess intact mechanistic checkpoints that ensure the proper progression of cell cycle and generate defect-free progeny. The cell cycle progression is nonlinear network of multiple proteins and pathways that create various feedback loops creating a scenario in which downstream events lie upstream of themselves briefly described below (Fig. 11).

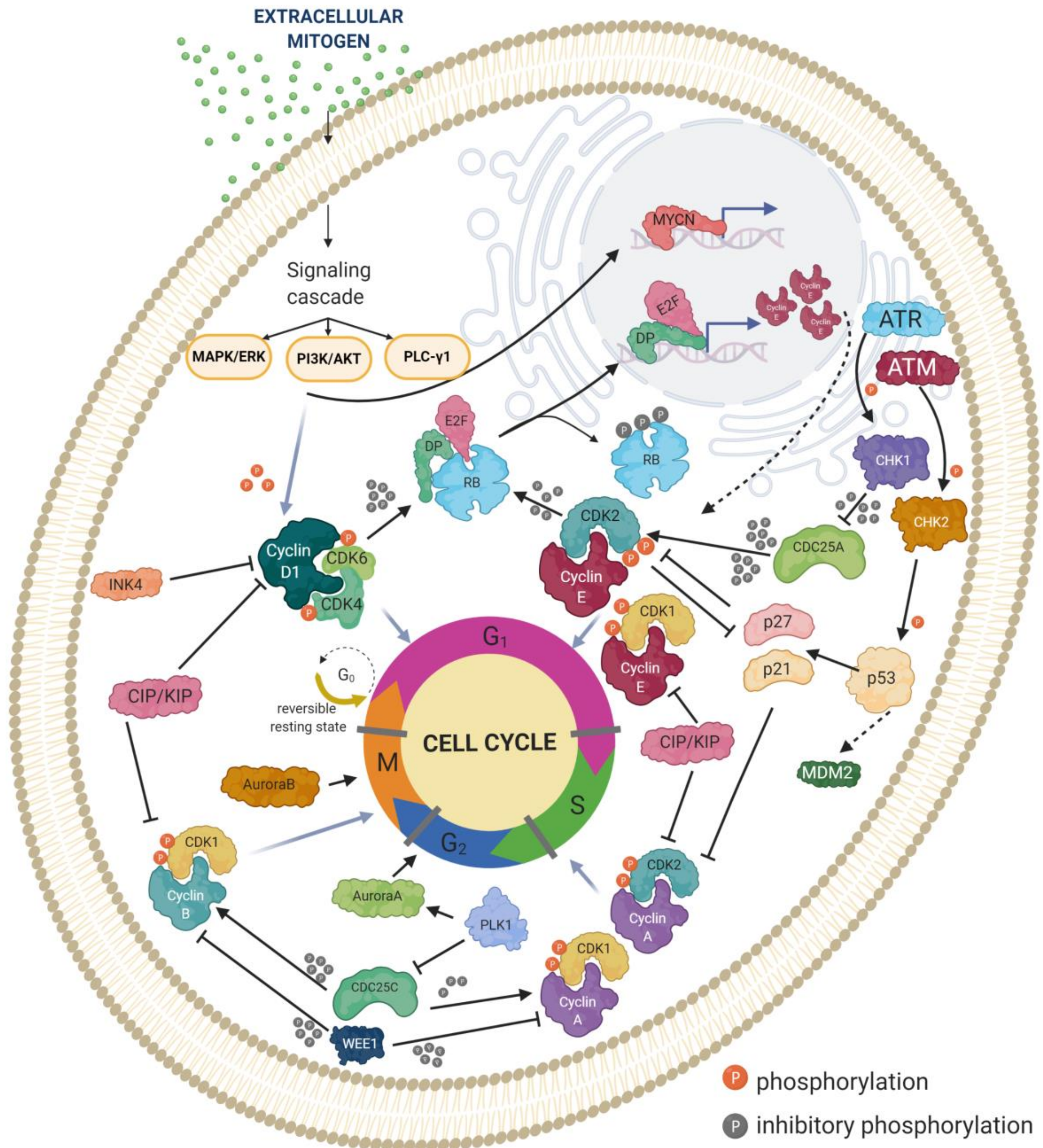

Fig. 11 Simplified illustration of a complex network regulating the cell cycle.

After re-entering the cell cycle, cells follow a sequential activation and deactivation of many cyclin-CDK complexes. This multilevel process is accompanied by CKI as well as controlled by tumour suppressor proteins, checkpoint kinases, and involves activation of many transcription factors. Adapted from (Blagosklonny and Pardee, 2002; Otto and Sicinski, 2017).

1.15.2. Cell cycle checkpoints

Cell cycle progression is orchestrated by checkpoints that are surveillance mechanisms to ensure that every step of the cell cycle is performed correctly and

completed prior to progression to the next phase of the cycle. Regulatory checkpoints comprise the G_1/S “restriction checkpoint”, the G_2/M- “DNA replication checkpoint” and the “metaphase/anaphase” or “spindle apparatus checkpoint”.

After re-entering the cell cycle, cells upregulate cyclin D1 by four distinct mechanisms: (1) induction of transcription, (2) protein stabilisation, (3) re-localisation to nucleus, and (4) assembly complex with CDK-4 and CDK-6. Assembly of cyclin D-CDK4/6 complexes is strictly regulated by the INK4 protein that can immediately cause cell cycle block by inhibition of CDK4/CDK6 monomers and lead to cyclin-D degradation. In unperturbed cells, the cyclin D-CDK4/6 complex phosphorylates the RB protein and causes E2F nuclear re-localisation and transcriptional activation of genes involved in nucleotide metabolism and DNA synthesis and also increases levels of cyclins E and A. Activated cyclin E-CDK2 complexes are essential to allow cell transition to S phase. During S phase cells assemble a multiprotein complex named the replisome, to perform DNA replication. This process is strictly controlled the by ATR kinase. In case of any DNA damage occurred upon DNA synthesis ATM/ATR signals via CHEK1/CHEK2 which primes protein phosphatase CDC25A for degradation. Upon DNA damage, ATM recognises double break strands (DBS) and phosphorylates p53. ATM phosphorylation of p53 leads to release a p53 negative regulator factor MDM2. Furthermore, p21 and p27, major targets of p53 transcriptional activation, can interact with cyclin-CDK complexes and result in cell cycle arrest at any stage. The presence of errors during replication such as lesions or stalled replication forks, prevents cells from entering G2 and starting preparation for mitosis. Cyclin B-CDK1 complex drives cells forward towards the M phase. WEE1 kinase controls cell size and can inhibit the mitotic entry by inhibitory phosphorylation of cyclin CDK1. The activation of polo-like kinase 1 (PLK1) toward the end of G2 executes mitotic entry associated with events such as centrosome maturation, Aurora phosphorylation, chromosome condensation and cytokinesis. In mitosis, cells proceed through 4 stages: prophase, metaphase, anaphase, and telophase, followed by cytokinesis. Cells need to ensure that equal distribution of sister chromatid took place and the chromosomes are properly attached to the mitotic spindle (Fig. 11) (Blagosklonny and Pardee, 2002; Otto and Sicinski, 2017). Recently, in 2018, Saldivar et al. proposed a fourth, intrinsic “S/G2 transition checkpoint” (Saldivar et al., 2018).

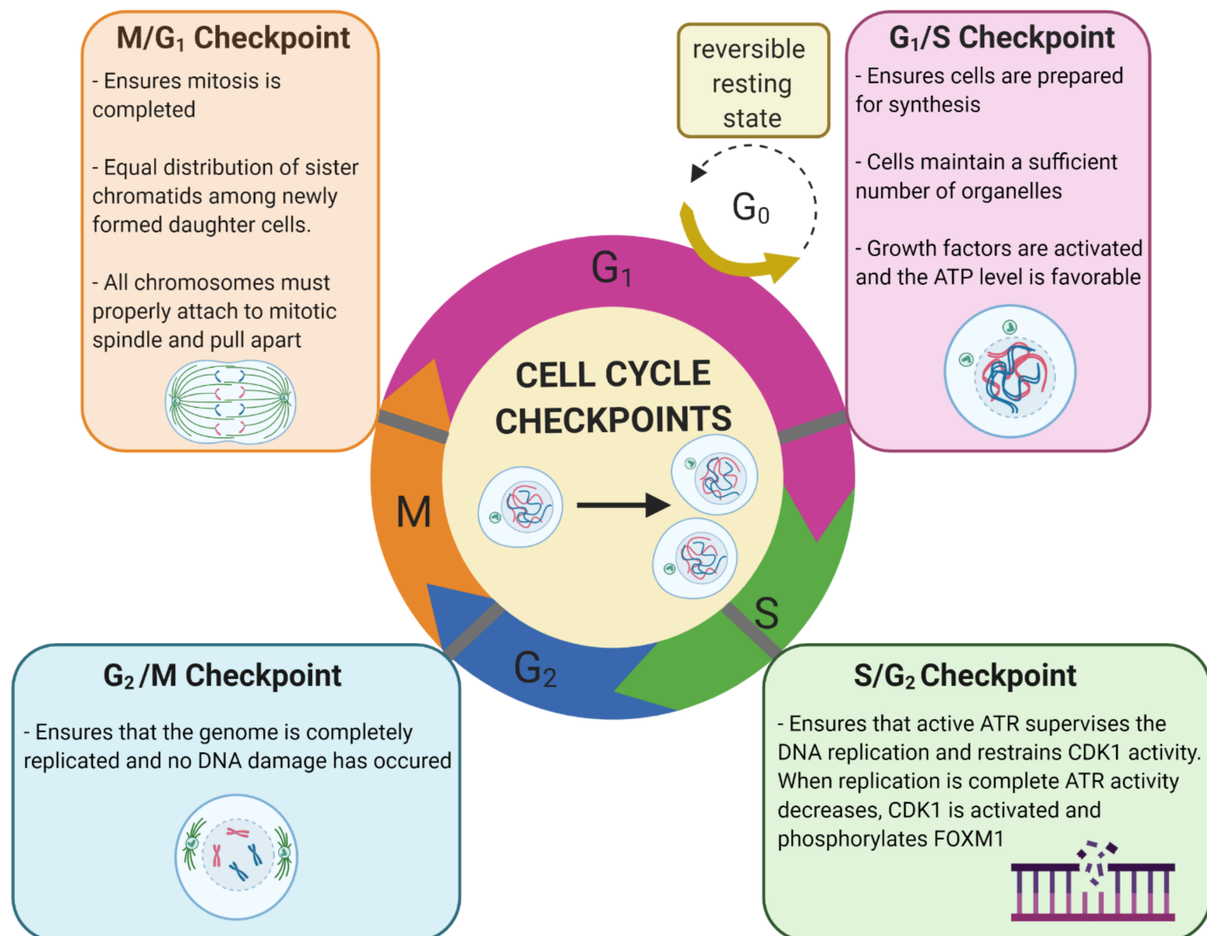

Fig. 12 Cell cycle checkpoints

Cell cycle progression is strictly regulated at the critical checkpoints by cyclins and CKI.

1.15.3. An intrinsic S/G2 checkpoint enforced by ATR

Upon completion of G1 phase, the conserved domain of ETAA1 potently and directly stimulates the ATR checkpoint kinase to block the S/G2 switch until S phase ends. During S phase, activated ATR assists ongoing DNA replication and blocks the CDK1-dependent FOXM1 phosphorylation switch. Upon the completion of DNA replication, ATR activity is no longer required, releasing CDK1 to rapidly phosphorylate and activate FOXM1 at the beginning of G2 phase. FOXM1 phosphorylation results in upregulation of a spectrum of mitotic genes involved in cell cycle progression to enter mitosis. ATR is responsible for ensuring that the G2/M progression is dependent upon the completion of S phase to preserve genome integrity, Salvador et al., refer to this pathway as the "intrinsic S/G2 checkpoint" (Fig. 12) (Saldivar et al., 2018).

1.15.4. The DNA damage response

DNA damage (DD) is defined as an alteration in DNA structure which occurs naturally as a result of cellular metabolism or is caused by factors such as exposure to genotoxic agents, such as ionizing radiation and chemotherapy. The damaged DNA can exist as single or multiple base damage, single strand breaks (SSBs) and less commonly as double strand breaks (DSBs). Observed damages are bases missing from the backbone of DNA, or chemically changed base/bases. Tens of thousands of such events occur every day in our cells, and indeed DD accumulation can lead to genomic instability, however this damage is simultaneously repaired by complex molecular machinery (Lindahl, 1993). To overcome DNA lesions cells have developed mechanisms to prevent it. The DNA damage response (DDR) is a multi-complex signaling cascade which consists of collection of different intra- and inter-cellular signaling events involving proteins that can detect and mediate the repair of damaged DNA, thereby ensuring that any perturbations are repaired and not transferred to the next generation (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). The process of DDR is orchestrated by over 700 proteins which are involved in processes that cause cell cycle arrest and control of DNA replication (Matsuoka et al., 2007). If the DNA damage is too massive and repair machinery cannot cope with DNA repair, the DDR impacts on downstream cell fate so that damaged cells are directed to cell death (apoptosis) or senescence (d'Adda di Fagagna et al., 2003; Freund et al., 2010; Kang et al., 2015). The efficiency of DNA repair appears to decrease with advancing age and leads to accumulation of DNA damage in tissues (Lombard et al., 2005). DDR defects are associated with human diseases such as cancer (Sancar et al., 2004).

1.15.5. Ataxia Telangiectasia Mutated and Rad3-related kinase

Ataxia Telangiectasia Mutated and Rad3-related belongs to the phosphatidylinositol 3-kinase-related (PIKK) serine/threonine kinase family which includes 5 other members such as: ATM (ataxia-telangiectasia mutated), PRKDC (DNA-PKcs, DNA-dependent protein kinase catalytic subunit), SMG1 (suppressor of morphogenesis in genitalia), TRRAP (transformation/transcription domain-associated protein) and MTOR (mammalian target of rapamycin) (Lempiäinen and Halazonetis, 2009). All PIKK kinases share similarities in their structure and in particular all contain a C-terminal FAT domain (Fig. 13). They are involved in cellular events such as proliferation, survival, metabolism, and differentiation (Ciccia and Elledge, 2010; Jackson and

Bartek, 2009; Lempiäinen and Halazonetis, 2009). ATR is a key player in a cellular response to ssDNA damage, a lesion frequently caused by replication stress (RS) at stalled replication forks. Single strand DNA damage can also occur due to exposure to anticancer chemotherapy and ionizing radiation (IR). The ATR phosphorylation cascade signals via CHK1 phosphorylation and controls cellular processes such as cell cycle arrest through stimulation of intra-S and G2/M checkpoints to orchestrate DNA damage repair (Reinhardt and Yaffe, 2009).

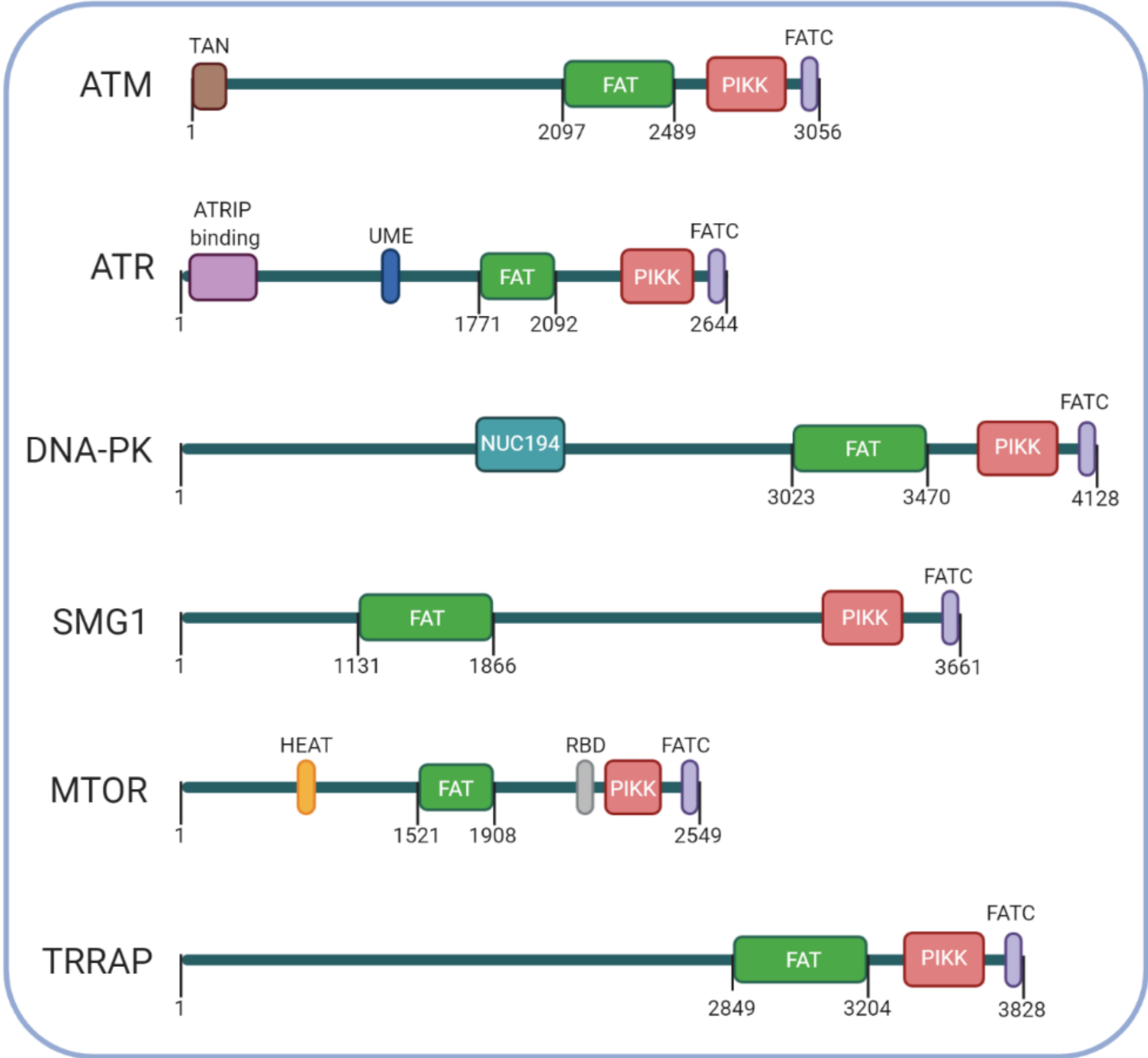

Fig. 13 PIKK family

The PIKK family consists of six members: ATM, ATR, DNA-PK, SMG1, MTOR and TRRAP. All PIKK possess a conserved kinase domain (PIKK), FRAP-ATM-TRRAP (FAT) domain, and an additional C-terminal, FRAP-ATM-TRRAP-C-terminal (FATC) domain. Adapted with permission from (Weber and Ryan, 2015).

1.15.6. Mutations in DDR related genes

Many cancers rely on DDR pathways to survive genomic instability, however many mutated DDR genes are frequently associated with diseases caused by developmental defects (Ruzankina et al., 2007). *ATR* knockout is lethal (Brown and Baltimore, 2000) and mice die early in development, subsequent to the blastocyst stage. In humans, reduced ATR function leads to developmental disorders, manifest as Seckel syndrome, characterised by microcephaly and short stature. (O'Driscoll et al., 2004). Inducible knockout of ATR in adult mice is tolerable with no major consequences (Murga et al., 2009; Ruzankina et al., 2007; Schoppy et al., 2012). Depletion of ATR in adult mice results in an age related phenotype characterised by hair graying, alopecia, osteoporosis, kyphosis, as well as stem cell loss (Ruzankina et al., 2007). These age-related phenotypes are unique in mice and humans with mutations in DDR genes. The list expands to pathologies associated with metabolic and cardiovascular abnormalities, increased incidences of malignancies and shortened lifespan (Hasty et al., 2003; Lombard et al., 2005). The tumour suppressor p53 plays a vital role in the cell intrinsic response to DNA damage (downstream phosphorylation of ATM), and its activation drives cell-cycle arrest, apoptosis, and senescence. Patients with tumours lacking specific DDR functions, such as mutations in p53, become more dependent on the S and G2/M checkpoints and therefore hypersensitive to inhibitors of ATR (Stewart and Weinberg, 2006).

1.15.7. ATR inhibitors

Accumulation of DNA damage contributes to genomic instability that is linked to the vast majority of human cancers. Cancer are dependent on DDR pathways to mitigate genomic instability by activating the ATR checkpoint kinase (Halazonetis et al., 2008). Therefore, targeted therapy against ATR will lead to impairment of DNA repair mechanisms but also halt checkpoint activation leading to premature cell cycle progression which can result in mitotic catastrophe. The fast proliferation of malignant cells compared to normal cells make the former more sensitive to DDR based therapies (Foote et al., 2018).

ATR kinase has recently become an interesting drug target because of its crucial function in repair of ssDNA damage since unrepaired damage can lead to fatal DSB. The process of developing ATR inhibitors started in 1990, however the first attempts resulted in neither potent nor specific compounds such as caffeine (IC_{50}= 1,1mM),

which also targets other PIKK family members like ATM (IC_{50}= 0.2 mM) (Cortez, 2003; Sarkaria et al., 1999) and a low potent wortmannin (with IC_{50}= 1.8 uM) (Knight et al., 2006). In recent years, highly potent and selective compounds have been synthesised and four of them, based on promising preclinical data, have entered human clinical trial assessment. ATR inhibitors such as AZD6738 (ceralasertib), M6620/VX-970 (berzosertib), M4344/VX-803, BAY1895344 entered human studies (Fig. 14). Currently, AZD6738, BAY1895344, VX-970 are in the clinical trials in 29, 6 and 16 different studies respectively, including monotherapies as well as combinatory therapies. M4344/VX-803 has been enrolled in one clinical study NCT02278250, however a second study including a PARP inhibitor niraparib is planned (https://clinicaltrials.gov/).

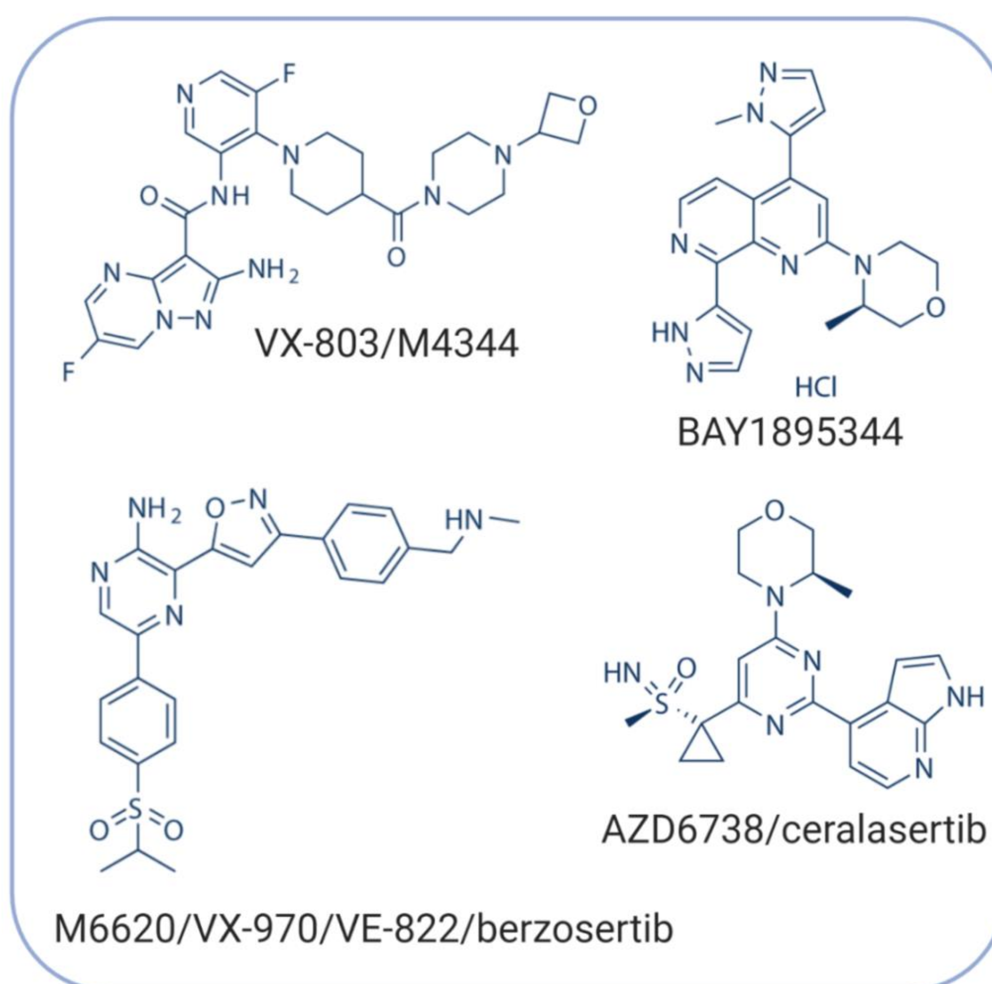

Fig. 14 ATR inhibitors in I/II phase clinical trials.

ATR inhibitors which are currently in clinical trials as a monotherapy as well as in combination treatments.

1.15.7.1. AZD6738, ceralasertib

AZD6738, ceralasertib, is an orally bioavailable, ATP competitive ATR kinase inhibitor developed by Astra Zeneca. AZD6738 inhibits ATR substrate CHK1 Ser345 phosphorylation in cells with IC_{50}= 74 nM (Foote et al., 2018). Ceralasertib is potent and selective against ATR kinase activity (Foote et al., 2018). Vandetti et al., showed that treatment with AZD6738 leads to cell death and senescence in NSCLC cell lines. Another study showed that, AZD6378 enhanced the cytotoxic effect in combination with gemcitabine in NSCLC cell lines. Ceralasertib treatment revealed a synergistic effect in combination with cisplatin to induce a rapid cell death in ATM-deficient NSCLC. Additionally, NSCLC cells with ATM knockdown were shown to demonstrate increased sensitivity to combination of cisplatin and AZD6738. Vandetti et al., tested two NSCLC grafts: H460 and H23 in *in vivo* studies. The treatment with AZD6738 resulted in tumour growth inhibition in the nude mice bearing H460 tumours. Also the treatment of mice with ATM- deficient H23 NSCLC grafts with AZD6738 in combination with cisplatin was well tolerated and caused rapid regression of ATM-deficient NSCLC tumours (Vendetti et al., 2015). Checkley et al., developed a cell-based model for predicting a tumour growth, as a supporting tool currently employed in phase I clinical trial design of AZD6738 monotherapy and combination with ionizing radiation (Checkley et al., 2015). Ceralasertib is at the moment in numerous phase II clinical studies as a single agent therapy and in combination with carboplatin (NCT02264678), paclitaxel (NCT02630199), radiotherapy (NCT02223923), and PARP inhibitor, olaparib (NCT03462342, NCT03330847), acalabrutinib (NCT03328273), and durvalumab (NCT03334617) (Dillon et al., 2018; Foote et al., 2018).

1.15.7.2. BAY1895344

BAY1895344 is an orally available, potent and highly selective ATR protein kinase inhibitor designed by Bayer AG. BAY1895344 selectively binds to and inhibits the activity of ATR kinase leading to abrogation downstream signaling molecules such as Chek1. BAY1895344 demonstrated potent inhibition in *in vitro* biochemical assays with IC_{50}= 7 nM and also inhibits hydroxyurea-induced H2AX phosphorylation in HT-29 cell with IC_{50}=36nM. This inhibitor also showed a potent, antineoplastic activity among a broad spectrum of human tumour cell lines, with median IC_{50} = 78 nM (Lücking et al., 2020). A study performed by Wengner et al., demonstrated a potent antiproliferative activity of BAY1895344 *in vitro* and *in vivo*. BAY1895344 inhibits cell growth of a panel

of 38 human cancer cell lines covering various types of cancer. As a single agent, BAY1895344 strongly abrogated the growth of cancer xenografts carrying DNA damage repair deficiencies. BAY1895344 exhibits synergistic antitumor efficacy in combination with external beam radiotherapy (EBRT) in colorectal cancer. The combination of BAY1895344 with olaparib in PARPi-sensitive BRCA1-deficient breast cancer and in PARP-inhibitor resistant prostate cancer showed synergistic antitumour growth *in vitro* and *in vivo.* In this comprehensive study, combination of BAY1895344 with the novel, nonsteroidal androgen receptor antagonist darolutamide showed significant reduction of the tumour growth in comparison to monotherapy treatment in hormone-dependent prostate cancer. Additional exposure to EBRT resulted in even further increased antiproliferative capability of the treatment (Wengner et al., 2020).
A recent study performed by the thorium conjugate research group from Bayer AS, employed targeted alpha therapy (TAT) in the combination with monoclonal antibodies of fibroblast growth factor receptor 2 (FGFR2). Several cancers including: triple negative breast cancer, gastric cancer, colorectal cancer exhibit overexpression of receptor tyrosine kinase FGFR2 (Wickstroem et al., 2019). The advantage of TAT is to deliver the radiobiological particle to the tumour via tumour-specific ligand, such as a monoclonal antibody, which in the case of this study was conjugated of fibroblast growth factor receptor 2 with targeted thorium-227 (FGFR2-TTC). *In vitro* analysis showed increased potency of FGFR2-TTC in combination with BAY1895344 with elevated level of histone H2AX that caused cell cycle arrest. Wickstroem et al., also performed *in vivo* study on MFM-223 breast cancer xenograft models and observed a synergistic effect of the FGFR2-TTC combination with BAY1895344 whereas single agent showed no effect (Wickstroem et al., 2019).

1.15.7.3. M6620 (formerly VE-822, VX-970 berzosertib)

VX-970 was identified by high-throughput screening as potent ATR inhibitor (Charrier et al., 2011). VX-970 sensitised cells to chemo- and radiotherapies in both *in vivo* and *in vitro* models of pancreatic ductal adenocarcinoma (PDAC). VX-970 significantly reduces survival of pancreatic cancer cells in combination with XRT and gemcitabine *in vitro* and *in vivo* and did not increased toxicity in healthy cells and tissues (Fokas et al., 2012). This inhibitor also significantly sensitized breast cancer PDXs to radiotherapy (Tu et al., 2018). In clinical trials, VX-970 performed safely and was well tolerated as a monotherapy leading to complete response in patient with colorectal cancer. Yap et

al., also tested VX-970 in combination with carboplatin, however this required a dose reduction of VX-970 in compare to monotherapy (Yap et al., 2020). In clinical studies, VX-970 in combination with topotecan seems particularly active and decreased the tumour size of platinum-refractory small-cell lung cancer, in contrast to single treatment with topotecan which was not that efficient (Thomas et al., 2018). Currently evaluated in clinical trials, VX-970 shows anticancer activity, both as a single agent and in the combination with chemotherapy or radiotherapy (NCT02157792, NCT02567409).

1.15.7.4. M4344 (VX-803)

VX-803 is an oral bioavailable, adenosine triphosphate (ATP)-competitive, highly specific and potent inhibitor of ATR. M4344 potently inhibits ATR-driven phosphorylation of direct downstream target CHEK1 kinase with an IC50 of 8 nM. This inhibitor is currently under clinical trial as a mono-therapeutic as well as in combination with carboplatin, gemcitabine and cisplatin. To date, there are no clinical data available (Moreno et al., 2020).

The range of ATR inhibitors enrolled into the clinical trials such as: AZD6738, M6620, M4344 and BAY1895344 present novel therapeutic strategies for treating patients with malignancies carrying defects in DDR genes, both in monotherapy as well in the combination with spectrum of DNA damage–inducing or –compromising cancer therapies (Wengner et al., 2020).

2. AIMS:

To better understand ALK signaling in NB, as well as to investigate potential novel therapeutic strategies, for NB treatment, we:

Paper I:

Performed comprehensive analysis of ALK signaling of by proteomic and RNAseq analysis to identify novel targets for treatment.

Paper II:

Investigated the potential of a recently developed ALK inhibitor, repotrectinib, in NB setting.

Paper III:

Explored ATR in NB, testing the effect of the BAY1895344 ATR kinase inhibitor on NB growth.

3. MATERIALS AND METHODS:

A brief introduction of the most commonly used methods to generate data are included. More details can be found in the papers and manuscript.

3.1. Cell culture

NB cells were cultured in RPMI-1640 medium supplemented with 10% FBS and grown at 37°C, 5% CO_2, 95% humidity. All ALK-addicted cell lines were cultured on collagen pre-coated dishes. PC-12 cells were maintained in MEM/EBSS medium supplemented with 3% FBS and 7% horse serum and a mixture of 1% penicillin/streptomycin at 37°C and 5% CO_2.

3.2. Inhibition of ALK activity in neuroblastoma cell lines

NB cells were seeded and treated with inhibitor as indicated. Cell lysates were collected after 1h of treatment and protein concentration was determined by BCA assay. Protein lysates were analysed by immunoblotting.

3.3. Immunoblotting

Cells were lysed on ice with RIPA buffer for 15 min and then centrifuged for 10 min at 4°C. Proteins were separated on 7.5% bis-acryl-tris gels, transferred to membranes, blocked in 5% bovine serum albumin (BSA) and immunoblotted with primary antibodies overnight at 4°C. Secondary antibodies were diluted 1:10 000 and incubated with shaking at room temperature for 1 hour. Enhanced chemiluminescence substrates were used for detection and membranes were scanned.

3.4. Immunofluorescence

NB cell lines were seeded on collagen precoated cover glasses. After 24 hours, cells were treated with inhibitor as indicated. Cells were fixed in 4% formaldehyde for 15 min at room temperature and then rinsed three times in PBS for 5 min each. Membranes were permeabilized with 1% Triton X-100 for 5 min and rinsed three times in PBS with Tween20 (PBST) for 5 min each. Samples were blocked in blocking buffer for 60 min (5% BSA in PBS) before application of primary antibody overnight at 4°C. Samples were rinsed three times in PBST (with 0.5% BSA) for 5 min each and incubated with fluorochrome-conjugated secondary antibody for 1 to 2 hours at room

temperature in the dark, then rinsed twice with PBST (with 0.5% BSA) for 5 min each, and rinsed once in 1. PBS for 5 min. Specimens were mounted with Fluoromount-G.

3.5. Apoptosis assay

Cells were seeded and treated with inhibitor at the indicated concentrations for 24 h. Cell lysates were collected using RIPA buffer and protein concentration was determined. Samples were immunoblotted with PARP antibody, which recognizes both full length and cleaved PARP1. Actin was used to normalize cleaved PARP1.

3.6. Proliferation assay

NB cell lines were seeded on 48-well plates. Next day, cells were treated for 5 days with increasing concentration of inhibitor. The experiment was analysed in either an Incucyte instrument, where images were taken every 24 hours, or by resazurine assay performed at day 5.

3.7. Neurite outgrowth assay

ALK constructs, either mutant or wild type, and pEGFPN1 were co-transfected into PC-12 cells. After transfection, cells were diluted in culture medium and seeded into 24-well plates. The next day cells were treated with inhibitor as indicated. Neurite outgrowth was analysed 48 h post transfection.

3.8. ALK phosphorylation in PC-12 cells

Cells were transfected by electroporation with ALK mutant constructs or the wild type ALK construct. After 48 h, cells were treated with serial dilutions of inhibitor for four hours. Cell lysates were collected and analysed by immunoblotting. Actin, phospho-ALK- 1604 and pan-ALK band intensity were determined.

3.9. RNA-seq sample preparation

For RNA-seq experiments, CLB-BAR, CLB-GE, cell lines were treated for 24 and 48 hours with BAY1895344 (50 nM). Total RNA was isolated using the Promega Total RNA Isolation Kit (Promega), and RNA samples were sent to Novogene for analysis. n = 1 biological replicate for each cell line and treatment condition.

3.10. RNA-seq data analysis

The quality of the data was examined with fastqc (version 0.11.2). TrimGalore (version 0.4.0) was used to trim away any remaining sequencing adapters together with ends with a lower quality phredscore than 20. Reads shorter than 30nt after the trimming were removed in the same step. The reads were aligned towards the human reference genome (GRCh38.90) using STAR (version 2.5.2b). The amount of reads mapped towards annotated features were quantified using featureCounts (version 1.6.4). The count matrix was imported into R (version 3.5.1) where the statistical analysis was performed using DESeq2.

3.11. Proteomic/Phosphoproteomic sample preparation:

Cells were seeded in FBS free media, next day treated with ATR inhibitor-BAY1895344 (ATRi) 50 nM for the indicated time. Prior to harvesting, cells were washed with cold PBS, then gently removed from the dish. Media with cells were pour into 50ml tube and centrifuge at 1500 rpm, for 5 min. Supernatant was poured off and pellets moved and suspended again in 1 ml of ice-cold PBS in 1.5 ml tube and repeated for 5 times, 1400 rpm, 4 degree. After the last wash, cells were suspended and aliquots were taken to perform western blotting. After last spin, supernatants were discarded and cell pellets frozen and stored at -80 degree.

3.12. Proteomic/Phosphoproteomic sample analysis:

Identification and relative quantification were performed using Proteome Discoverer version 2.4 (Thermo Fisher Scientific). The database search was performed using the Mascot search engine v. 2.5.1 (Matrix Science, London, UK) against the Swiss-Prot *Homo sapiens* database. Trypsin was used as a cleavage rule with no missed cleavages allowed; methylthiolation on cysteine residues, TMTpro at peptide N-termini and on lysine side chains were set as static modifications, and oxidation on methionine was set as a dynamic modification. For the total proteome analysis, precursor mass tolerance was set at 5 ppm and fragment ion tolerance at 0.6 Da. For the phosphopeptide analysis, precursor mass tolerance was set at 5 ppm and fragment ion tolerance at 30 mmu; phosphorylation on serine, threonine, and tyrosine was set as an additional dynamic modification. Percolator was used for PSM validation with the strict FDR threshold of 1% in both cases. Quantification was performed in Proteome Discoverer 2.4. TMT reporter ions were identified with 3 mmu mass tolerance in the

MS2 HCD spectra for the phosphopeptide experiment or in the MS3 HCD spectra for the total proteome experiment, and the TMT reporter S/N values for each sample were normalized within Proteome Discoverer 2.4 on the total peptide amount. Only the unique identified peptides were taken into account for the protein quantification.

3.13. Xenograft neuroblastoma model

Female BALB/cAnNRj-Foxn1nu mice 4-6 weeks old were subcutaneously injected into the left flank with 1 × 106 CLB-BAR cells. Once the tumour reached a volume of 150 mm^3, mice were randomized to orally administrated inhibitor treatment. Xenograft tumours were harvested after 14 days of treatment and fixed in 4% paraformaldehyde for 72 h. Fixed tumour tissue was imbedded in paraffin blocks for sectioning.

3.14. Software for data presentation

To analyse data we used different software packages. Western blotting membranes were visualized with immobilon Forte Western HRP substrate in an Odyssey Fc system, and band intensity was determined using Image Studio Lite software. Images were cropped using Adobe Photoshop CS6 and the final version generated in Illustrator CS6. Neurite formation was determined with a Zeiss Axiovert 40 CFL microscope. GraphPad Prism 7 was used to represent all numeric data sets: cell proliferation curves, determination of IC50 values, combination treatment and calculate statistic.

3.15. Statistical analysis

One way-ANOVA followed by the appropriate post hoc test for multiple comparisons were used to determine differences in apoptosis, immunohistochemistry and in the xenograft experiments at significance level of 0.05.

4. RESULTS AND DISSCUSION:

4.1. Paper I:

"Phosphoproteome and gene expression profiling of ALK inhibition in neuroblastoma cell lines reveals conserved oncogenic pathways."

In Paper I we established both phospoproteomic and gene signature profiles of ALK activity from several different NB cells comparing treatment with the first and third generation ALK inhibitors. Crizotinib was one of the first ALK TKIs to be described; however the response in patients with ALK positive NB was poor (Mossé et al., 2013). Lorlatinib is a third generation ALK TKI with very positive responses in NSCLC (Solomon et al., 2018). We treated two ALK-addicted cell lines, CLB-BAR and CLB-GE, as well one ALK non-addicted (SKNAS) NB cell line with either crizotinib or lorlatinib and performed phosphoproteomic and RNAseq profiling to identify targets for future combinational treatment for patients. The profiling of CLB-BAR and CLB-GE NB cell lines led to the identification of 3345 and 2252 phosphoproteins, respectively. In ALK-addicted cells phosphoproteomic analysis revealed more than 50 proteins that were dephosphorylated upon treatment with ALK TKIs. Phosphorylation of the ALK receptor itself and its downstream targets was decreased in both lines upon treatment with crizotinib or lorlatinib. In parallel, RNAseq profiling was performed at 24 hrs of NB cells. Comparison with non-treated controls revealed 19 232 differently expressed genes. More than 400 genes were downregulated, and more than 600 were upregulated, upon treatment with either crizotinib or lorlatinib. As an outcome we obtained a list of predicted targets for further analysis. We focused on validation of downstream signaling molecules such as: transcription factors FOXO3a/4, a member of the dual specificity protein phosphatase subfamily DUSP4 and the transcriptional repressors ETV3/4 which modulates, ALK signaling. This analysis has unveiled a number of important leads for novel combinatorial treatment strategies for NB patients as well as an increased understanding of ALK dependant signaling processes.

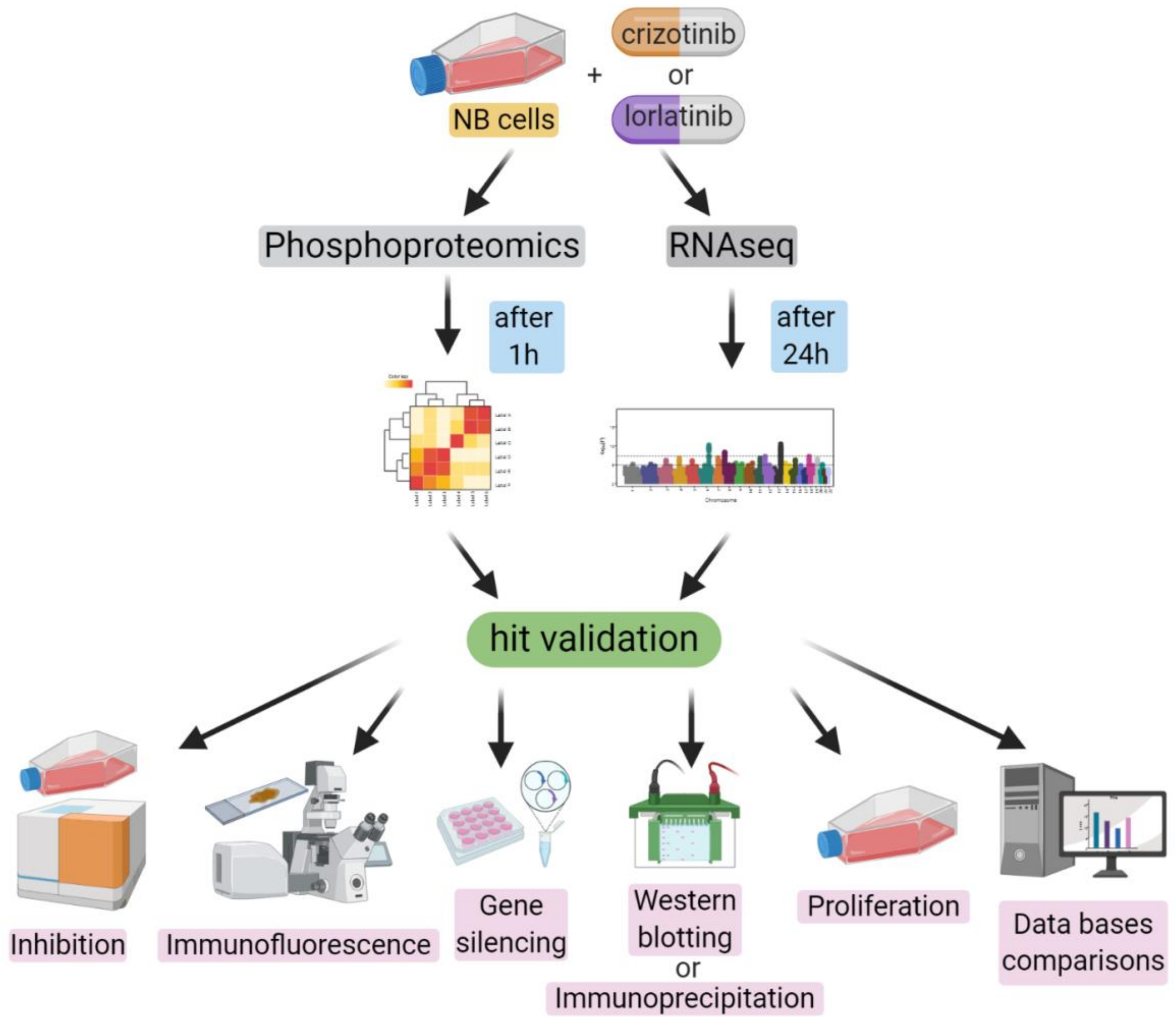

Fig. 15 Workflow of project I.

Advance analysis of oncogenic ALK signaling events in neuroblastoma.

4.2. Paper II:

“Repotrectinib (TPX-0005), effectively reduces growth of ALK driven neuroblastoma cells.”

A spectrum of small-molecule TKI inhibitors (TKIs) has been FDA approved for use in ALK-driven cancers including NSCLC and ALCL. Most ALK-dependent tumors initially respond to TKIs, but drug resistance often develops (Choi et al., 2010; Rotow and Bivona, 2017). In the present study we investigated the effects of repotrectinib (TPX-0005), a novel TKI, in an NB setting *in vitro* and *in vivo*. This rationally designed selective and highly potent TKI against ROS1, TRKA-C, and ALK possesses low-molecular weight and a three-dimensional macrocyclic structure that precisely anchors in the adenine binding site. Repotrectinib overcomes resistance acquired due to solvent-front mutation G1202R and is currently in clinical trial for adult patient with solid tumours. To investigate the effect of repotrectinib in an NB setting, a range of NB cells were treated to test if the drug can inhibit ALK and to determine its effect on proliferation. PC12 cells transfected with different ALK mutant variants indicated the efficacy of repotrectinib to block ALK activation/signaling. We also tested the effect of repotrectinib *in vivo* in a NB xenograft model. Our results show that repotrectinib is capable of inhibiting signaling activity of a range of ALK mutant variants and importantly it exhibits strong antitumour effects in xenograft NB models. Repotrectinib is superior to crizotinib in abrogating xenograft tumour growth, likely due to its pharmacology properties, and also perhaps reflecting that repotrectinib is a potent inhibitor with a broader target kinase range despite similar IC50 cell proliferation values in cell culture models. To conclude, our experimental analysis supports repotrectinib as a new potent ALK inhibitor with potential for clinical use in NB.

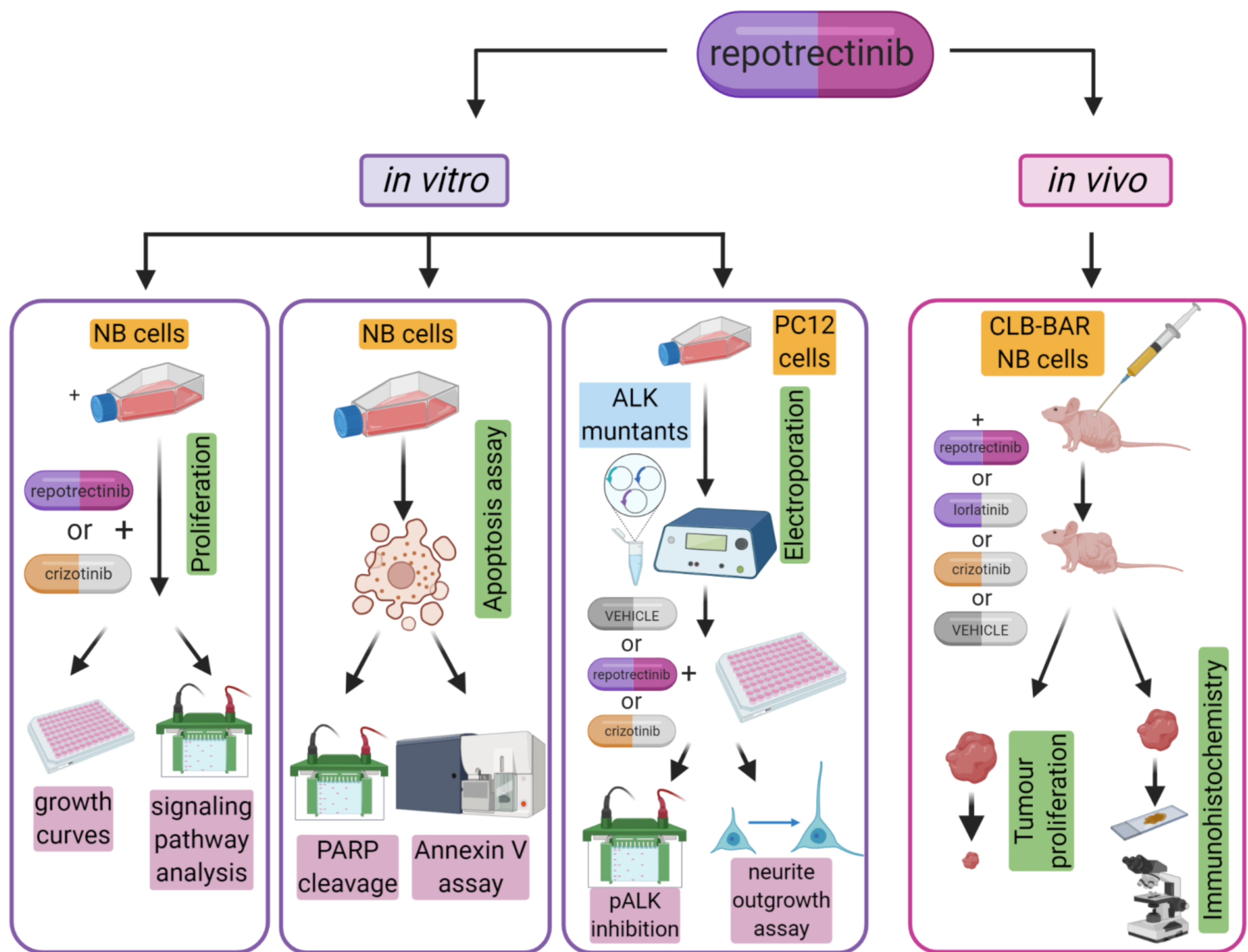

Fig. 16 Workflow of project II.

Preclinical analysis of the novel tyrosine kinase inhibitor repotrectinib in a neuroblastoma setting.

4.3. Paper III:

“Modulation of SUN2 phosphorylation downstream of ALK pathway identifies a role for ATR in neuroblastoma cell survival”

Further investigation of ALK downstream targets pathways performed in the study I identified ATR as a potential candidate. In study III, we investigated ATR and its therapeutic potential in NB. ATR protein kinase is one of the key mediators in maintaining genome integrity and coordination of the DDR. Inhibition of ATR prevents Chk1 pathway from stalled replication forks and enhances replication stress and premature mitotic entry. First, we tested two independent ATR inhibitors, AZD6738 and BAY1895344, noting that the BAY1895344 compound exhibited lower IC50 values on both CLB-BAR and CLB-GE NB cell lines leading us to choose this inhibitor for further study. In addition, BAY1895344 is the first ATR kinase inhibitor in clinical trial NCT03188965. BAY1895344 was highly effective in preventing growth of both ALK-addicted and non-addicted NB cell lines, exhibiting an apoptotic response and reduced proliferation. These findings suggest that ATR signaling is required for survival in NB cells. We also confirmed that the ATR intrinsic regulation mechanism observed and published by Saldivar in S/G2 is conserved in an NB setting. To better understand the mechanism of action of the BAY1895344 inhibitor we performed proteomic, phosphoproteomics and RNAseq analysis. Our ‘omnix’ analysis identified a broad landscape of DDR related genes which were both downregulated and upregulated, including Chk1 and p53 tumour suppressor as well as FANC genes, and apoptotic regulatory proteins. In this study we also show that BAY1895344 is effective in xenograft models of NB.

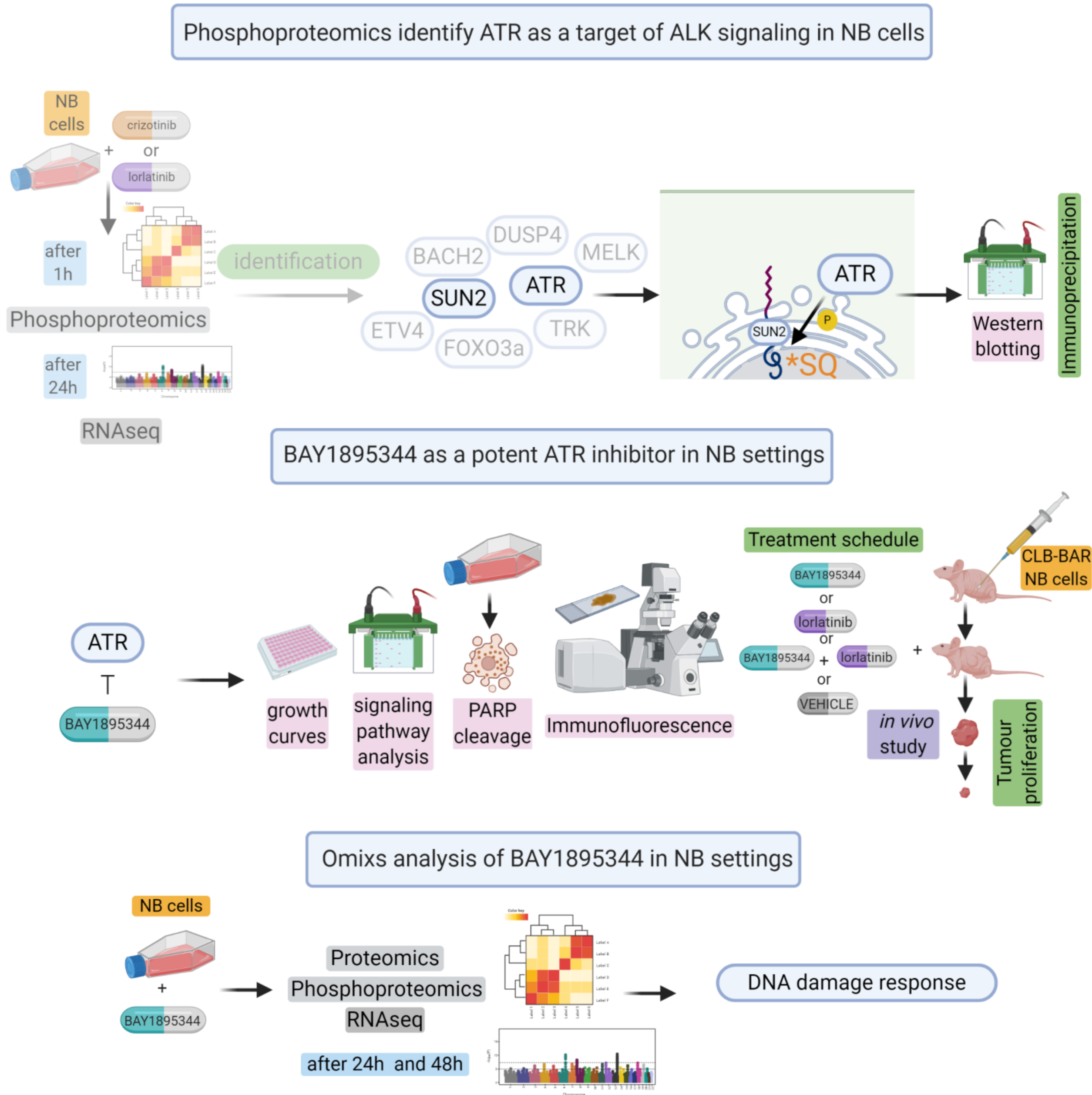

Fig. 17 Workflow of project III.

Investigation of ATR in neuroblastoma signaling.

5. CONCLUSIONS:

5.1. Paper I:

- In this study we established and compared phosphoproteomic and gene signature profiles of ALK activity in NB cells employing first and third generation ALK TKIs.
- Phosphoproteomic analysis of CLB-BAR and CLB-GE NB cells pointed to identification of 3345 and 2252 phosphoproteins, respectively.
- Phosphorylation of the ALK receptor itself was decreased in both CLB-BAR and CLB-GE lines upon treatment with crizotinib or lorlatinib.
- In ALK addicted cells, phosphoproteomic analysis revealed more than 50 proteins that were dephosphorylated upon treatment with ALK TKIs.
- RNAseq comparison with non-treated controls revealed 19 232 differently expressed genes.
- RNAseq profiling of ALK addicted CLB-BAR and CLB-GE cell lines led to the identification of more than 400 downregulated genes and more than 600 upregulated genes, upon treatment with either crizotinib or lorlatinib.
- Omics analysis lead to successful identification of well-known downstream signaling partners of ALK as well as a novel for further validation such as: ETS family, FOXO family and DUSP4.
- Validation of ETS family member ETV3 and ETV4 shows that they are regulated by oncogenic ALK.
- ALK inhibition reduces DUSP4 protein levels in an ALK-dependant manner
- ALK regulates subcellular localization of FOXO3a.
- This comprehensive analysis has increased our understanding of the ALK signaling pathway and revealed a number of important targets, including ATR as a novel treatment strategy for NB patients.

5.2. Paper II:

- We showed that repotrectinib inhibits cell proliferation in NB cells that are dependent on ALK for growth such as CLB-BAR, CLB-GE and Kelly cell lines.
- Repotrectinib abrogates ALK activity in *in vitro* biochemical assays in a manner comparable to crizotinib.

- Repotrectinib induces an apoptotic response in ALK-addicted cell lines in contrast to ALK-non-addicted cell lines.
- Our results show that repotrectinib is capable of inhibiting signaling activity of a range of ALK mutant variants.
- Repotrectinib inhibits ALK driven neurite outgrowth in PC-12 cell line.
- Treatment with repotrectinib as a single agent in human ALK-addicted NB xenografts resulted in robust tumour growth reduction.
- The antitumor effect of repotrectinib was stronger than that observed with a first generation TKI crizotinib in a xenograft model of NB.
- Repotrectinib treatment of NB xenografts leads to a decrease in the proliferation marker Ki-67 in histochemically stained tumour samples.
- We confirmed that repotrectinib is a potent ALK TKI for further exploration in NB settings.

5.3. Paper III:

- Phosphoproteomic and RNAseq analysis identified ATR as a target of ALK signaling in NB cells.
- BAY1895344 is a potent (IC50 in a range of 50-60nM) and specific ATR inhibitor for both ALK-addicted and non-addicted cell lines.
- A wide range of NB cell lines are sensitive to ATR inhibition, exhibiting an apoptotic response and reduced proliferation.
- ATR signaling is required for survival in NB cells.
- ATR regulates the S/G2 checkpoint in NB cells.
- RNAseq analysis of NB cells upon treatment with BAY1895344 identified a broad landscape of the DDR related genes which were both downregulated and upregulated and will be in used for further validation.
- Proteomic and phosphoproteomic analysis lead to identification of p53 and DDR pathway enrichment.
- BAY1895344 inhibits tumour growth in an NB xenograft model.

6. ACKNOWLEGMENTS:

"Ever tried. Ever failed. No matter. Try again. Fail again. Fail better."
Samuel Beckett

If you are reading this thesis now, it means that you are very special to me.

My journey started in a small village in a post communistic eastern European country. Insecurities, uncertainties and lack of resources was the world in which I grew up. My strong interest in science, hunger for change and vision of a better life was an engine to overcome all obstacles. However, my dream to become a successful scientist is a life-long multistep process that would never be possible without the support of many amazing people. I would therefore like to take a moment to thank those who have made this PhD adventure of mine truly special and memorable.

First and foremost, to my supervisor **Ruth**. Thank you for seeing potential in me and the offer to join your team. It has been a privilege and pleasure to work together with you in the exciting field of ALK and neuroblastoma. Thank you for years of guidance, for always being encouraging and enthusiastic. I admire your inspirational commitment to science! With your attention to details and high standards you push me to always give the maximum of my abilities. You are an excellent example of how to be a powerful woman, scientist and mother. You have been a tremendous support and there is so much that I have learnt from you. Thank you!

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Hisae, Tack för all din hjälp att hålla labbet organiserat. Du är en mycket varm och hjälpsam person och jag önskar dig och din familj allt gott! Jag kommer aldrig glömma kakorna av röda bönor som du bakade till mig.

Marcus, good luck with your medical career and PhD!

To all new members of the lab, **Edith**, I wish you all the best. **Malik**, good luck with your studies. **Jonatan**, your journey just stated, have a good one! Good luck with your studies! **Badrul** all the best to you! **Vimala**, I hope you will become *in silico* hero in the lab! All the best!

Thank you to all the former **Biomedicine-mates!** To **Piyush**, **Martin**, **Ganesh**, **Diana**, **Hannah** and **Maite**. It was nice to meet you all. Thanks for your valuable comments during lab meetings! Maite thanks for being my concert buddy! I love your music taste! I am very much looking forward to some great gigs with you!

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7. LIST OF FIGURES:

All figures (except Fig. 8 and Fig. 9) have been created with BioRender.com

Fig. 1 Cancer initiation and progression. 1
Fig. 2 Hallmarks of cancer. 4
Fig. 3 Neural crest cells migration 8
Fig. 4 Schematic representation of SCV in NB patients. 12
Fig. 5 Telomere biology in cells. 21
Fig. 6 Architecture of ALK receptor. 24
Fig. 7 Signaling downstream of ALK. 27
Fig. 8 Crystal structure of crizotinib binding to the KD of ALK. 31
Fig. 9 Crystal structure of lorlatinib binding to the KD of ALK. 32
Fig. 10 Comparison of the structures of crizotinib, lorlatinib and repotrectinib. 33
Fig. 11 Simplified illustration of a complex network regulating the cell cycle. 35
Fig. 12 Cell cycle checkpoints 37
Fig. 13 PIKK family 39
Fig. 14 ATR inhibitors in I/II phase clinical trials. 41
Fig. 15 Workflow of project I. 51
Fig. 16 Workflow of project II. 53
Fig. 17 Workflow of project III. 55

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