The role of KMT2A in zebrafish development

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**NANYANG
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THE ROLE OF *KMT2A* IN ZEBRAFISH DEVELOPMENT

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ABSTRACT

The Histone-lysine N-methyltransferase2A (KMT2A) acts as a global regulator of gene expression. KMT2A is responsible for methylation of lysine 4 of histone 3 (H3K4) on a small subset of genes, including HOX genes. The methylation of H3K4 neutralizes lysine positive charge thus making associated genes available for transcription. Having a general cellular function in histone methylation, KMT2A plays important roles in a variety of different cell processes and embryonic development. In medicine *KMT2A* is known to form fusions with other genes as a result of chromosomal translocations. The products of these fusions are known as a major cause of infant acute myeloid and lymphoblastic leukaemia.

In spite of these extremely important biological roles, the molecular mechanisms of KMT2A action are poorly understood. A better understanding of the action of KMT2A in embryonic development will help shed light on its basic cell functions and on the molecular mechanisms of the oncogenicity of KMT2A-fusions.

To study the role of KMT2A in embryonic development, we used morpholino (MO) knock-down and CRISPR-Cas9 knock-out approaches in zebrafish embryos. By phenotypic observation of *kmt2a* morphants and mutants, we found that downregulation or loss of function of *kmt2a* cause severe defects in blood circulation, defects in the development of some tissues derived from the neural plate border (NPB) and defects in eye development.

We revealed severe perturbations of primitive erythropoiesis and vascularization in *kmt2a* knock-down embryos and established the position of *kmt2a* in the genetic hierarchy regulating embryonic erythropoiesis and vasculogenesis. *kmt2a* downregulation also was shown to affect development of NPB derivatives (craniofacial cartilages, Rohon-Beard cells, trigeminal ganglia). We also detected transcription factors regulating development NPB and its derivatives neural crest (NC) and Rohon-Beard (RB), the expression of which is affected by *kmt2a* knock-down. The novel role of *kmt2a* in the bilateral splitting of forebrain into left and right fields and morphogenesis of optic stalk and optic cup was also shown by downregulation and loss-of-function approaches.

The study thus revealed novel roles for *kmt2a* in zebrafish primitive and definitive hematopoiesis, vasculo- and angiogenesis, NPB, forebrain and eye development and provides multiple avenues by which deeper understanding of the molecular functions of *kmt2a* can be further explored.

1. INTRODUCTION

1.1. The histone-lysine N-methyltransferase *KMT2* gene family

Histones can undergo a variety of biochemical modifications such as phosphorylation, acetylation, ubiquitylation, methylation, which influence their interaction with DNA and other proteins thus acting as an important regulating mechanism of gene expression. Methylation of histones can occur on arginines, lysines and histidines and influence gene expression in both activating and suppressing ways ¹. Based on the modern nomenclature system proteins responsible for the transfer of methyl group to lysine residues are grouped into KMT families ².

Histone-lysine N-methyltransferase 2 (*KMT2*) is the second out of eight *KMT* mammalian gene families known to date. *KMT2* contains six genes playing major roles in transcriptional regulation. *KMT2* members play their regulative role by transferring a methyl group from S-adenosyl-methionine to lysine 4 of histone 3 ³. This transfer neutralizes the positive charge of lysine and contributes to the release of negatively charged DNA from the nucleosome making it available for transcriptional machinery. Thus *KMT2* act as positive transcriptional regulators ⁴. The six members of the *KMT2* family have diverse structures but all contain highly evolutionary conserved SET (Su(var)3-9/Enhancer of zeste/trithorax) domain which is responsible for methyl transfer ²⁻⁴. *KMT2* members are known to be involved in a variety of critically important cell processes such as X-chromosome inactivation, apoptosis and cell cycle regulation ⁵⁻⁷. Recently their bookmarking role in mitotic cell memory was discovered ⁸⁻¹¹. In medicine, malfunctioning *KMT2* members are associated with a variety of cancers ^{3,12}.

The best-studied member of the family is *KMT2A*, a human homologue of *Drosophila trithorax (Trx)* whose best-known function is the maintenance of *HOX* (homeobox containing) gene expression in a developing embryo ^{13,14}. *KMT2A* and *HOX* play important roles in the regulation of embryonic body patterning along the antero-posterior axis ¹⁵⁻¹⁷, embryonic and adult hematopoiesis ¹⁸⁻²⁰, and patterning and functioning of neural system ²¹⁻²⁴. *KMT2A* is also well known in the medical field as the mixed lineage leukemia gene *MLL1*. As can be concluded from the name, the rearrangements in the gene are strongly associated with leukemia ^{19,25-28}. The critical biological functions of *KMT2* family members and their active involvement in the development of common human diseases make it extremely important to understand their role and the molecular mechanisms of action.

1.2. Discovery and evolutionary aspects of *KMT2* gene family

The first SET-domain containing protein Trx was identified in 1980 by Ingham and Whittle as a spontaneous mutation in *Drosophila* which leads to improper localization of different organs along the embryonic antero-posterior axis (transformation of halteres into wings, leg transformations, appearance of additional pair of wings). It is thought to be a “rediscovery” of a mutation which was found by Astauroff in 1930. Astauroff described similar phenotypic changes and mapped the gene, to which he gave the name “*tetraptera*”, to the same chromosomal locus^{16,29}.

The phenotype of Trx-mutated flies was similar to that which had been described for certain *hox* gene mutants, suggesting that these genes possibly share a common function. Subsequently, the role of Trx in the maintenance of *hox* gene expression pattern was established^{15,16}. Today a diverse number of Trx *hox*-dependent and *hox*-independent functions are being discovered, however the developmental role of Trx in the maintenance of *hox* expression remains the foundational and the most well-known one. Discovery of Trx led to further studies which identified a group of proteins functioning in big complexes and associated with gene activation. With reference to the founder protein, the group was named the Trithorax group (TrxG)^{13,17,30}.

TrxG proteins are subdivided into three different classes according to their function. The first class is represented by histone methyltransferases which modify histone structures through methylation of histone tails. The second class includes ATP-dependent chromatin-remodeling factors which recognize methylation marks and are attracted to the targeted chromatin. The third class, Trithorax, binds specific DNA sequences and recruits chromatin-remodeling and histone-modifying complexes. The SET domain is directly responsible for adding methyl groups to all histone lysines (except H3K79) that are known to be the targets of methylation (H3K4, H3K9, H3K27, H3K36, H4K20). The importance of methylation of lysine residues on histones defines the critical role of TrxG for basic cellular processes¹³.

The six members of the mammalian *KMT2* family arose as a result of whole genome duplication. Mammals has two paralogous gene in each group, *KMT2A* and *KMT2B* homologous to *Trithorax*, *KMT2C* and *KMT2D* homologous to *Trithorax-related*, *KMT2F* and *KMT2G* homologous to *dSet1*^{20,31} (Figure 1). All six members of the family (*KMT2A*, *KMT2B*, *KMT2C*, *KMT2D*, *KMT2F* and *KMT2G*) are translated into products which contain a SET domain. The SET domain is highly conserved in evolution through yeast to humans and plays the actual methyltransferase role²⁰. SET domain-containing proteins transfer a methyl

group from s-adenosylmethionine to histones within large multi-subunit complexes named COMPASS or COMPASS-like (complex proteins associated with Set1) ^{13,32,33}.

Figure 1. KMT2 family members and their functional domains.

Adapted from ³.

1.3. HOX genes and their biological role

1.3.1. Discovery and evolutionary aspects of the HOX gene family

Trithorax and its mammalian homologs KMT2A and KMT2B are known to be responsible for methylation of H3K4 at a small subset of genes that include *HOX* genes ³². *HOX* genes encode homeodomain (HD)-containing proteins which act as transcriptional regulators. The homeodomain containing proteins comprise a very diverse group with around 100 members described to date in different species. The HOX are grouped in a single HD-containing class of proteins based on specific functions and specific structural characteristics ³⁴.

The history of *Hox* discovery can be traced back as early as 1894 when W. Bateson first defined the phenotype by saying that when “something is changed into the likeness of something else” it is a “homeotic” transformation. In 1915, Calvin Bridges identified a mutation in flies in which the anterior part of the third thoracic segment of *Drosophila* developed as a copy of the anterior part of the second thoracic segment. That was the first homeotic mutation identified and was given the name “*bithorax*” based on the observed phenotype. Later, the second (*bithoraxoid*) and third (*Ultrabithorax*) homeotic mutations affecting different segments of the *Drosophila* body plan were discovered ^{35,36}. The phenotypic changes characterized by the development of some organs rather than others (homeotic transformations) were similar for all discovered mutations. Further research revealed that *bithorax* and *bithoraxoid* were located at the same chromosomal locus and led to a similar phenotype when mutated. That observation highlighted the complexity of their genetic interactions which was characterized as “pseudoallelism”.

The majority of the work aimed at understanding pseudoallelism was done by Lewis. He spent 30 years studying phenotypic changes provoked by linked genes using *Hox* genes as a model. He identified more than 100 mutations in the *bithorax* complex and in 1978 formulated the hypothesis that homeotic genes had arisen through genome duplication events and were evolutionary conserved for bilaterian species¹⁵. He mapped and grouped into clusters the genes which when mutated caused homeotic transformations in different thoracic segments of *Drosophila* along the antero-posterior axis. Later on, the sequencing of genes from the discovered clusters revealed the sequence of the commonly shared *homeobox* which was further identified in a variety of related genes in numerous animal species³⁷⁻³⁹.

Today the *Hox* genes are known to be present in all bilaterians starting from the first ancestor, where they play regulatory roles in the patterning of the organs, tissues and structures along the antero-posterior axis during development⁴⁰. The genes are most often grouped in clusters which are thought to result from genome duplication and inheritance. Land vertebrates typically have four clusters of *Hox* genes. As a result of a genome duplication event in the teleost lineage, zebrafish have seven *hox* clusters⁴¹⁻⁴³.

1.3.2. Collinearity phenomena of *Hox* expression

The *Hox* gene expression pattern along the antero-posterior axis is characterized by a set of fascinating features first noticed by Lewis, and described as “*collinearity*”¹⁵. *Hox* gene expression is spatially, temporally and quantitatively collinear. Spatial collinearity of *Hox* genes means that their expression along the antero-posterior axis is collinear with their order in the chromosomal locus; this means that the first gene of the cluster (closest to the 3' end with respect to the direction of transcription) expresses most anteriorly, the expression of the next gene is observed at the following body segment (or antero-posterior level) and so on along the cluster and antero-posterior body axis. Temporal collinearity describes the sequential order of *Hox* gene activation in time as observed in vertebrates. Quantitative collinearity was used to describe the development of tissues in the genitalia of mice. It was shown that if two neighboring *Hox* genes are co-expressed in the region, the expression of the more posterior gene is always stronger than the more anterior one⁴⁴⁻⁴⁶.

1.3.3. The establishment and maintenance of *Hox* gene expression

To orchestrate developmental events along the antero-posterior axis, *Hox* genes first should be collinearly activated. The expression of *Hox* genes is known to be induced in response to complex signaling cues⁴⁷⁻⁴⁹. The regulatory network governing the initiation of *Hox* gene expression is a complex series of events which differ in time, germ layer and along

the antero-posterior axis. In mesoderm, *Hox* expression was shown to directly or indirectly depend on early Wnt and Fgf signaling during the formation of the primitive streak^{50,51}. Fgf, Wnt and Notch also regulate initiation of *Hox* expression in mesoderm during segmentation⁵²⁻⁵⁴. Retinoic acid (RA) and retinoids also participate at both gastrula and segmentation stages^{48,55}.

The patterning of *Hox* expression in the neuroectodermally derived hindbrain is directly regulated by retinoids and RA through the presence of retinoic acid responsive elements (RAREs) in associated enhancer regions. RAREs are short DNA sequences specifically recognized by the retinoic acid receptors. Binding of RA to the receptor can activate or repress transcription of associated genes. In neuroectodermal patterning, RAREs flanking some *Hox* genes become activated in time windows and presumptive body segments which are collinear with the initiation of the corresponding *Hox* gene expression pattern⁵⁶⁻⁶¹.

There are also transcription factors directly involved in the initiation of specific *Hox* expression. Among these, Krox20 regulates *Hox* in neuroectoderm, and Kreisler is specific for the expression of certain *Hox* genes in the 5th and the 6th rhombomeres. Among the known mesodermal direct regulators of *Hox* are Gdf1⁶² and the recently implicated t-box16⁶³. The *Cdx* gene family, whose members also have a homeobox and are thought to share a common ancestral cluster with the *Hox* genes, are also translated into proteins which are direct regulators in both mesoderm and neuroectoderm^{64,65}.

After the initiation, other systems are responsible for the regulation and maintenance of *Hox* expression. These include such regulatory mechanisms as chromatin remodeling, regulation by non-coding RNAs and the action of other transcription factors at *Hox* enhancers. HOX themselves exert auto- and cross-regulatory control over their own expression⁶⁶⁻⁶⁸. Recently the role of non-coding RNAs in the establishing and maintenance of *Hox* pattern was described⁶⁹⁻⁷¹. The other important mechanism regulating initiation and patterning of *Hox* expression are dynamic chromatin modifications such as chromatin looping out and de-condensation^{65,66}.

Polycomb (Pc-G) and Trithorax group (Trx-G) are the most well-known chromatin modifiers responsible for the maintenance of *Hox* gene expression patterns. These two families play opposite regulatory roles on *Hox*. Trx-G (KMT2A in humans) maintains the expression of previously activated *Hox* genes, while Pc-G maintains the repression of inactivated *Hox* genes. Both groups are recruited to DNA by direct binding to corresponding responsive elements (PRE for PcG and TRE for trxG), interaction with ncRNAs and

recruitment by other proteins and recognition of histone marks. The recruited Pc-G complex methylates H3K27 and H3K9, the common feature of silenced chromatin, keeping cis-regulatory elements of *Hox* genes in an inactive state. TrxG methylates H3K4, thereby maintaining an open chromatin configuration and ensuring that *Hox* regulatory regions are available for other transcriptional activators and the transcriptional machinery³⁰.

1.3.4. Molecular mechanism of HOX action and the HOX cofactors MEIS, PREP and PBX

To play their regulatory role, HOX bind corresponding elements at target genes to activate or repress transcription. The single DNA binding HD in HOX proteins, along with the rather short and degenerate HD recognition motif, raises the question as to how HOX manage to act in such a highly specific manner. The sequence outside of the HD is divergent between different HOX proteins, making it possible for them to interact with a variety of other proteins. Indeed, three cofactors PBX, MEIS and PREP are known to directly interact with HOX proteins and to influence DNA binding⁷². Several permutations of HOX-cofactor interaction on DNA have been described including HOX-PBX, HOX-MEIS/PREP and HOX-PBX-MEIS/PREP, with one of the first *Hox* autoregulatory elements described in vertebrates having three HOX-PBX binding sites⁷³. The other suggested mechanism defining HOX specificity is a so called latent specificity mechanism^{74,75}. The enhancers including those which are targeted by HOX along with the high-affinity binding sites contain multiple low-affinity binding sites. These low-affinity sites were shown to define specificity of the targeting of corresponding enhancers by different members of the HOX family⁷⁶. As noted for many other transcription factors, HOX-complexes exert their regulative roles at least in part through the recruitment of histone modifiers (e.g. CBP, HDAC1, HDAC3), which do not bind DNA^{36,72}.

1.3.5. *Hox* in embryonic development

As in *Drosophila*, vertebrate *Hox* genes are also responsible for axial morphogenesis⁷⁷. Mutations in *Hox* genes cause defects in derivatives of all three germ layers and are characterized by skeletal abnormalities, neural and hematopoietic defects, and altered morphogenesis of neural crest derivatives. The most well studied is the role of *Hox* in somite morphogenesis, especially with regard to the development of the axial skeleton⁷⁸⁻⁸⁰. Analogous to the somitic column, the hindbrain is segmented along the antero-posterior axis into rhombomeres which are patterned by differential *Hox* expression. Patterning of the

antero-posterior and dorso-ventral axes of the spinal cord is also under Hox gene regulation^{81,82}.

Hox also regulate the development of craniofacial structures by patterning of NC cells and the associated pharyngeal arches into which they migrate. NC cells adjacent to the hindbrain rhombomeres migrate into the pharyngeal arches which will develop into cranial skeletal tissue, connective tissue, ganglia, pharyngeal glands and bones of the middle ear⁸³. In fish, pharyngeal arches develop into the mandibular and hyoid bones of the jaw and the ceratohyal bones of the gills^{84,85}. There are two possible mechanisms for regulation of NC cells of pharyngeal arches by Hox. First, the rhombomeres can influence adjacent NC cell morphology; second, the NC cells themselves can express different patterns of Hox genes (except the NC cell of the first pharyngeal arch which is known to be Hox-negative)⁸⁶⁻⁸⁸.

Multiple studies done on zebrafish and mice describe the involvement of *Hox* genes in embryonic and adult hematopoiesis⁸⁹⁻⁹¹. The well-known involvement of *HOX* in human leukemia gives additional evidence for the important role of the genes in hematopoiesis^{18,92}. To add to this body of knowledge, research from our team has revealed *hoxd4a* plays a role in zebrafish primitive and definitive hematopoiesis⁹³ and neurogenesis (data not published).

1.3.6. *HOX* in human diseases

Clinically, *HOX* are mostly known as one of the causes of acute myeloid leukemia. As a result of chromosomal translocation, they can form gene fusions resulting in the production of chimeric oncogenic proteins. One of the most frequent fusion partners is *NUP98*, which can be fused with eight different *HOX* genes including *HOXA9*, *HOXA11*, *HOXA13*, *HOXD11* and *HOXD13*. *HOX* deregulation also makes an important contribution in *KMT2A*-induced leukemia¹⁸. There is also a list of rare genetic disorders caused by inborn mutations in different *HOX* members. The phenotypic features for a majority of them include, but are not limited to, limb malformations, craniofacial abnormalities, and neurological and hematopoietic defects⁹⁴.

1.4. *KMT2* genes and their biological role

1.4.1. The biological role of *KMT2* and their mechanism of action

KMT2 family members are involved in a variety of biological processes influencing the function of cells, tissues and the whole organism. Apart from the developmental role in the maintenance of *HOX* expression, they are known to be involved in the regulation of cell memory (*KMT2A* stays on the mitotic chromatin of a dividing cell and plays a

“bookmarking” role to restore the epigenetic program of the cell after division), mitosis, apoptosis, hematopoiesis, and neurogenesis^{8–11,13,14,18,23,24}.

As for *HOX*, the most clinically relevant member of the *KMT2* family, *KMT2A*, has achieved its fame due to its ability to cause mixed lineage leukemia following chromosome translocation^{26,95}. Recent research has also revealed the involvement of *KMT2* members in a variety of rare and common cancers^{3,12,96}. *KMT2* may also be involved in such medical conditions as dementia, memory loss, autism, depression and schizophrenia and in proper brain functionality generally²². A better understanding of *KMT2* functions and mechanisms of action is thus of great biological and clinical interest.

1.4.2. *KMT2A* structure and partners within COMPASS-like functional complex

KMT2A is a large protein containing 3,969 amino acids and multiple functional domains¹⁹. After translation, it undergoes cleavage by taspase I into N- and C-terminal products that re-associate to form the functional protein. The SET domain, as well as protein-protein interaction domains that mediate formation of the COMPASS-like complex, are located at the C-terminus. The N-terminus contains important protein-protein interaction domains, including a menin-binding motif that is responsible for interaction with the tumor suppressor Menin. Menin is a unique subunit of COMPASS-like structures containing *KMT2A* and is important for the localization of the complex on chromatin^{97,98}. The N-terminus also contains three AT-hook motifs and a CXXC domain that are not represented in the *Drosophila* homologue Trithorax. The AT-hook motifs are thought to mediate direct binding of *KMT2A* to AT-rich sequences via the minor groove of DNA. The CXXC domain is highly conserved within a small group of proteins, including *KMT2A*, DNA methyltransferase and methyl-CpG-binding protein and is thought to recognize CpG rich DNA regions^{13,29,99}. All the functional domains and the mature cleaved *KMT2A* along with its main partners within the COMPASS-like complex are represented on Figure 2.

Such multi-domain structure determines the complex biological roles of *KMT2A* beyond the SET domain H3K4 methylation function. When isolated from *KMT2A* and COMPASS proteins, the SET domain possesses only weak monomethylase activity, which significantly increases after addition of other COMPASS components (WDR5, RbBP5, Ash2L, Dpy30)³². Moreover, removal of WDR5, RbBP5 or Ash2L impairs the ability of COMPASS to di- or tri-methylate H3K4, while monomethylation stays unchanged. *KMT2A* removal also affects the ability of COMPASS-like to dimethylate H3K4¹⁹. Based on these observations, it was suggested that the interaction of *KMT2A* with its partners is necessary to

establish an active structural conformation of the SET domain¹⁰⁰. Another model suggests that non-SET-containing regions of KMT2A contribute to an independent methylase active site within the COMPASS complex¹⁹.

Figure 2. Schematic representation of KMT2A and its main partners within COMPASS-like complex.

Adapted from¹⁹. MBM - menin binding motif, LBD - lens epithelium-derived growth factor binding domain or (light red), DNA binding AT-hooks, homologous to DNA methyltransferases CXXC motifs, PHD plant homeodomain fingers, BD - bromodomain, FYR - phenylalanine-tyrosine rich regions, Win - WDR5 interaction motif, SET domain. a) The full-length MLL1 protein b) cleaved MLL1 and re-associated MLL-N and MLL-C fragments with partners - menin, lens epithelium derived growth factor, nuclear cyclophilin (Cyp33), host cell factor (HCF); CREB binding protein (CBP); histone acetyltransferase MOF; WD-40 repeat containing protein-5 (WDR5); retinoblastoma binding protein-5 (RbBP5); absent, small, homeotic disc-2 like (Ash2L); DPY-30.

1.4.3. KMT2 as regulators of cellular processes

Consistent with their size, multi-domain structure and inclusion within multi-subunit complexes, SET-domain-containing proteins are involved in a variety of cellular processes such as transcription regulation, mitosis, apoptosis, cell memory, DNA replication, recombination and repair^{4,101,102}.

Recent studies suggest a possible role of KMT2A as well as other family members in regulation of mitosis^{9,103}. During mitosis, KMT2A stays associated with DNA on condensed chromatin playing a bookmarking role in the dividing cell, and is therefore responsible for cell memory⁸⁻¹¹.

Studies in yeast demonstrated that mutation of set1 results in the death of the yeast culture within six days. Cell death was shown to be the result of activation of apoptotic pathways associated with the loss of H3K4 methylation in Δ set1 culture¹⁰⁴. The earlier study

also demonstrated involvement of KMT2A and H3K4 methylation into the apoptotic processes mediated by E2F1 transcription factor ⁶. Additional research done on yeast demonstrated involvement of set1 into the NHEJ repair. The set1 complex was shown to methylate H3K4 at the DSB points making it available for transcriptional machinery ¹⁰⁵.

1.4.4. KMT2A in embryo development

As a regulator of *Hox* gene patterns, *Kmt2a* shares common functions with *Hox* in embryonic development. Analogous to *Hox* it is important for skeletal patterning along the antero-posterior axis and proper neurogenesis and hematopoiesis. Both heterozygous and homozygous mutations of *Kmt2a* result in altered *Hox* gene pattern and resemble the major phenotypic defects characteristic to the ones induced by *Hox* mutations. The characterization of *Kmt2a*^{-/-} mice showed that *kmt2a* loss of function is embryonic lethal, while *Kmt2a*^{+/-} mice showed segmental abnormalities, multiple defects in embryonic hematopoiesis ^{106–109} and cranial ganglia development (neural crest derivative) ¹¹⁰.

Interestingly, mice exclusively lacking the SET domain of KMT2A are viable and fertile, demonstrating only skeletal and hematopoietic abnormalities. Loss of the SET domain disturbs mono- and di-methylation patterns of H3K4 at specific *Hox* gene promoters and the phenotype is similar to those caused by *Hox* gene mutations ²⁹. A conditional knock-out model with deletion of exons 8 and 9 of *Kmt2a* in adult mice using the Vav-Cre system led to a disability of hematopoietic stem cells (HSC) to maintain self-renewal, but did not cause any severe defects in hematopoiesis. Deletion of exons 3 and 4 using the MX1-Cre system led to bone marrow degradation and depletion of HSC ¹¹¹.

kmt2a knock-down studies on zebrafish revealed similar phenotypic defects. The fish with downregulated *kmt2a* developed defects in primitive and definitive hematopoiesis ^{112,113}. The gene was also shown to play an important role in the specification of neural cells at the stage of neural tube formation, their survival and proliferation abilities and further development into glial lineage ²⁴. In addition to hematopoietic, skeletal and neural abnormalities, *kmt2a* was also shown to regulate development of cardio-vascular derivatives of the mesoderm ¹¹⁴.

1.5. KMT2 in human diseases

1.5.1. Structure of leukemogenic fusions of KMT2A

The most well-known implication of *KMT2A* in medical condition is its involvement in adult and infant leukemia. *KMT2A* is known to form fusions with other genes as a result of

chromosome 11 translocations. These fusion proteins are the main cause of infant acute myeloid leukemia and lymphoblastic leukemia (>70% of cases) and one of the reasons for adult acute myeloid leukemia (10%). *KMT2A* can form fusions with more than 70 different genes. Interestingly, in the products of all these fusions, the SET domain, Win motif, PHD and TAD domains and cleavage site of *KMT2A* are lost, which emphasizes the importance of the *KMT2A* N-terminal region in leukemogenesis. *KMT2A* can also undergo small rearrangements undetectable by cytological methods, such as internal deletions and partial or whole gene duplications^{19,108}. *KMT2A* fusions and partial duplications are schematically represented in Figure 3.

Figure 3. Schematic representation of *KMT2A* fusions and partial duplication.

Adapted from¹⁹. (A) wild-type *KMT2A* with indication of the break point region, (B) schematic representation of *KMT2A*-fusion proteins, (C) example of partial tandem duplications (PTDs) in *KMT2A*.

1.5.2. Molecular mechanism of *KMT2A*-fusions leukemogenic action

As a result of chromosomal structural modifications, the *KMT2A* sequence can change in the way which results into coding sequences for proteins with oncogenic activity. *KMT2A* can undergo different chromosomal rearrangements, such as direct or reciprocal fusions with other genes, internal duplications, inversions or deletions of chromosome 11 and two-directional insertions between chromosome 11 and other chromosomes, which result in the formation of coding sequences for oncogenic protein fusions. The most frequent rearrangement is the fusion of *KMT2A* with other genes, up to 79 of which are characterized at the molecular level²⁶.

It is well-known that a subset of *HOX* genes (*HOXA9*, *HOXA10*, *HOXA7*) and *MEIS1* are overexpressed in the majority of *KMT2A*-fusion-induced leukemia cases. Moreover, overexpression of some *HOX* genes individually or in combination with *MEIS1* leads to leukemogenic transformation of hematopoietic stem cells (HSC) that are phenotypically similar to the stem cells of *KMT2A* fusion-induced leukemia^{19,95,108}. However the mechanism of wild-type *KMT2A* on *HOX* genes differs from the one run by *KMT2A* fusions as they lose the sequence encoding SET domain and as a result the protein does not have methyltransferase activity.

In general the mechanism by which *KMT2A* fusions are leukemogenic just recently started becoming clear. However, there is scarce novel data available explaining different malignant mechanisms triggered by the *KMT2A* fusions, the existing body of which will be detailed below. Some *KMT2A* fusion partners are implicated in the formation of the super elongation complex (SEC) and regulate the elongation step of transcription in healthy cells. The products of *KMT2A* fusion genes contain the N-terminus of KMT2A and part of its fusion partner. It was suggested that they misregulate transcription of corresponding targets by overactivating the elongation step of targeted genes^{19,115,116}. The N-terminus of both wild-type KMT2A and its fusions interact with multiple endocrine neoplasia 1 (MEN1) and the lens epithelium-derived growth factor (LEDGF/p75). The interaction of these three proteins were shown to be responsible for binding KMT2A or KMT2A-fusion complexes to chromatin and is essential for the induction of oncogenic processes in leukemic tissues^{97,117–120}.

It was also shown that the presence of wild-type KMT2A is required for the leukemogenic action of KMT2A-fusions and both wild-type and fusion proteins are recruited to the *HOX* promoters in leukemic tissues¹²¹. The suggested model explaining *HOX* overexpression is that KMT2A-fusion protein causes overexpression of the targeted gene and then wild-type KMT2A according to its regular biological function maintains the active state of these overexpressed targets thus inducing malignant transformation of the cells³. In particular KMT2A-fusion-induced overexpression of *HOXA9* gene is thought to be guided by this mechanism, as destabilization of SEC in KMT2A-fusion-containing leukemic cells leads to loss of *HOXA9* overexpression¹¹⁶. Recently it was confirmed that some of the fusions complexes recruit elongation factor complexes to *HOXA9* stimulating its transcription through this mechanism¹²². At the same time it was also demonstrated that deletion of wild-type KMT2A in MLL-AF9 induced leukemic tissues does not alter MLL-AF9 leukemogenic

ability¹²³. So the precise molecular mechanisms by which KMT2A fusions alter *HOX* gene expression in leukemic tissues are still not fully understood.

Moreover, KMT2A fusion proteins do not only deregulate expression of wild-type KMT2A targets. It is known that KMT2A fusions are preferentially bound to a small subset of wild-type KMT2A targets, however only a small percentage of these genes increase their expression in response to the action of KMT2A fusions. This is consistent with the observation that only 3% of wild-type KMT2A targets decrease their expression in response to the removal of KMT2A⁹⁵. Some of the specific targets of KMT2A fusion proteins were recently discovered. Among them are frequently rearranged in advanced T-cell lymphomas genes 1 and 2 (*Frat1* and *Frat2*). Another gene being deregulated under control of KMT2A fusions is *RUVBL2*, which is important for transcriptional regulation, DNA repair and chromatin remodeling. One more direct target is *RUNX1*, an important transcriptional regulator of myeloid lineages.

The other interesting discovery on the molecular action of KMT2 fusions is the involvement of miRNAs in the pathogenic process. In particular, the expression of miR-196b was shown to be activated by the fusions. The miR-196b was shown to play both pro- and anti-leukemogenic functions in leukemic tissues by regulating the expression of *Hoxa9*, *Meis1* and tumor-suppressor (*Fas*) genes. KMT2A fusions downregulate such miRNAs as let-7b and miR-150, and both of these are strongly linked to a variety of cancers. The data on another miR495 demonstrated its ability to suppress leukemogenic processes when overexpressed by downregulation of *MEIS1* and *PBX3*^{122,124}. The other suggested mechanisms of KMT2A leukemogenic action are based on deregulation of chromatin structure by different chromatin modifying mechanisms, including aberrant histone methylation, recruitment of chromatin remodeling complex, etc.¹⁹.

1.5.3. *KMT2* roles in other cancers

The *KMT2* role in oncology has mostly been studied based on knowledge of leukemia induced by rearranged *KMT2A*. However, recent research revealed overwhelming data on the role of the whole *KMT* family in a very diverse group of common and rare cancers. *KMT2A* by itself was shown to be mutated in different solid tumors including colon, lung, bladder, endometrium and breast. A majority of mutations resulted in a truncated KMT2A protein which was analogous to leukemogenic KMT2A fusions that lack SET domain. Other mutations affecting functional domains of KMT2A were also detailed in the research³.

The *KMT2B* mutations mostly in coding regions were detected in endometrial, large intestine, lung, glioma, and liver tumors. In liver tumor cells *KMT2B* was shown to be inactivated by Hepatitis B virus or fused with it. The screening of tumors for *MLL3/KMT2C* and *KMT2D* mutations resulted in an overwhelming number of different mutations found in different tumors. The summary presented by D. Ford and A. Dingwall lists 26 different tumors where *KMT2C* and *KMT2D* were mutated, demonstrating that these two *KMT2* members are the most important for further cancer research. *KMT2F* and *KMT2G* are known to massively methylate H3K4 in most cells. *KMT2F* is found to be mutated in 62% of large intestine, lung, endometrial carcinomas, and malignant melanoma tissue samples, and *KMT2G* mutations were detected in 63% of intestine, kidney, lung, endometrial cancers and malignant melanoma^{3,12}. Thus, the evidence strongly supports the critical role of *KMT2* mutations in malignant tissue formation.

1.5.4. Other medical conditions associated with *KMT2* functions

Inborn genetic disorders caused by mutations in both methyltransferases and demethylases of H3K4 have also been identified. Haploid insufficiency of *KMT2A* was shown to cause the rare genetic condition Wiedemann–Steiner syndrome ([OMIM] # 605130). The phenotype of affected individuals includes microcephaly, short stature, facial and skeletal dysmorphism, developmental delay, autism, aggression, and intellectual disability¹²⁵. Mutations in *KMT2C* are also known to cause Kleefstra syndrome ([OMIM] # 610253), which has been diagnosed in more than 400 children worldwide. It is also a neurodevelopmental disease with brachy(micro)cephaly, severe intellectual disability, autism, aphasia, anxiety and epileptic seizures. The last known inborn disease caused by mutations in a *KMT* family member is Kabuki syndrome (MIM no. 147920) which is caused by mutation in *KMT2D*. The clinical manifestation also includes a spectrum of neurological abnormalities such as autism, seizures, microcephaly and cortical atrophy^{21,22}. The phenotypic features of the identified syndromes and the role of *KMT* in neurogenesis shown on animal models point to the important general role of the protein family in brain development and functioning.

The latest research focused on the neurological processes associated with H3K4 methylation show possible involvement of *KMT2F* in schizophrenia (MIM no. 18150)²². The study of *Kmt2a* in the mouse revealed its critical role in the proper functioning of the forebrain and adult prefrontal cortex. Animals with impaired function of *Kmt2a* demonstrated loss of memory, increased anxiety and other cognitive defects. Interestingly, *Meis1* knock-down in the prefrontal cortex resulted in a similar phenotype pointing to the possible shared role of the two proteins in brain function²³.

Disturbed mono-, di- and tri- methylation patterns on H3K4 have been seen in the cerebral cortex of subjects affected by autism, schizophrenia and depression. However, whether it is the primary reason for the medical conditions or a consequence of the development of the diseases are yet to be understood. The molecular mechanisms which link the clinical symptoms with the chromatin condition have not been explained yet ²¹.

1.6. Zebrafish as a model for study of *KMT2A* function

Zebrafish are widely used to study vertebrate biology and disease. A zebrafish facility is easier and cheaper to maintain compared to those for such animals as mice, rats and rabbits. Zebrafish eggs are externally fertilized and the embryos are transparent which makes it very convenient for microscopic observations and manipulation. Zebrafish is an especially convenient model for the study of embryonic hematopoiesis, as at the early stages of development embryos can obtain sufficient oxygen from the water, so they survive for several days despite severe hematopoietic abnormalities. On the genetic level, zebrafish have homologs of the majority of mammalian hematopoietic transcription factors, which has led to it being adopted as a research model for human hematopoiesis and hematopoietic malignancies ^{126–128}.

Zebrafish and human *KMT2A* share 46.4% amino acid identity. The sequence similarity within functional domains reaches 68–100%. To date there are three papers published on *mlf1* functions in zebrafish ^{24,112,129}.

1.6.1. Zebrafish hematopoiesis and vasculogenesis

The zebrafish is an attractive model to study vertebrate hematopoiesis as it has all the blood lineages presented in mammals and shares a lot of similarity in regulation of their differentiation. Also analogous to mammals, zebrafish blood development occurs in two stages – primitive and definitive hematopoiesis. In between primitive and definitive waves of hematopoiesis there is also a transient wave, taking place in posterior blood island (PBI). PBI tissues can differentiate in both erythroid and myeloid lineages ^{126,127,130,131}.

The primitive hematopoietic cells as well as vascular endothelial cells emerge from the anterior lateral mesoderm (ALM) and posterior lateral mesoderm (PLM). The population of hematopoietic and angiogenic progenitors can be first identified at the two somite stage in the ALM and PLM. Previously these populations were thought to be a single population of multi-potent cells (hemangioblasts) which gave rise to both angioblasts and hematopoietic cells. The latest data is consistent with hemogenic and endothelial lineages differentiating

from two separate embryonic progenitors. However, the possible existence of a multi-potent hemangioblast population comparable to what is seen in mammals has not been excluded^{132,133}. First definitive hematopoietic progenitors specify after 24 hpf at the ventral wall of dorsal aorta from an endothelial population¹³⁴.

1.6.1.1. Induction of hematopoietic and angiogenic fates in lateral mesoderm

The earliest regulatory events specifying hematopoietic and vascular progenitors happen in the mesodermal germ layer under regulation of the Bmp signaling pathway at gastrulation. At this developmental stage the Bmp gradient is established along the dorsal-ventral axis defining the fate of the future anterior and posterior cells. The more anterior structures will be formed from the dorsal side of early gastrula with the lowest Bmp level and more posterior from the mesoderm of the ventral side with the highest Bmp levels. At the post-gastrula stage ventral mesoderm also under Bmp gradient regulation patterns into lateral and intermediate tissues. Lateral mesoderm with the highest Bmp levels will give rise to hematopoietic and vasculogenic lineages, while intermediate ventral mesoderm will develop into pronephrons^{135,136}.

The specification of lateral mesoderm into hematopoietic and vasculogenic progenitors is regulated by a number of transcription factors. *cloche* is the one which is known to take the highest position in the genetic hierarchy specifying progenitors of hematopoietic and angiogenic lineages in lateral mesoderm^{137,138}. Downregulation of *cloche* causes defects in both hematopoiesis and vasculogenesis and results in decreased expression in the earliest known markers of hematopoietic and endothelial lineages *etv2* and *sc11*¹³⁷. The role of *cloche* in the top position of the genetic hierarchy regulating embryonic hematopoiesis and vasculogenesis was confirmed by loss-of-function and gain-of-function approaches¹³⁸. Two other homeobox genes *cdx1* and *cdx4* also act very early in the specification of hematopoietic and vasculogenic progenitors. The genes take high position in the genetic hierarchy acting upstream of *sc11* which is one of the earliest markers of hematopoietic and vascular lineage^{139,140 139}.

Our group and a number of other studies have revealed an implication for a subset of *hox* genes and their cofactors *meis1.1* and *pbx* at early stage of hematopoietic and endothelial tissue specification. Downregulation of the genes resulted in morphological defects in blood and vasculature formation as well as in downregulation of specific hematopoietic and angiogenic markers *scl*, *gata1*, *fli1*, but not *cdx*^{93,141,142}. Our research for the first time

showed that *hoxd4a* is also an important regulator of specification of hematopoietic and angiogenic lineages acting upstream of *meis1*, *scl*, *gata1* and *fli1* but downstream of *cdx*⁹³.

kmt2a was also shown to play an important role in zebrafish embryonic hematopoiesis. Downregulation of *kmt2a* resulted in defects of blood flow and decreased expression of *scl*, *gata1*, *fli1* and other markers of hematopoietic lineages. *cdx* expression was not affected by downregulation of *kmt2a* thus putting *kmt2a* downstream of *cdx* genes. The effect of *kmt2a* on zebrafish hematopoiesis may be partially through its *hox* regulative function, as a subset of *hox* genes known to be involved in mammalian hematopoiesis (*hoxa9a*, *hoxb4a*, *hoxb6b*, and *hoxd3a*) was downregulated in embryos with reduced expression of *kmt2a*. However only *hoxa9a* and *hoxd3a* mRNA could partially rescue expression of *scl*, *gata1*, and *pu.1* in embryos with downregulated *kmt2a* and none of *hox* genes mRNA could fully rescue the phenotype. As such, *kmt2a* presumably acts downstream of *cdx* genes in specification of hematopoietic progenitors and might regulate development of the hematopoietic lineages as through regulation of its *hox* targets as through regulation of other unknown targets¹¹².

This body of existing evidence allows us to suggest a more refined model of transcription factors regulating the development of hematopoietic and angiogenic lineages in zebrafish. Specification of lateral mesoderm starts under regulation of consequent events initiated by *cloche* and *cdx* genes and followed by *hox*, *kmt2a*, *meis1.1* and *pbx*, which further activate the expression of a subset of *hox* genes and first specific hematopoietic and endothelial markers *scl1*, *lmo2* and *fli1* (Figure 4). However there is still a lack of data on mechanisms of action of *cloche*, *cdx*, *kmt2a*, *meis1.1*, *pbx* and *hox*. It is still unknown whether they act in a synergistic or independent manner in regulating downstream targets as well as which positions they take in the genetic hierarchy relative to each other. Better understanding of the regulative role and mechanism of action of these transcription factors in zebrafish hematopoiesis would help to reveal their role in mammalian development as well as better understand molecular mechanisms of development of leukemia and cardiovascular diseases.

1.6.1.2. Development of primitive and definitive hematopoietic lineages

After specification of hematopoietic progenitors expressing *scl1*, *lmo2* and *fli1* in lateral mesoderm the primitive hematopoietic cells further develop in erythropoietic and myeloid lineages. Erythroid progenitors differentiate from PLM and express erythroid marker

gata1 starting from two somite stages. Myeloid primitive cells differentiate from ALM and are marked by expression of myeloid lineage marker *spi1* (*pu.1*). Primitive zebrafish blood also contains thrombocytes and neutrophils, however the data on their progenitor lineage are still contradictory^{113,127,131}.

At 12 somite stage lateral mesoderm cells migrate to the midline where they form an intermediate cell mass (ICM) and rostral blood island. ICM is the major site of zebrafish primitive erythropoiesis, while rostral blood island provides embryos with cells of myeloid lineage. After 24 hpf primitive blood cells exit ICM into formed vasculature where they continue differentiating into mature cells. The primitive hematopoiesis provides the embryo with blood cells until 3.5 dpf^{113,127,131,143}.

The onset of definitive hematopoiesis in the AGM is marked by the specification of HSCs which provides fish with blood cells of all lineages throughout the life of a vertebrate. Definitive hematopoiesis in zebrafish starts by specification of subset of endothelial cells (hemogenic endothelium) of the ventral wall of the dorsal aorta into hematopoietic stem cells (HSC). The first HSC can be detected at 26 hpf. They are characterized by the expression of hematopoietic stem cells markers *runx1* and *cmyb*. These cells then migrate into caudal hematopoietic tissue (CHT). The CHT is known to be a transient site of hematopoiesis and at 3.5 dpf it starts expressing the first definitive erythrocytes, thrombocytes and myeloid cells. The HSC also migrate to the thymus and kidney, establishing definitive hematopoiesis sites. The thymus is a source of T cells for adult zebrafish and kidney marrow is a hematopoietic organ producing myeloid cells, B cells, erythrocytes and thrombocytes^{126,127}.

Analogous to the primitive blood lineages definitive erythrocytes are marked by *gata1* expression and myeloid cells by *pu.1*. Lymphoid progenitors can be identified in the bilateral thymii at 72 hpf and marked by *ikzf1* expression^{126,134}. *cdx*, *meis1.1*, *pbx*, *hox* and *kmt2a* were shown to affect both primitive and definitive hematopoietic lineages, however their precise role in definitive hematopoiesis is still unclear. These transcription factors may affect definitive hematopoiesis through their early regulative role in the development of hematopoietic and angiogenic progenitors in lateral mesoderm, and they may also be involved in the regulation HSC maturation. Better understanding the possible different roles of the regulative network in specifying early embryonic hematopoietic progenitors and differentiation of HSC into mature blood lineages can shed light on a number of developmental and medical questions, including pathological processes in leukemia and other hematological diseases.

The schematic representation of primitive and definitive hematopoiesis and their major regulators in zebrafish are presented in Figure 4 and 5.

Figure 4. Genetic hierarchy regulating development of hematopoietic and endothelial lineages.

Figure 5. Development of hematopoietic and angiogenic lineages in zebrafish.

Adapted from ¹³⁰ and ¹⁴⁴. (A) specification of angiogenic and hematopoietic progenitors at six somite stage, ALM – anterior lateral mesoderm, MLM – medial lateral mesoderm, ICM – intermediate cell mass, AGM – aorta-gonad mesonephros, PLM – posterior lateral mesoderm; (B, left) sites of primitive hematopoiesis at 24 hpf; (B, right) sites of definitive hematopoiesis at 72 hpf; CHT – caudal hematopoietic tissues.

1.6.1.3. Zebrafish vasculogenesis

Zebrafish are widely used to study vasculogenesis as vasculature formation in zebrafish embryos share a lot of similarity with other vertebrates. In particular, similarly to

mammals, formation of the vasculature network in zebrafish includes two different mechanisms. De-novo assembly of large vessels from endothelial cells called vasculogenesis and sprouting of smaller vessels as a result of major vessels structure remodeling called angiogenesis.

The first vascular progenitors develop at the two somite stage in the ALM and PLM and are marked by the expression of *scl*, *lmo*, *fli1* and endothelial specific genes *flk1/vegfr2*^{137,145}. After specification, angioblasts migrate ventrally and start assembling into the primary vessels along the midline. In the trunk area PLM angioblasts assemble into a vascular cord which further gives rise to the dorsal aorta (DA) and consequently to the posterior cardinal vein (PCV). In the cranial region, angioblasts of the ALM assemble into two lateral dorsal aortae LDA primordia, which further connect with each other and develop into the paired LDA. LDA then connects with the trunk DA through angiogenesis^{146,147}. The first cranial veins formed through vasculogenesis are the primordial hindbrain channels (PHBC). In the hindbrain the first artery is the basilar artery (BA) which forms in between two PHBC. Later two central arteries are formed (CtAs).^{148,149} An overview of zebrafish vasculature at 2 dpf is presented in Figure 6.

Cranial and trunk vessel assembly goes through the same mechanisms and from the same progenitor type; however, differences in the regulation of anterior vs posterior vasculogenesis and angiogenesis have been reported. In particular, mutation in *sox32* results in delayed but normal formation of DA, whereas the LDA are not formed. Mutations in chemokine receptor *cxc4a* or its endodermal ligand *cxc112*, which is specifically expressed in the endoderm underlying the LDA, both result in the absence of LDA. These results demonstrate the involvement of different mechanisms and the important role played by surrounding tissues on the regulation of vasculogenesis at different spatial locations in the body^{147,150}.

hox, *meis1.1*, *pbx*^{93,113} and *kmt2a*¹¹² have been shown to affect vasculogenesis and their role at the early stages of angioblasts specification in lateral mesoderm has also been reported. However their targets, mechanism of action, and role in the pathways regulating different processes of vessel formation (as described below) at later stages have not been studied yet.

Figure 6. Vasculature of zebrafish embryo at 2 dpf¹⁴⁶.

CA – caudal artery; CV – caudal vein; Se – intersegmental vessel; PCV – posterior (caudal) cardinal vein; DA – dorsal aorta; DLAV – dorsal longitudinal anastomotic vessel; SIV – subintestinal vein; CCV – common cardinal vein; PHBC – primordial hindbrain channel; CtA – central artery; PCeV – posterior (caudal) cerebral vein; DLV – dorsal longitudinal vein; MsV – mesencephalic vein; PrA – prosencephalic artery; NCA – nasal ciliary artery; IOC – inner optic circle; PHS – primary head sinus; VA – ventral aorta; AA – mandibular arch; H – heart.

1.6.1.4. Acquisition of arterial vs. venous fate

The mechanism by which primary vessels acquire either arterial or venous fate is still not well understood. Previously it had been suggested that the vessels differentiate into the veins and arteries after the initiation of blood flow and under its influence. However, it was later shown that after differentiation in the lateral mesoderm and migration to midline angioblasts, it starts assembling into a vascular cord located between the hypochord and mesoderm. This primordial vessel will further give rise to arterial and then vein cell populations. After the assembly of the vascular cord, Shh signals from notochord activate the expression of *vegfa* in somites. Vegf-a from somites binds Kdr1 receptor on angioblasts resulting in activation of a Notch signaling pathway. The activated Notch signaling together with other transcription factors (Efnb2) induce differentiation of dorsally located angioblasts into artery cells which will form DA. The other group of cells which do not respond to Vegf-a signalling, have a lower level of Notch activation and express the specific marker *ephB4*, will acquire venous fate, migrate ventrally from vascular chord ventrally and assemble into CV and PCV^{151,152}. *sox7* and *sox18* were also shown to participate in this differentiation process acting downstream of Shh and Vegf signaling, but upstream of Efnb2/EphB4¹⁵³.

1.6.1.5. Development of the vasculature network through angiogenesis

Once formed, the major vessels develop smaller veins, arteries and microvasculature through angiogenesis. During angiogenesis, the bigger vessels form endothelial sprouts which

grow in different directions, establishing connections with other vessels that shape the developed vascular network.

The DA sprouts dorsally to form the inter-segmental vessels (ISV) which then branch and fuse at the dorsal-most point of their trajectory to form the dorsal longitudinal anastomotic vessel (DLAV). The DA also extends ventral sprouts which group around blood cells to form the caudal vein (CV). The brain vessels sprout into microvasculature in the same way.^{148,149,154}

The sprouting process is driven by the tip-cells of the sprouts. Tip-cells differ from the other cells of the sprouts phenotypically and molecularly. The behavior of these cells is similar to axonal growth cones in the nervous system. During sprouting, both utilize similar signaling pathways like VEGF and Notch signaling and express the same signaling molecules like Semaphorins, Netrins, and Ephrins^{155,156}.

1.6.1.6. Lumenization of large vessels and microvasculature

The other important morphological process in developing vasculature is lumen formation. Lumenization of biological tubes can occur in different ways, but for vasculature the cord or cell hollowing are the most common¹⁵⁷. Large vessels like DA are first formed as a cord and then develop the lumen through a cord hollowing mechanism. The cells of the cord change their apical–basal polarity which causes separation of the membranes and consequent formation of the lumen. The microvasculature forms lumen through the fusion of vacuoles from neighboring cells and the formation of a long tube surrounded by a single membrane, a process known as cell hollowing. The CV exploits a different mechanism first identified on the zebrafish model by which it is assembled as a tube around blood cells¹⁴⁹. The process of lumen formation is controlled by different regulative molecules, including integrins, Cdc42, Rac, Pak2, Pak4, Par3, Par6 and PKCz¹⁴⁸.

1.6.2. Development of zebrafish neural plate border

The neural border is specified from the ectodermal germ layer as the intermediate area between neural and non-neural ectoderm. Further, it develops into a neural crest (NC) and Rohon-Beard lineages giving rise to craniofacial organs and peripheral nervous system. Schematic representation of ectoderm development presented on Figure 7.

Figure 7. Schematic representation of cellular fates of neural ectoderm.

The first NC cells appear in paired dorso-lateral stripes along the forming neural keel, whereupon they spread bilaterally away from the neural keel under the overlying ectoderm and migrate to different locations in the body. Cranial neural crest cells form separate streams along the antero-posterior axis, each contributing to the development of different craniofacial structures. Diencephalon and anterior mesencephalon NC form mandibular arches which migrate into the frontonasal process and populate cranial mesenchyme. The next three streams originate from midbrain and hindbrain and migrate laterally. These streams populate branchial arches and further develop in craniofacial bones, ganglia, and some tissues of the eye. In zebrafish NC originating from midbrain, r1 and r2 will contribute to the first pharyngeal arch, from r5 and 4 will seed the second arch and r6 and r7 NC cells contribute to the third arch. One of the major derivatives of zebrafish the cranial pharyngeal arches are gills cartilages. The mandibular arch develops in Meckel's cartilage, the first pharyngeal arch develops into palatoquadrate cartilage, the second pharyngeal arch forms hyosymplectic, ceratohyal and basihyal cartilages and the third pharyngeal arch develops into ceratobranchial and basibranchial cartilages⁸³. Trunk NC cells give rise to such tissues as dorsal root ganglia, sympathetic ganglia, enteric neurons and glia, melanocytes, peripheral neurons, connective tissue, and mesenchyme smooth muscle cells (SMC) of the cardiovascular system.

Specification, development and migration of NC and RB cells are controlled by a combination of complex events. From the molecular regulatory perspective, the NPB is specified in the ectoderm by intermediate levels of Bmp signaling in conjunction with inputs from the Wnt, Fgf and Notch signaling pathways which start the expression of specific genes. The earliest transcription factors regulating NPB development are *zic*, *pax*, *dlx*, *msx* and *prdm1a*¹⁵⁸. After the establishment of NPB such NC specifiers start expressing in the NC

subpopulation *slug/snail*, *foxd3*, *sox9*, *ap-2*, *foxd3*. With further development and during migration NC cells express among others *zic2*, *tfap2*, *sox10*^{159,160}.

The segmentation of cranial NC into different arches is regulated by different mechanisms from corresponding brain segments. Analogous to the mechanism of hindbrain patterning, the segmentation of NC that has originated from different rhombomers into different streams along the antero-posterior axis is regulated by *hox* genes. Alterations of *hox* functions in the cranial region results in homeotic transformations of the facial bones and cartilages analogous to the ones observed in trunk skeleton of *hox*-mutants. *Hoxa2* has been shown to play a role in the formation of second branchial arches. Mice deficient in *Hoxa1* and *Hoxb1* lack the second arch of NC at all^{159,161,162}.

Less is known about RB neurons, which are the transient embryonic population of sensory neurons that undergo apoptosis later in development. They are also derived from the NPB, but do not undergo the same extensive migration as NC cells¹⁶³. The difference in molecular regulation of specification of RB cells and NC cells is still not well-understood. Such genes as *neurogenin1* and *neuroD* have been shown to induce RB fate and later at development they are marked by expression of *islet1*.

A summary of the molecular markers specifying NPB and its derivatives, NC and RB cells, is presented on Figure 8 B.

Figure 8. Morphological and molecular overview of NC and RB development.

Adapted from P.Z. Myers, January, 2008

(<http://scienceblogs.com/pharyngula/author/pharyngula/>). (A) development of neural tube and NC; (B) molecular specifiers of NPB, NC and RB cells development.

KMT2A association with neurological diseases²¹ together with the paucity of available studies showing that *Meis1* and *Kmt2a* play a role in the development of neural crest lineages^{24,164}, brings the question of *kmt2a* role in the development of zebrafish NPB derivatives into the forefront of our research objective.

1.6.3. Zebrafish eye development

The vertebrate eye is a complex structure containing derivatives of neuroepithelium, embryonic ectoderm and a neural crest. The development of the eye includes a variety of complex processes responsible for the proper structuring of all morphological components.

The morphogenesis of the eye starts with the specification of the eye field from forebrain neuroepithelium. During the conversion of anterior neural plate into neural keel, the cells which give rise to the primordial eye migrate more slowly, taking a more lateral position. When the neural keel forms these cells are located around the forebrain midline. Then these outer cells change migratory direction and start moving laterally toward the future left and right eyefield. In addition to this lateral migration, the proper segregation into right and left eye fields is facilitated by the anterior progression of diencephalon. The left and right eye fields further develop into left and right optic vesicles. Outgrowth of the optic vesicles is accompanied by proximo-distal regionalization into the optic stalk and optic cup. Morphogenesis of the laterally growing optic cup and formation of the lens are initiated when the optic vesicle reaches and contacts the overlying ectoderm. The signaling cascade from the optic vesicle induces the thickening of ectoderm at the place of contact, which further develop into the lens and fully separates from the ectoderm becoming an eye structure. In the optic cup this contact induces invagination of its distal part which results in the formation of a bilayer structure. The internal layer of the optic cup then develops into a retina and the outer into retinal pigmented epithelium (RPE). At the same time the signals from the midline and/or optic stalk induce neurogenesis in retina starting in the ventro-nasal quadrant of the retina which further spreads out through the entire retina. The invagination of the optic cup also results in the formation of a choroid fissure, a cleft which is normally transient and essential for vascularization of the eye. At later stages of eye development, the choroid fissure closes, finally shaping the developed optic cup and optic stalk^{165,166}. The brief overview of eye morphogenesis and its molecular regulators are presented on Figure 9.

The genetic regulation of eye development is orchestrated by a number of interacting signaling pathways. As the neural eye progenitors surround the forebrain primordia their growth in bilateral directions results in the formation of a cell-depleted region in the midline of the future forebrain. This cell-depleted region is further occupied by anteriorly growing diencephalon driven by TGF- β -related nodal and non-canonical Wnt signaling pathways. The mutations in the genes encoding signaling molecules of Shh and Nodal result in such defects of bilateral brain and eye separation as holoprosencephaly and cyclopia, affecting diencephalon anterior progression and consequently bilateral splitting of the forebrain and eyefield ¹⁶⁷.

The major mechanism by which Nodal contributes to diencephalic anterior progression and further eye development is activation of the sonic hedgehog (Shh) pathway in the ventral midline of diencephalon ¹⁶⁷. Additionally to Nodal, Shh is upregulated through retinoic acid (RA) signaling ¹⁶⁸. The other known players in nodal and Shh regulation are *zic1*, which activates both Nodal and RA pathways, and *six3* acting directly on Shh ¹⁶⁹. Activated in the ventral midline of diencephalon, Shh promotes the anterior migration of the diencephalon. Then diencephalic cells occupy the cell-depleted region of the forebrain and continue expressing Shh. Shh diffuses away from its site of synthesis at the diencephalic midline thus establishing a concentration gradient of the signaling molecule in bilateral directions from the midline and towards the lateral side of each eye field. This gradient further orchestrates genetic events regulating the development of the optic cup and optic stalk. The higher levels of Shh induce the optic stalk from more proximal cells of the optic vesicle, while distal cells receive much lower levels of Shh and develop as the optic cup. The higher concentrations of Shh activate expression of optic stalk regulators *pax2*, *vax1*, *vax2* and *zic2a*. The higher concentrations of Shh define the optic cup fate activating *pax6* and *vsx2* at the lateral regions of the eye fields (Figure 9 F) ^{170–172}. The non-canonical Wnt pathway and the fibroblast growth factor (Fgf) pathway regulates retina and RPE development with the participation of such transcription factors as Otx1, Otx2, Pax6 and Mitf. The neural differentiation in the retina is initiated by Atoh7 ^{173,174}.

Hox genes were never shown to be involved in eye development, however their well-known partners *Pbx* and *Meis1* play important roles in embryonic eye organogenesis ^{93,175}. In zebrafish some of the *hox* genes express starting from 1 cell stage (e.g. *hoxd4a*) ⁹³ and are known regulators of the development of neural ectoderm and NC. *kmt2a* is ubiquitously expressed in zebrafish embryo from 1 cell till 16 hpf and is an important regulator of brain development ²⁴. Based on this, we assume that there might be an

undiscovered role of *hox*, *meis1.1*, *pbx* and *kmt2a* action in eye development and our research seeks to further develop this assumption.

Figure 9. Morphological and molecular overview of eye development.

Adapted from ¹⁷⁶⁻¹⁷⁸. (A-E) Patterning of eye structures, (F) molecular pathways regulating eye morphogenesis.

1.6.4. Morpholino knock-down and CRISPR-Cas9 knock-out technologies

Morpholino knock-down technology has been widely used to study gene function in the zebrafish model since it was introduced by Nasevicius and Ekker in 2000 ¹⁷⁹. The limitations of morpholino technology, such as off-target effects and non-specific activation of the p53 apoptotic pathway, have been thoroughly discussed ^{180,181}. However, with careful use of controls, morpholino technology is still a powerful tool for providing robust and reliable data. In particular, non-specific cell-death due to activation of p53 apoptotic pathway can be assessed by acridine orange staining, the caspase3 assay, the TUNEL assay, and concomitant

knock-down of p53 together with the targeted locus. The specificity of knock-down can be further confirmed by the use of two different specific morpholinos or by co-injecting morpholino with mRNA of a targeted gene to rescue the knock-down effect. The use of this last control is not possible for *kmt2a* as any transcripts spanning the open reading frame would be more than 12 kb long and transcripts of such length cannot be synthesized *in vitro* with current methods.

The generation of animals carrying mutations in the gene of interest can provide researchers with more robust and reliable information about the gene function and can be used to confirm the data obtained with morpholino techniques. Gene editing in zebrafish can be done by three approaches: TALEN (Transcription Activator-Like Effector Nucleases), Zinc-finger nucleases (ZFNs) and recently developed CRISPR-Cas9 technology^{182,183}. All three approaches induce double strand break (DSB) in the targeted DNA region, which is repaired by NHEJ or HEJ mechanisms. Mutations occur as a result of mistakes during NHEJ or specifically designed nucleotide sequences can be introduced by providing donor DNA if HEJ utilized^{182–184}.

TALEN and ZFN approaches are precise and specific, however they both require complex and high-cost construction of chimeric proteins containing specifically designed DNA binding domain and catalytic domain of restriction endonuclease. In addition to the procedural complexity, they also demonstrated lower efficiency in comparison with CRISPR-Cas9 method¹⁸². Given these considerations, we have chosen to employ CRISPR-Cas9 technology.

CRISPR-Cas9 technique is much easier to apply as it does not require construction of chimeric proteins, but only guiding RNA sequences. The approach is based on the Bacteria and archaea immune response to foreign nucleic acid. When e.g. viral DNA enters the bacterial cell it is cut into fragments which are further inserted into clustered regularly interspaced short palindromic repeats (CRISPR) loci and then transcribed into short CRISPR RNAs (crRNAs). The crRNA is then annealed to transactivating crRNA (tracrRNA) forming single guide RNA (sgRNA). SgRNA binds to Cas proteins through tracrRNA sequence and the formed system binds the viral DNA strands based on the complementarity of crRNA. Cas9 is then attracted to the site and destroys the virus by breaking apart both DNA strands (Figure 10)¹⁸⁵.

Figure 10. SgRNA and Cas9 protein complex.

Adapted from ¹⁸⁶. PAM – protospacer adjacent motif, crRNAs – short CRISPR RNAs, tracrRNA – transactivating crRNA.

The system was adapted for scientific research in different organisms and cell lines and is based on introducing Cas9 RNA or protein into cells/organisms together with sgRNA, crRNA sequence of which is complementary to the targeting region of DNA. Analogous to the bacteria cells, such introduction induces a double strand break (DSB) in the targeted sequence which is then repaired by NHEJ mechanism. Mistakes in NHEJ or utilization of HEJ by introducing extra donor DNA with arms homologous to the broken region results in mutations in the targeted sequence ^{184,185}.

High efficiency, simple implementation, and lower cost makes CRISPR-Cas9 the preeminent method of choice in DNA editing experiments. The possibility of off-target action can be minimized through the careful choice of target sites with low similarity to other genomic regions or use of modified Cas9 protein ^{184,185,187}.

The relative ease of genome editing provided by the CRISPR-Cas9 technology has allowed the reassessment of many morpholino-based results. Surprisingly, initial data sets revealed that for a number of genes the results obtained by morpholino-mediated knock-down were not reproduced in mutants derived using CRISPR-Cas9 approaches ¹⁸⁸. This raised considerable concern regarding the reliability of morpholino-based approaches ¹⁸⁹. Nonetheless, recent work strongly suggests the inconsistency of the results yielded by the two different methods can be explained by differences in induction of genetic compensation mechanisms by knock-down and knock-out ¹⁹⁰. Our research addresses this methodological debate by employing both methods to examine downregulated functionality and loss-of-function of *kmt2a* in zebrafish.

2. AIM AND OBJECTIVES

The aim of this PhD research project is to study the role of *kmt2a* in embryo development, and detect its molecular targets and position in the genetic hierarchy regulating the developmental processes on the zebrafish model.

To reach the aim we have developed the following objectives:

1. Characterize primitive hematopoiesis and changes in the level of expression of transcription factors regulating hematopoiesis in zebrafish embryos with downregulated functionality of *kmt2a*.

2. Examine and characterize vasculogenic defects and changes in the level of expression of transcription factors regulating embryonic vasculogenesis in zebrafish embryos with downregulated functionality of *kmt2a*.

3. Characterize the effect of *kmt2a* downregulation on the development of NPB derivatives and expression of known transcription factors regulating the development of NPB, RB-cells and NCC.

4. Examine eye morphology in embryos with downregulated *kmt2a* expression and establish its role in eye development (if any).

5. Generate two *kmt2a*^{-/-} fishlines, targeting two different regions of *kmt2a*, to confirm results of morpholino knock-down approach with loss-of-function methodology.

3. MATERIALS AND METHODS

3.1. Ethics Statement

All animal work conformed to the Institutional Animal Care and Use Committee (IACUC) guidelines at Nanyang Technological University and was reviewed and approved under protocol number ARF SBS/NIE-A 0144 AZ.

3.2. Plasmid constructs

3.2.1. Plasmids used for *in situ* hybridization probe synthesis

RNA probes for were synthesized using plasmids generously gifted by the following investigators: *fli1* from Professor Roger Patient ¹⁹¹, *meis1.1*, *cdx4*, *ephB4a*, *efnb2a* from Professor Takeshi Suzuki ¹⁴¹; *scl* from Dr. Karuna Sampath ¹⁹²; *gata1* and *hbbe1* from Professor Low Boon Chuan ¹⁹³; *sox10* from Dr. Vincent Cunliffe ¹⁹⁴; *islet1* from Professors Bernard and Christine Thisse ¹⁹⁵; *myod* from Professor Philip Ingham ¹⁹⁶.

3.2.2. Plasmids used for synthesis of mRNA in rescue experiments

Plasmids encoding *fli1* and *scl* mRNA were a kind gift from Dr. Aldo Ciau-Uitz ¹⁹⁷, plasmid encoding *meis1.1* mRNA were kindly gifted by Professor Andrew Waskiewicz ¹⁴².

3.2.3. Plasmids used for cloning sgRNAs and synthesis of Cas9 mRNA

For sgRNAs cloning and Cas9 mRNA synthesis we used plasmid constructed by W. Hwang ¹⁸⁴ ordered from Addgene (SgRNA: DR274, item #42250; Cas9: MLM3613, item #42251).

3.3. Chemicals, reagents and antibodies

3.3.1. Antibodies used in this study

We used antibodies in immunohistochemical assay and *in situ* hybridization (ISH). As a primary we used antibody against acetylated α -tubulin (Catalog No.T6793; Sigma-Aldrich, St. Louis, MO) and secondary antibody Alexa Fluor® 546 rabbit anti-mouse IgG (H+L) (Catalog No.A11060; Invitrogen, Carlsbad, CA).

In ISH we used anti-digoxigenin antibodies, conjugated to alkaline phosphatase (anti-DIG-AP Ab, Roshe # 11093274910).

3.3.2. DNA primers and oligos

Primers were used in qRT-PCR and screening of CRISPR-Cas9 mutants. Oligos were used for cloning target sequence for future synthesis of sgRNA and for preparation of donor DNA for future homologous recombination

Table 1. List of primers used in qRT-PCR

Primer name	Forward (5'-3')	Reverse (5'-3')
meis1	GAGGACACACATCGCACAGT	GAGCCATGCCCTCATAATGT
Gata1	AAGATGGGACAGGCCACTAC	TGCTGACAATCAGCCTCTTTT
hoxd4a	ACCCCTAGCCCTTTCCCTG	GGTCTTTGTGTTTTGTTGTTGTCC
scl1	CTATTAACCGTGGTTTTGCTGG	CCATCGTTGATTTCAACCTCAT
fli1	CAACGGATCCAGAGAGTCG	CCATGTAGCCAGTATAGTTCATCTG
prdm1a	GGATACTCACCAGCAGGCTC	CAGGGTAGTGGTGCATGAGG
tfap2a	GCTCCTCCGCTGTCTCATAC	GACTGGGCATGCAGAGAGTT
zic2a	GGATGTGATCGACGCTTTGC	TGCTTTCGAAGAGAGCTGGG
islet1	GGTGCTGGTGACCCATTAAG	GGAGCTGTTTTTCGTTGAGG
sox10	TCCATGTCCCCTGGTCACTC	CTTCCTCGTCGGACTTCACC

Table 2. List of primers used screening of CRISPR-Cas9 mutants

Primer name	Forward (5'-3')	Reverse (5'-3')
TgE3_1kb	GCCCTGGAAGAACGTCAATA	TGGCATCAGTGCGTTTAGAG
TgE1_1kb	CTTTATGCAGTTGCGTTTGG	CAGAGAAGCAGCAACACCAG
TgE3N	GTCATGGCGTTTAAACCTTAAT	TTACGTCAGAGCCCTCGCCA
TgE1N	GTCATGGCGTTTAAACCTTAAT	CTGTCAGTGGTGGTCCCATA

Table 3. List of oligos used for SgRNA screening of CRISPR-Cas9 mutants

Target name	Oligo1	Oligo2
SgRNA_TgE3	TAGGACTGGAGCTGCACTCCTG	AAACCAGGAGTGCAGCTCCAGT
SgRNA_TgE1	TAGGTTCCCTGCTCGGCCCGGA	AAACTCCGGGCGGAGCAGGGAA

Table 4. List of oligos used for donor DNA for HEJ for generation of *kmt2a*+/- line by CRISPR-Cas9 method

Target name	Oligo1
SgRNA_TgE3	AGGGTAGAGAAGTCCCACAGGTCATGGCGTTTAAACCTTAATTAAGCTG TTGTAGGAGTGCAGCTCCAGTCCTGT
SgRNA_TgE1	GGCGGTTCCCTGCTCGGCCCGTCATGGCGTTTAAACCTTAATTAAGCTGT TGTAGGGAGGGAGCAGCAGCTCGGG

3.3.3. Zebrafish strains

Transgenic fish lines *fli1*-EGFP and *gata1*-dsRED were kindly shared with us by Professor Vladimir Korzh (IMCB, A*STAR). Wild-type zebrafish is AB genotype/strain (<http://zebrafish.org/zirc/fish/lineAll.php>). Zebrafish were maintained as described ¹⁹⁸.

3.4. Staging of embryos

Staging of embryos was defined based on relevant morphological landmarks as described ¹⁹⁹.

3.5. Morpholino injections

Morpholino (MO) sequences against zebrafish *kmt2a* were taken from ¹¹² and ordered from Gene Tools. We used two different MO's – the first targets the AUG translational start codon (*kmt2a*-MO1) and the second targets the exon1-intron1 splicing junction site (*kmt2a*-MO2). As a control, we used Vivo-MO standard MO, Gene Tools.

MO injections were done using a Milli Pulse Pressure Injector (MPPI)-2 (Applied Scientific Instrumentation, Inc.). Needles were prepared from glass capillaries (Sutter Instrument) using a Laser micropipette puller (Sutter Instrument). MO was diluted to a concentration of 8 ng/nl and needles were calibrated to inject the appropriate volume by injecting MO solution with addition of phenol red into mineral oil on a micrometer scale under the microscope. The injected volume was defined based on measurement of the diameter of the MO drop in oil according to the equation $V=4\pi r^3/3$. Adjustments of volume were done by changing the pulse duration on the injector. To deliver 8 ng MO, 1 nl of MO solution was required, so pulse duration was adjusted until the diameter of the injected drop reached 0.125 mm; for 4 ng delivery 0.5 nl injection was required so pulse duration was adjusted until the diameter of the drop was 0.1 mm.

3.6. Acridine orange staining

The embryos were anesthetized in 3-amino benzoic acid ethyl ester (Tricaine) solution Sigma (Cat.# A-5040) (stock solution was prepared by dissolving 400 mg of tricaine in 97.9 ml of distilled water, pH was adjusted to ~7 by adding 1 M Tris (pH 9); for working solution 4.2 ml of stock was added to 100 ml of water. The fish get anesthetized within 0.5-3 minutes depending on the developmental stage). Acridine Orange (ThermoFisher Scientific, cat # A1301) was dissolved in distilled water to the concentration of 5 $\mu\text{g/ml}$ ²⁰⁰. Anesthetized embryos were submerged for 10 minutes in the solution of Acridine Orange and then visualized and photographed under a FITC filter with a Zeiss Lumar stereomicroscope V12.

3.7. O-dianisidine staining of RBC

For the staining of RBC, embryos at 22 hpf were first treated with 0.0012% (w/v) phenylthiourea (PTU) solution (Sigma, P7629) in egg water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl_2 , 0.33 mM MgSO_4 , 0.0002% methylene blue) to prevent pigmentation. At 72 hpf, embryos were fixed overnight at room temperature (RT) in 4% paraformaldehyde solution and then washed three times in PBS (phosphate buffered saline) followed by incubation in the staining buffer (0.6 mg/ml o-dianisidine, 10 mM sodium acetate (pH 5.2), 0.65% hydrogen peroxide, and 40% ethanol) for 15 min in the dark²⁰¹. After staining, embryos were washed 3 times for 5 minute each in PBS, mounted in glycerol and visualized and photographed under brightfield with a Zeiss Lumar stereomicroscope V12.

3.8. Alkaline phosphatase staining of vasculature

Embryos in which pigmentation had been blocked (see above) were fixed at 72 hpf for 30 min at RT in 4% paraformaldehyde solution in PBS. Then embryos were treated with acetone at -20°C for 30 min, followed by two rinses in PBS (5 min each). Rinsed embryos were equilibrated with NTMT buffer (100 mM Tris pH 9.5, 50 mM MgCl_2 , 100 mM NaCl, 0.1% Tween 20) 3 times for 15 min each at RT and then stained with NBT/BCIP (Roche #11681451001) for 30 min²⁰². The stained vasculature was visualized under brightfield light and photographed using a Zeiss Lumar stereomicroscope V12.

3.9. Alcian blue staining of cartilage

To stain cartilage, 5 dpf embryos were euthanized by adding of 20 ml of stock tricaine solution to 100 ml of and then fixed overnight in 4% paraformaldehyde (PFA) solution. Next day, fixed embryos were washed 3 times with a solution of 0.1% Tween 20 in

PBS (PBT) followed by bleaching in 30% hydrogen peroxide for 2 h for pigment removal. After removal of hydrogen peroxide, embryos were washed in PBT and stained overnight in filtered Alcian blue solution (1% concentrated hydrochloric acid, 70% ethanol, 0.1% Alcian blue). After staining, tissues were cleared for 2 hours in 100% methanol and washed in PBS²⁰³. Embryos were mounted in glycerol and visualized and photographed under brightfield light using a Zeiss Lumar stereomicroscope V12.

3.10. Immunohistochemical staining of trigeminal ganglia and RB cells

For staining of RB and trigeminal ganglia, embryos were manually dechorionated at 28 hpf and fixed overnight in 4% paraformaldehyde solution. After fixing, embryos were washed in PBS and blocked overnight in blocking buffer (PBS, 1% Triton, 10% FBS, 0.5% BSA, 1% DMSO). Then embryos were incubated with primary mouse monoclonal antibody against acetylated α -tubulin (Catalog No.T6793; Sigma-Aldrich, St. Louis, MO) (1:1000 dilution) overnight at 4°C. Next, embryos were washed 6 times with PBT and incubated with secondary antibody Alexa Fluor® 546 rabbit anti-mouse IgG (H+L) diluted 1:1000 overnight at 4°C²⁰⁴. After incubation, embryos were washed five times with PBT, deyolked manually, oriented dorsally for visualizing RB cells and laterally for trigeminal ganglia. Image stacks along z-axis were taken on a scanning confocal microscope ZEISS LSM 710.

3.11. qRT-PCR

For total RNA isolation, 30 embryos were homogenized with Trizol reagent (Invitrogen cat. # 18080051) in 1 ml of Trizol. RNA was extracted by adding 0.2 ml of chloroform followed by vortexing, 3 minutes incubation at RT and centrifugation at 12000 g for 15 min at 4°C. The upper aqueous phase containing RNA was collected and RNA was precipitated by adding 0.5 ml of isopropanol, followed by 10 min incubation at RT and centrifugation at 12000 g for 10 min at 4°C. Supernatant was completely removed and the RNA precipitate was washed twice with 1 ml of 75% ethanol. After washing, ethanol was completely removed, the RNA pellet was air-dried for 10 minutes and then dissolved in 30 – 40 μ l of RNAase-free water¹⁹⁸.

First strand cDNA was prepared using reagent kit SuperScript III First-Strand Synthesis System (Invitrogen cat. # 18080051) according to the manufacturer's instruction. Before synthesis, genomic DNA was removed by RNase-free DNaseI treatment (Thermo Scientific cat. # EN0521).

qRT-PCR was done on BIO-Rad iQ-5 platform using SYBR Green dye (Invitrogen) with accordance to manufacture instructions. Analysis of results was done with BIO-Rad iQ-5 software. A two-tailed students' t-test was carried out to calculate the statistical significance of the observed changes in gene expression in the morphants.

3.12. *In situ* hybridization

In situ hybridization analysis including synthesis of RNA probes, preparation of embryos and staining was done according to the previously described protocol ²⁰⁵.

3.12.1. Synthesis of labelled antisense RNA probes

The plasmid containing the sequence of the corresponding probe flanked by upstream and downstream bacteriophage promoters was linearized with restriction enzymes at 3' end of the probe sequence. The linearized plasmid was purified using the phenol-chloroform purification method. The RNA probe was synthesized from the linearized plasmid using DIG RNA Labelling Kit (Roche) according to the manufacturer instructions.

3.12.2. Preparation of zebrafish embryos

Embryos at the appropriate developmental stage were fixed overnight at 4°C in 4% PFA solution. If needed, they were manually dechorionated before fixation. After fixation, embryos were dehydrated by rinsing with methanol 3 times for 10 minutes each and kept in methanol in -20°C.

3.12.3. ISH staining

To rehydrate, embryos were transferred for 5 min each through serial dilutions of methanol in PBST (0.1% of Tween20 in PBS) starting from 75% of methanol, then 50% of methanol, then 25% of methanol and then PBST. To increase tissue permeabilization, embryos were treated with 10 µg/ml of proteinase K in PBST. Treatment was at RT for 1 min for embryos before the 6 somite stage, 3 min for embryos before the 18 somite stage and for 10 min for embryos from 24 to 36 hpf. After permeabilization, embryos were fixed in 4% PFA for 20 min followed by 5 washings in PBST, 5 min each. After washing, embryos were transferred into Hyb(plus) buffer (50% formamide, 5xSSC, 0.1% Tween 20, 50 µg/ml heparin and 500 µg/ml yeast tRNA in DEPC treated water, 0.05% DEPC in distilled water). For 50 embryos, we took 700 µl of buffer and kept them at 65°C for 2 hours. After this prehybridization step, the buffer was discarded and embryos incubated overnight in 20 µl of Hyb(plus) buffer with 30-100 ng RNA probe at 65-70°C. After incubation, the solution of

probe in Hyb(plus) buffer was removed and stored at -20°C as it can be reused for a limited number of times. The embryos were washed in serial dilutions of Hyb(minus) (50% to 60% Formamide, 5x SSC, 0.1% Tween-20) in SSCT for 10 min each. The first wash was done in 75% Hyb(minus) in 2x SSCT (0.05%), followed by 50% Hyb(minus) in 2x SSC, then 25% Hyb(minus) in 2x SSC and finally 2x SSC. After washing, embryos were equilibrated two times in 0.2x SSC (0.05%) at 70°C for 30 min each. Embryos were then washed in serial dilutions of SSC in PBST at RT for 5 min each. The first wash was in 75% 0.2x SSC in PBST (0.05%), followed by 50% 0.2x SSC in PBST, then 25% 0.2x SSC in PBST and finishing with PBST. After the washing, embryos were incubated in 0.5 ml of blocking solution (prepared from 5x stock: 2 mg/ml BSA, 2 to 5% sheep serum in PBST) at RT, 1 hr. Then the embryos were incubated in 0.5 to 1 ml blocking solution with anti-digoxigenin antibodies, conjugated to alkaline phosphatase (anti-DIG-AP Ab, Roshe # 11093274910) with dilution from 1:2000 to 1:10,000 at 4°C , overnight. After the incubation, embryos were washed in PBST 6 times at RT, 15 min each. Then embryos were washed 3 times for 5 min each in freshly prepared staining buffer (0.1 M Tris-Cl, pH 9.5, 50 mM MgCl_2 , 0.1 M NaCl, 0.1% Tween-20) and then stained in the staining buffer containing NBT/X-phosphate until the signal became visible under the microscope. After the signal developed, staining buffer was removed and embryos washed with PBST for 6 times at RT for 5 minutes each, followed by fixation in 4% PFA for 2 hours at RT or overnight at 4°C . After fixation, embryos were washed with increasing concentrations of methanol in PBST (25%, 50% and 75% methanol). Embryos were then incubated in 100% methanol until the signal became clear and blue and achieved optimal contrast. After incubation embryos were rehydrated by washing in serial dilutions of methanol in PBST (75%, 50% and 25% methanol followed by pure PBST). The stained embryos were mounted in 80% glycerol for consequent storage or visualization and photography under microscope. The stained area represents the tissue expressing the gene of interest and intensity of staining represents the level of the gene expression.

3.13. Flow cytometry

Embryos of the *gatal*-dsRed transgenic fish line were dechorionated manually at 56 hpf. We analyzed three batches of 150 embryos each. After dechorionation, embryos were washed in calcium-free Ringer's solution (116 mM NaCl, 2.9 mM KCl, 5.0 mM HEPES pH 7.2) for 15 min and deyolked by triturating through a 200 μl micropipette tip. The recovered tissue was treated with 0.25% trypsin and 1 mM EDTA for 60 min at 28.5°C with trituration through a 200 μl pipette tip every 10 min to dissociate the mixture into a single-cell suspension. The obtained suspension was centrifuged at 1000 g for 9 min at 4°C , the

supernatant discarded, and the cell-containing pellet resuspended in ice cold 0.9× PBS supplemented with 5% fetal bovine serum. The mix was subsequently filtered through a 40 µm nylon mesh filter²⁰⁶. Flow cytometry was performed on a BD LSII instrument (BD Biosciences). To quantify RBC, we compared gata1-dsRED embryos injected with a control morpholino, gata1-dsRED embryos injected with *kmt2a*-MO1. Wild type zebrafish embryos were used as a negative control.

3.14. Cryosectioning

Embryos at 5 dpf were anesthetized with tricaine solution and fixed at 4% PFA overnight at 4°C. Embryos were then washed in PBS and transferred into 30% sucrose in PBS and filtered through a 0.4 µm filter. The embryos were kept in the sucrose solution until sinking or overnight. Embryos were then oriented within an embedding chamber filled with OCT cryostat embedding medium. The embryos were oriented in such a way that they were in the middle of the block equi-distant from all edges of the embedding chamber. The chamber plus oriented embryos was slowly frozen in liquid nitrogen and then stored at -70°C or immediately used cryosectioning. The sectioning was done at -20°C with 20 µm thickness¹⁹⁸. Sections were transferred to microscopic slides and photographed under NICON microscope.

3.15. Generation of *kmt2a* mutant zebrafish lines using CRISPR-Cas9 technology

3.15.1. Design of target sites and cloning of sgRNAs

The targets and corresponding sgRNAs were designed using <https://chopchop.rc.fas.harvard.edu/>²⁰⁷ software and selected based on the highest specificity (lowest number of possible off-targets sites and if any, with 3 or more mismatches). sgRNA was cloned into DR274 and Cas9 mRNA message was synthesized from MLM3613 following the presented protocol¹⁸⁶. The plasmids were ordered from Addgene (<http://www.addgene.org/crispr/jounglab>).

For sgRNA cloning, 2 µg of each oligonucleotide were resuspended in 50 µl of annealing buffer (10 mM Tris pH 7.5-8.0, 50 mM NaCl, 0.5 M EDTA) and kept at 95°C in a heating block for 5 min. The block was then removed from the heater and gradually cooled at RT for 40-45 min. DR274 plasmid was linearized with BSAI enzyme and purified by phenol-chloroform extraction. For ligation, 300 ng of annealed oligos were mixed with 100 ng of the linearized plasmid, 2 µl of T4 ligase buffer, 1 µl of T4 ligase and water for a total volume of 20 µl. The mixture was incubated at RT for 1 hr. After incubation, 10 µl of the ligation mix

were mixed with competent Top10 *E. coli* cells. The cells were kept on ice for 30 minutes, heat-shocked at 42°C for 45 sec and chilled on ice for 3 min. Then 250 µl of LB broth were added to the cells and the culture shaken for 1 hr at 37°C. The cells were plated on agarose plates containing kanamycin and grown overnight at 37°C. Next day single colonies were picked and transferred into 4 ml of LB broth with added 4 µl of kanamycin (50 µg/mL) and shaken overnight at 37°C. The following day the DNA was extracted from the culture using PureLink® Quick Plasmid Miniprep Kit (Invitrogen catalogue # K210011) and sent to 1st BASE for DNA sequencing service to check for positive clones.

The plasmids bearing correct sgRNA sequences were linearized with the *DraI* restriction enzyme and purified by the phenol-chloroform method. The sgRNA was synthesized using the Ambion MEGAscript® reagent kit with T7 polymerase according to the manufacturer's instructions.

To generate Cas9 mRNA, the MLM3613 plasmid was linearized with the *PmeI* restriction enzyme and purified by phenol-chloroform extraction. The RNA was synthesized using mMESSAGE mMACHINE® Kit using T7 polymerase according to the manufacturer's instructions.

3.15.2. Design of a template for HEJ approach

CRISPR-Cas9 system can produce mutations by utilizing non-homologous end joining (NHEJ) and homologous end joining (HEJ) mechanisms. NHEJ allows the generation of mosaic F₀ generation animals bearing heterogeneous mutations. Following improvements in CRISPR-Cas9 technology, we also used a second approach suggested by the Gagnon group²⁰⁷. These authors reported that the efficiency of mutagenesis and the frequency of frame-shift mutations can be increased by exploiting the HEJ repair mechanism following injection of Cas9 protein rather than mRNA. We followed the suggested protocol and designed single strand donor cassettes with repetitive stop codons and homologous arms complementary to 20 bp stretches flanking the 3' and 5' ends of the presumed double-strand break DSB point (3 bp away from protospacer adjacent motif (PAM) for both targets (Table 2.4. , Figure 29 C, D).

3.15.3. Injections

For mutagenesis employing NHEJ, we injected 1 nl/embryo of a solution at 50 ng/µl of sgRNA and 300 ng/µl of Cas9 mRNA.

For mutagenesis employing HEJ, the injection mix contained 1 μ l of Cas9 protein at 600 ng/ μ l, 1 μ l of sgRNA at 300 ng/ μ l, 1 μ l of 3 μ M donor DNA and 0.3 μ l of phenol-red dye. 300 embryos were injected with the solution.

3.16. Screening for the efficiency of mutagenesis using T7 endonuclease assay, insert-specific PCR and sequencing

To assess the efficiency of mutagenesis in the F_0 population and to screen for *kmt2a*^{+/-} F_1 animals, we used three approaches: the T7 endonuclease assay, PCR using an insert-specific primer, and sequencing. Genomic DNA used in all three approaches was extracted using DNeasy® Blood & Tissue Kits (Qiagen, catalogue # 69506) following the manufacture's instructions. The extraction worked sufficiently well to recover usable DNA from a single embryo at 24 hpf.

3.16.1. T7 endonuclease assay

Genomic DNA was amplified using TgE3_1kb or TgE1_1kb primers corresponding to the targeted site. PCR product was purified by QIAquick PCR Purification Kit (Qiagen, catalogue # 28104) according to the manufacturer's instructions. Five hundred ng of purified PCR product was mixed with 2 μ l of NEB buffer and MilliQ H₂O to a total volume of 19 μ l. The mix was placed into PCR cyclor with the following program: heating at 95°C for 5 min, cooling to 85°C at a rate of 2°C/s and further to 25°C at a rate of 0.1°C/s, and maintenance at 4°C. Then T7 endonuclease was added and the mixture kept at 37°C for 15 min. The reaction was stopped by adding 2 μ l of 0.25M EDTA and immediately loaded on a 1-1.5 % agarose gel.

3.16.2. PCR using primer-specific insert

For screening of mutations in the NHEJ approach, we designed primers with forward or reverse complementary to the donor DNA TgE3N and TgE1N (Table 2.). After amplification of a 1 kb region, we used the PCR product for nested PCR. The forward primer used for nested PCR was complementary to the sequence of the insert which should be artificially introduced into *kmt2a* by NHEJ.

3.16.3. Sequencing

We used the commercial services provided by 1st Base (Axil Scientific Pte Ltd, 41 Science Park Road #04-08, The Gemini, Singapore Science Park II, Singapore 117610) to

sequence the amplified PCR products. For the F_0 generation, we sequenced both 1 kb and nested PCR products. For the F_1 generation, we sequenced nested PCR products.

4. RESULTS

4.1. *kmt2a* knock-down embryos have defects in blood flow, pigmentation, eye and body shape.

To determine the optimal amount of MO, embryos were injected with *kmt2a*-MO1 or *kmt2a*-MO2 at two different concentrations — 4 ng and 8 ng per embryo. Injection of lower amounts (4 ng) of *kmt2a*-MO1 produced slight changes in phenotype, whereas 4 ng of *kmt2a*-MO2 did not lead to any obvious change. Previously reported effect of *kmt2a* downregulation on zebrafish embryonic phenotype included curved body, smaller eyes, heart edema, abnormal head, decrease in circulating blood cells and blood flow¹¹². In our hands injections with 8 ng of either MO reproduced the reported data and additionally we observed decrease in pigmentation and eye coloboma. At 24 hpf we can observe heart edema, deformed bodies, defects in eye and head shape (Figure 11 A-F). At 48 hpf, the phenotype was persistent with more obvious eye defects, blood pooling in the heart area and slight shortening of body length (Figure 11 G-J). The measurements of length were done in ImageJ software, the full embryo length was measured from the most anterior till the most posterior points of the embryo, the length of head was measured from the most anterior point of the embryo till the anterior border of otic vesicle. At this developmental stage, we noticed that the shortening of body length is rather due to the shortening of anterior part of the embryo. If the whole embryo were shorter than control by 4.3% for *kmt2a*-MO1 injected embryo and 12.8% for *kmt2a*-MO2 injected embryo (Figure 11 J), the head length of morphants was shorter than control embryo by 11.5% for *kmt2a*-MO1 and 8.3% for *kmt2a*-MO2 (Figure 11 H). The eyes of *kmt2a*-MO injected embryos were less pigmented, deformed and smaller in size (Figure 12 E-H).

At 72 hpf, the phenotype remained persistent with more obvious eye defects represented by coloboma, microphthalmia and blowout-like phenotype (Figure 13 A-C). The *kmt2a*-MO1-induced phenotype was more severe than that provoked by *kmt2a*-MO2. The phenotype persisted till 8 dpf (Figure 13 F, G), which is the actual lifespan of *kmt2a*-MO injected embryos.

Phenotypic changes caused by *kmt2a*-MO injection are summarized in Figure 14. The figure represent number of embryos injected with *kmt2a*-MO1 which have specific phenotypic defects. For each experiment we characterized 60 embryos and experiments were done in three biological replicates. Measurements were done for single embryos for better representation of subjectively registered phenotypic changes.

Figure 11. Phenotype of *kmt2a* knock-down embryos.

(A-E) 24 hpf, (A) control embryo, (B) embryo injected with 4 ng of *kmt2a*-MO1, (C) embryo injected with 8 ng of *kmt2a*-MO1, (D) embryo injected with 4 ng of *kmt2a*-MO2, (E) embryo injected with 8 ng of *kmt2a*-MO2, (F) body length at 24 hpf of development, (G-I) 48 hpf, (G) control embryo, (H) embryo injected with 8 ng of *kmt2a*-MO1, (I) embryo injected with 8 ng of *kmt2a*-MO2, (J) body length at 24 hpf of development.

Figure 12. Head and eye defects in *kmt2a* morphants at 48 hpf.

(A) control embryo, (B) embryo injected with 8 ng of *kmt2a*-MO1, (C) embryo injected with 8 ng of *kmt2a*-MO2, red line represent the distance from extreme anterior till otic vesicle, (D) measured distance, (E) control embryo, (F) embryo injected with 8 ng of *kmt2a*-MO1, (G) embryo injected with 8 ng of *kmt2a*-MO2, eye is outlined by yellow, (H) measured eye area.

Figure 13. Head and eye defects in *kmt2a* morphants at 72 hpf and 8 dpf.

(A-C) 72 hpf, (A) control embryo, (B) embryo injected with 8 ng of *kmt2a*-MO1, (C) embryo injected with 8 ng of *kmt2a*-MO2, red line represents the distance from the extreme anterior to the otic vesicle, (D) eye surface area, (E) head length, (F-G) 8 dpf, (F) embryo injected with 8 ng of *kmt2a*-MO1, (G) embryo injected with 8 ng of *kmt2a*-MO2, red arrows point blowout-like eye.

Figure 14. Summary of phenotypic characteristics caused by *kmt2a* knock-down comparative to the wild type.

The numbers represent the mean number of embryos representing corresponding phenotypic characteristics. Experiment was done in three biological replicates. For each experiment 60 embryos were injected with 8 ng of *kmt2a*-MO1 and 60 embryos with 8 ng of control-MO. Significance of proportion of embryos with phenotypic changes was compared using two-tailed Fisher's exact test. Phenotype is considered significant if $p < 0.05$. P-values for all phenotypic changes were less than $< 2.2e-16$.

4.2. The observed phenotype is not caused by morpholinos-induced increase in apoptosis due to non-specific activation of the p53-apoptotic pathway

Morpholino technology is known to give non-specific craniofacial and neurogenic phenotypes in 15–20% of cases by activation of the p53 apoptotic pathway¹⁸¹. This possibility was excluded by evaluating apoptosis in morphants using acridine orange staining and by co-injection of the *kmt2a*-MO1 along with anti-p53 morpholino.

Acridine orange staining did not show evidence of increased apoptosis (Figure 15 A, B). The double knock-down of *kmt2a* along with *p53* resulted in the same phenotypic changes as observed for the single *kmt2a*-MO1 injection (Figure 15 C). The results are consistent with those reported by others¹¹² and validate the specificity of the observed phenotype.

Figure 15. Evaluation of activation of p35 apoptotic pathway by *kmt2a*-MO's.

(A-B) acridine orange staining, (A) control embryo, (B) *kmt2a*-MO1, (C) embryo coinjected with *kmt2a*-MO1 and anti-p53-MO at 48 hpf.

4.3. Effect of *kmt2a* knock-down on zebrafish haematopoiesis and vasculogenesis.

Following published data describing the important role of *kmt2a* in zebrafish primitive and definitive haematopoiesis¹¹² and based on our initial observation of blood pooling and slower blood flow in *kmt2a* knock-down embryos, we conducted further experiments to study the effect of *kmt2a* knock-down on haematopoiesis and vasculogenesis of zebrafish embryos.

4.3.1. *kmt2a* knock-down results into hematopoietic and vasculogenic defects in zebrafish embryos

Defect in formation of mature RBC at 48 hpf due to downregulation of *kmt2a* was previously shown in o-dianisidine staining done by Wan's group¹¹². Based on phenotypic observations the authors also reported the recovery of blood flow in *kmt2a* morphants after 36 hpf following by its decrease after 4 dpf. To check whether erythropoiesis also recovers we visualized RBC by o-dianisidine staining at 72 hpf. Our results still showed significant decrease in RBC in *kmt2a*-MO1 compared to control (Figure 16 A, B) at this developmental stage, leading us to the conclusion that despite of visual recovery of blood flow, number of mature RBC stays decreased in *kmt2* morphants¹¹².

The same group also reported contradictory data on the role of *kmt2a* in zebrafish vasculogenesis. In particular, expression of the endothelial marker *flk1* and artery marker *ephb2a* was reduced in *kmt2a* morphants at 24 hpf, however the observation of *flk*-GFP transgenic fish injected with *kmt2a* MO under fluorescent microscope did not show defects in formed vasculature after 36 hpf. To check whether vessels formation is not affected by downregulation of *kmt2a*, we visualized the vessels by alkaline phosphatase staining at 72 hpf. Surprisingly, contradictory to the published data describing no defects in vasculature of *kmt2a* morphants, we observed obvious defects in large and small vessels. At 72 hpf alkaline phosphatase staining showed obvious dosage-dependent defects in development of the SIV for both *kmt2a*-MO1 and *kmt2a*-MO2 morphants (Figure 16 C-G). The ISV were formed, however this method does not allow to evaluate small vessels properly. The persistent vasculature defect was further confirmed by injection of a *fli1*-EGFP transgenic line with 8 ng of *kmt2a*-MO1 and visualization of the vasculature at 72 hpf by light-sheet fluorescence microscopy (Figure 17 A-D). Major vessels were still formed while the microvasculature (especially anteriorly) was severely defective (Figure 17 A, B). Posterior small vessels (ISV) did not show any morphological defects (Figure 17 C, D), however, the fluorescence signal representing the level of *fli1* expression was much lower in *kmt2a*-MO1-injected than in control embryos. To visualize the vessels in *kmt2a*-MO1, we need to increase either laser power or exposure time (Figure 17 E). Figure 17 represents the overlap of more than 200 slices based on maximum intensity projection.

Figure 16. Alkaline phosphatase staining of vessels and o-dianisidine staining of RBC of *kmt2a*-MO's.

(A-B) o-dianisidine staining, (A) control embryo, (B) embryo injected with 8 ng of *kmt2a*-MO1, (C-G) alkaline phosphatase staining, (C) control embryo, (D) embryo injected with 4 ng of *kmt2a*-MO1, (E) embryo injected with 8 ng of *kmt2a*-MO1, (F) embryo injected with 4 ng of *kmt2a*-MO2, (G) embryo injected with 8 ng of *kmt2a*-MO2.

To confirm deficiency in RBC, we injected *gata1*-dsRed transgenic embryos with 8 ng of *kmt2a*-MO1 and observed morphants under the light-sheet fluorescence microscope (Figure 17 F-I). Anteriorly in the head region we observed a complete lack of circulating RBC (Figure 17 F, G). Posteriorly RBC were circulating only in the major vessels (DA and CV) while only single cells were able to enter the ISV (Figure 17 H, I). At the same time, contrary to the results obtained by o-dianisidine staining, the intensity of the signal (which represents either number of *gata1* expressing cells or the level of *gata1* expression) was not lower in *kmt2a*-MO1 than in control embryos. The microscope was able to capture *dsRED* signal from *gata1*-expressing RBC with the same exposure time and laser power as for morphants as for control embryos. All together our and previously published data allow to conclude, that downregulation of *kmt2a* affects blood circulation in zebrafish embryo and decrease in number of mature RBC. Our additional observation of vessels development points that the observed phenotype is caused by both – defects in haematopoiesis and vasculogenesis.

Figure 17. Light-sheet microscopy of *fli1*-EGFP and *gata1*-dsRed transgenic lines injected with *kmt2a*-MO1.

(A-D) *fli1*-EGFP line, (A, C) control embryo, (B, D) embryo injected with 8 ng of *kmt2a*-MO1, (E) laser power and exposure time used to capture gfp signal, (F-I) *gata1*-dsRed line, (F,H) control embryo, (G, I) embryo injected with 8 ng of *kmt2a*-MO1, A – anterior, P – posterior.

4.3.2. *kmt2a* is required for specification of hemangioblasts in the lateral mesoderm prior their commitment to endothelial, hematopoietic and hemogenic endothelial lineages

Decrease in expression of hematopoietic progenitors *scl* and *gata1* in *kmt2a* morphants at 10 somite stage was previously reported¹¹². Along with our novel observation of defective vessels formation in *kmt2a* morphants we supposed that *kmt2a* regulates not only hematopoietic progenitors, but hemangioblasts specification at the stage of their specification from lateral mesoderm. To test the hypothesis we performed qRT-PCR at 10 and 12 hpf (1-4 somite) and *in situ* hybridization at 12 hpf of *scl*, *gata1* and *fli1* at, when they can be first detected in lateral mesoderm. Consistent with published data¹¹² and our expectations based on previously obtained results, the expression of all three markers was reduced at 10 and 12 hpf (Figure 18 A-F).

To clarify the position of *kmt2a* in the genetic hierarchy regulating specification of the hemangioblast (Figure 4), we checked the expression of *meis1.1*, *hoxd4a* and *cdx4* which are known to lie upstream of *scl*, *gata1* and *fli1* in the regulatory network⁹³ (Figure 18 G-J). *meis1* and *hox* genes are well known targets of *kmt2a* and its pathogenic fusions^{14,20}. All three genes are known to be expressed in the early stages of development of zebrafish embryo in the ventral marginal mesoderm and all have been shown to play important roles in zebrafish haematopoiesis and vasculogenesis^{93,112}.

qRT-PCR revealed a significant decrease in *meis1.1* expression in ATG-MO *mll1* knock-down embryos at 12 hpf, while *hoxd4a* and *cdx4* expression was not significantly changed (Figure 18 K). The results for *cdx4* and *meis1* were confirmed by *in situ* hybridization (Figure 18 G-J).

Figure 18. Effect of *kmt2a* knock-down on expression of hemangioblast markers at the stage of their specification in lateral mesoderm (12 hpf).

(A) expression of *gata1*, control (B) expression of *gata1*, embryo injected with 8 ng of *kmt2a*-MO1, (C) expression of *fli1* and *myod* (to confirm identity of developmental stages of control and morphant), control (D) expression of *fli1* and *myod*, embryo injected with 8 ng of *kmt2a*-MO1 (E) expression of *scl*, control (F) expression of *scl*, embryo injected with 8 ng of *kmt2a*-MO1 (G) expression of *meis1*, control (H) expression of *meis1*, embryo injected with 8 ng of *kmt2a*-MO1 (I) expression of *cdx4*, control (J) expression of *meis1*, embryo injected with 8 ng of *kmt2a*-MO1, ALM – anterior lateral mesoderm, PLM – posterior lateral mesoderm, A – anterior, P – posterior, (K) qRT-PCR of *gata1*, *fli1*, *scl*, *hoxd4a*, *meis1*, *cdx4*. In the figure, p<0.05 is shown as *, p< 0.01 as **, p< 0.001 as ***, p< 0.0001 as ****.

4.3.3. Endothelial lineage of *kmt2a* morphants remains defective at the stages of specification of ICM and formation of the first blood vessels

The controversy surrounding whether hemangioblasts are the multipotent progenitors of the endothelial and hematopoietic lineages or rather a mix of two already specified hematopoietic and endothelial progenitors is actively ongoing. In any case, the latest understanding regarding this question is that the hemangioblasts specified in the lateral mesoderm first migrate to the ICM and only then commit to different cellular fates. Within the ICM, they develop into angioblasts, which form vessels, into primitive hematopoietic lineages and into hemogenic endothelium which is the progenitor of definitive hematopoietic tissues^{132,134}.

To confirm defects in vasculogenesis from angioblasts at the stage of when the first vessels form, we carried out *in situ* hybridization for *fli1*, the arterial marker ephrinB2 (*efnb2*) and the venous marker ephrin type-B receptor 4 (*ephb4*). In accordance with the results of alkaline-phosphatase staining and light-sheet microscopy of *kmt2a*-MO1-injected *fli1*-EGFP transgenic embryos, the results of *in situ* hybridization of vasculature markers demonstrated that the vessel formation was defective. The intensity of staining for all three markers was much lower in *kmt2a*-MO1 morphants compared to control embryos (Figure 19).

Figure 19. Effect of *kmt2a* knock-down on vasculature formation (24 hpf).

(A) expression of *fli1*, control (B) expression of *fli1*, embryo injected with 8 ng of *kmt2a*-MO1, (C) expression of *ephB4a*, control (D) expression of *ephB4a*, embryo injected with 8 ng of *kmt2a*-MO1 (E) expression of *efnb2a*, control (F) expression of *efnb2a*, embryo injected with 8 ng of *kmt2a*-MO1.

4.3.4. *kmt2a* is required for the commitment of hemangioblasts to become primitive erythrocytes

Our preliminary data on the effect of *kmt2a* knock-down on primitive erythropoiesis were apparently contradictory. The expression of hematopoietic markers in ALM and PLM at 12 hpf was significantly decreased in *kmt2a* knock-down (Figure 18 A-F). However starting from 24 hpf we and Wans group¹¹² observed the recovery of blood flow. Our additional experiments revealed that blood flow recovers only in major vessels (Figure 17), the number of *gata-1* expressing cells is not decreased in *kmt2a* morphants (Figure 17), however downregulation of *kmt2a* still results in the deficiency of mature erythrocytes (Figure 16).

To clarify this apparent contradiction, we checked the expression of erythroid markers *scl*, *gata1* and *hbbe1* at 24 hpf by *in situ* hybridization and qRT-PCR. While Wans group reported decrease of the markers based on the data of *in situ* hybridization experiments¹¹², our results of *in situ* hybridization did not show any decrease in the area or intensity of the staining for all three markers, and even increase in the intensity of staining and stained area for *hbbe1* (Figure 20). Moreover as for my eye, the figures presented by Wans group shows decrease in the stained area along with total decrease of embryo size, but no decrease in the intensity of staining¹¹². However, both experiments, ours and Wans group, showed that *scl*, *gata1* and *hbbe1* expressing hematopoietic progenitors accumulated in ICM and did not enter the forming vasculature.

To confirm that the level of expression of *scl* and *gata1* did not decreased at 24 hpf, we performed qRT-PCR for these markers. The results showed that *scl* is equally expressed in morphants and control embryos while, surprisingly, *gata1* expression was increased in *kmt2a*-morphants at this developmental stage (Figure 20 C). To obtain additional quantitative information on the level of *gata1* expression at later developmental stages, we quantified *gata1* expressing cells by flow cytometry of a cell suspension from *gata1-dsRED* transgenic embryos at 56 hpf. The results demonstrated a 20 fold increase in the number of *gata-1* expressing cells in *kmt2a*-morphants (Figure 21 A, B). Interestingly, in parallel with the increase in the number of *gata-1* expressing cells, the number of cells with very low signal at *dsRED* emission wavelength 525-575 nm, so called “background” cells was significantly decreased (Figure 21 C, D). We infer that these latter cells are haemoglobin-expressing mature erythrocytes. This assumption is based on the following. First, the cells with low fluorescence signal fall into the same scattering range as the *gata1* expressing cells, so they represent the same population. Moreover, auto-fluorescence of mature erythrocytes may fall into the same wavelength range as for *dsRED*²⁰⁸. So a possible explanation of the results is

that at 56 hpf along with a significant increase in immature RBC, as *gata1* is a marker of immature erythrocytes, we observed significant decrease in mature erythrocytes in *kmt2a* morphants.

Moreover at 72 hpf *gata1* expressing immature RBC were circulation inside the major vessels without any evidence of decrease in the amount of cells or intensity of *gata1* expression (Figure 17 F-I). And at the same time o-dianisidine staining revealed a significant decrease in mature RBC at 72 hpf. O-dianisidine binds haemoglobin and mature erythrocytes are more enriched with this protein (Figure 16 A-C).

The observations suggest that *kmt2a* plays two distinct functions in primitive hematopoiesis. At the stage of specification of hematopoietic progenitors in lateral mesoderm tissues *kmt2a* is necessary for the proper development of these progenitors. Starting from 24 hpf the generation of erythropoietic tissues recovers and even gets intensified at 56 and 72 hpf. However the primitive erythrocytes do not develop into mature RBC containing haemoglobin. We assume that *kmt2a* first regulates specification of early haematopoietic progenitors in ALM and PLM and secondly regulates RBC maturation.

Figure 20. Effect of *kmt2a* knock-down on expression of hematopoietic markers at 24 hpf.

(A) expression of *gata1*, control, (B) expression of *gata1*, embryo injected with 8 ng of *kmt2a*-MO1, (C) expression of *scl*, control, (D) expression of *scl*, embryo injected with 8 ng of *kmt2a*-MO1, (E) expression of *hbbe1*, control, (F) expression of *hbbe1*, embryo injected with 8 ng of *kmt2a*-MO1.

Figure 21. Quantitative evaluation of the effect of *kmt2a* knock-down on expression of *scl* and *gata1* at 24 hpf and *gata1* at 56 hpf.

(A, C) flow cytometry of cell suspension form *gata1-dsRED* transgenic line, control (B, D) flow cytometry of cell suspension form *gata1-dsRED* transgenic line, embryo injected with 8 ng of *kmt2a*-MO1, green – *gata1* expressing cells, red – other cells (E) qRT-PCR of *gata1* and *scl* at 24 hpf. In the figure, $p < 0.05$ is shown as *, $p < 0.01$ as **, $p < 0.001$ as ***, $p < 0.0001$ as ****.

4.3.5. Vascular and hematopoietic defects if *kmt2a* knock-down embryos can be rescued by *fli1* and *meis1* mRNA but not by *scl* mRNA

Downregulation of expression of *scl*, *meis1*, *fli1* and *gata1* in *kmt2a* morphants support a model in which *kmt2a* acts upstream or in parallel with *meis1* in the genetic hierarchy regulating specification of hematopoietic and vascular lineages (Figure 4). To test this hypothesis, we tested the ability of the mRNA for the hematopoietic regulators *fli1*, *meis1* or *scl* for their ability to rescue zebrafish embryos co-injected with *kmt2a*-MO1.

Alkaline-phosphatase and o-dianisidine staining of the co-injected embryos revealed that *fli1* mRNA can rescue both the vasculature and RBC production; *meis1* mRNA also rescues both phenotypic features but the vessels are still smaller and the number of RBC is less than in *fli1* rescue, while *scl* mRNA does not rescue either of the developmental pathways (Figure 22, 23).

Figure 22. Alkaline-phosphatase staining of *kmt2a* morphants rescued with *fli1*, *meis1* or *scl* mRNA.

A) control, (B) embryo injected with 8 ng of *kmt2a*-MO1, (C) embryo co-injected with 8 ng of *kmt2a*-MO1 and 100 pg of *scl* mRNA, (D) embryo co-injected with 8 ng of *kmt2a*-MO1 and 100 pg of *meis1* mRNA, (E) embryo co-injected with 8 ng of *kmt2a*-MO1 and 50 pg of *fli1* mRNA.

Figure 23. O-dianisidine staining of *kmt2a* morphants rescued with *fli1*, *meis1* or *scl* mRNA.

(A) control, (B) embryo injected with 8 ng of *kmt2a*-MO1, (C) embryo co-injected with 8 ng of *kmt2a*-MO1 and 100 pg of *meis1* mRNA, (D) embryo co-injected with 8 ng of *kmt2a*-MO1 and 100 pg of *scl* mRNA, (E) embryo co-injected with 8 ng of *kmt2a*-MO1 and 50 pg of *fli1* mRNA.

4.4. Effect of *kmt2a* knock-down on zebrafish neurogenesis

Such phenotypic observations as an eye defect, a decrease in skin pigment and a smaller head in *kmt2a* knock-down embryos pointed to a possible disturbance of neurogenic processes. Melanocytes, or pigment skin cells are derived from neural crest cells (NCC). NCC give rise to a variety of tissues such as peripheral neurons and glial cells, craniofacial cartilage and bones, connective tissue, mesenchyme and smooth muscle cells (SMC) of the cardiovascular system¹⁶⁰. Recent studies demonstrated an important role of H3K4 methylation in brain development and function²¹. There are also a few clinical cases described where the neurological phenotype of individuals is caused by *KMT2A de novo* mutations¹²⁵, the important role of *kmt2a* in neural development was also shown in mice and zebrafish models^{23,24}. Taking all this into consideration, we continue our investigation into the neurological phenotype of *kmt2a* knock-down embryos.

4.4.1. *kmt2a* knock-down results in defective formation of organs derived from neural crest

To check whether there are any defects in other neural crest derivatives, we stained the cartilage of developing embryos at 5 dpf with alcian blue. *kmt2a*-MO1-injected embryos showed obvious dosage dependent defects in cartilage development (deformed Meckel's and ceratohyal cartilage, and reduction in length and quantity of ceratobranchials) (Figure 24 A-C).

The development of the trigeminal ganglion, another neural crest derivative, was checked by immunohistochemistry using an antibody against acetylated α -tubulin with further visualization by confocal microscopy. The trigeminal ganglia of *kmt2a*-MO1-injected embryos was much smaller than wild-type, with no nerve sprouts, and required higher laser power or exposure time for visualization (Figure 24 D, E).

Figure 24. Alcian blue staining of craniofacial cartilage and immunohistochemical staining of trigeminal ganglia of *kmt2a* morphants

(A-B) – alcian blue staining of gills cartilage, dorsal view, (A) control (B) embryo injected with 4 ng of *kmt2a*-MO1, (C) embryo injected with 8 ng of *kmt2a*-MO1, dorsal view, Meckel's cartilage (mc), ceratohyal cartilage (ch), palatoquadrate (pq); ceratobranchials (cb). White dots represents ceratobranchials, (D – E) immunohistochemical staining of trigeminal ganglia using antibody against acetylated α tubulin, lateral view, (D) control (E) embryo injected with 8 ng of *kmt2a*-MO1, TG - trigeminal ganglia, A – anterior, P – posterior.

4.4.2. *kmt2a* knock-down affects development of neural plate border derivatives of non-NC origin

To understand whether *kmt2a* plays a role only in NCC -derived tissue, or has a broader function in neurogenesis, we checked if RB cells, which are the non-NCC derivatives of the NPB, are defective in *kmt2a* morphants. The results revealed a decrease in the number of RB cells in *kmt2a* morphants (Figure 25).

Figure 25. Confocal microscopy of Immunohistochemical staining of RB cells of *kmt2a*-MO1 injected embryos with antibody against acetylated tubulin

(A) control (B) embryo injected with 8 ng of *kmt2a*-MO1.

4.4.3. *kmt2a* knock-down decreases expression of genetic markers of NPB, NCC and RB cells

Neurogenesis starts with the segregation of ectoderm into three different regions under a complex regulatory interplay of BMP, WNT, FGF and NOTCH signaling pathways^{159,209,210,160}. This interplay activates the expression of epidermal specifiers in non-neural ectoderm, neural specifiers in neural ectoderm and neural plate border specifiers at the junction between neural and non-neural tissues. A summary of the molecular markers specifying NPB and its derivatives, NC and RB cells, is presented in Section 1.5.2., Figure 8.

To establish a role for *kmt2a* in NPB specification and development, we used qRT-PCR to evaluate the level of expression of a set of molecular markers specifying neural tissues at early developmental stages in *kmt2a* morphants (Figure 26). To confirm effect of *kmt2a* knock-down on the development of the NCC lineage, we examined *zic2a*, *tfap2a* and *sox10* at 70% epiboly, 10 hpf and 12 hpf. All three markers regulate NCC specification at the earliest stages^{159,211–213} and were expressed at lower levels in *kmt2a* morphants at all stages examined (Figure 26).

For RB cells, the other lineage of NPB origin, we checked the expression of *isll*. *isll* is expressed in a set of neural tissues, including RB neurons^{159,214,215}. The expression in neural progenitors starts around 10 hpf in the rostral neural plate, and later at 12 hpf *isll* is also expressed in motor neurons and RB cell progenitors²¹⁴. We did qRT-PCR at 12 hpf stage and revealed decreased *isll* expression in *kmt2a* morphants (Figure 26 C).

Figure 26. qRT-PCR of NPB, NC and RB neurons markers in *kmt2a* morphants at 70% epiboly, 10 and 12 hpf.

(A) expression of *prdm1a*, *zic2a*, *tfap2a* and *sox10* at 70% epiboly developmental stage, (B) expression of *prdm1a*, *zic2a*, *tfap2a* and *sox10* at 10 hpf, expression of *prdm1a*, *zic2a*, *tfap2a*, *sox10* and *isll* at 12 hpf. In the figure, $p < 0.05$ is shown as *, $p < 0.01$ as **, $p < 0.001$ as ***, $p < 0.0001$ as ****.

To follow the development of NCC and RB cell lineage and to confirm qRT-PCR data, we did *in situ* hybridization of *sox10* and *isll* at 12 and 24 hpf (Figure 27). Interestingly, *in situ* hybridization of *sox10* did not show any decrease in the intensity of staining in *kmt2a* morphants at 12 hpf. However, the posterior domain of *sox10* expression cells was reduced in *kmt2a* knock-down (Figure 27 A, B). Results of *in situ* hybridization of *sox10* at 24 hpf demonstrated decreased *sox10* expression posteriorly (Figure 27 C, D). So *kmt2a* knock-down may decrease the number of NCC at early times before they start expressing *sox10*,

especially posteriorly, but does not influence the level of *sox10* expression in individual NCC once formed. Expression of *isll* was significantly decreased in motor neurons and RB cells at both developmental stages (Figure 27 F, H). These results confirm our preliminary data showing that *kmt2a* plays an important role for the development of both NCC and RB lineages.

To check whether *kmt2a* plays an early role in NPB specification, we assessed the expression of *prdm1a* by qRT-PCR. *prdm1a* is one of the earliest specifiers of NPB, with transcripts first appearing during mid-gastrulation^{158,216}, and lies upstream of *zic2a*, *tfap2a*, *sox10* and *isll* in the genetic hierarchy (Section 1.5.2., Figure 8). The results demonstrated decrease in *prdm1a* expression at 70% epiboly, 10 hpf and 12 hpf (Figure 26), suggesting that the earliest steps in NPB specification are compromised in *kmt2a* morphants.

It was also shown previously that *meis1a* plays a role in embryonic neurogenesis^{164,217,218}. Our group also demonstrated that *hoxd4a* is expressed in migrating anterior NCC⁹³, and *hoxd4a* knock-down results in defective development of NCC derivatives (data not published). This leads us to suggest that *meis1a* and *hoxd4a* participate in regulatory mechanisms of neurogenesis and act upstream of *prdm1a*. *kmt2a* is a well-known regulator of *meis1a* and *hox* genes^{14,20} and *kmt2a* knock-down results in decreased expression of *meis1a* but not of *hoxd4a*.

All together, these results confirm our preliminary data pointing to an important role for *kmt2a* in the earliest stages of neurogenesis. In the genetic hierarchy regulating NPB specification, *kmt2a* lies upstream of *prdm1a*, and acts in cooperation with or through the regulation of *meis1a*.

Figure 27. Effect of *kmt2a* knock-down on expression of neuronal markers at 12 and 24 hpf.

(A) expression of *sox10*, control 12 hpf (B) expression of *sox10*, embryo injected with 8 ng of *kmt2a*-MO1 12 hpf, (C) expression of *sox10*, control 24 hpf (D) expression of *sox10*, embryo injected with 8 ng of *kmt2a*-MO1 24 hpf (E) expression of *isl1*, control 12 hpf (F) expression of *isl1*, embryo injected with 8 ng of *kmt2a*-MO1 12 hpf (G) expression of *isl1*, control 24 hpf (H) expression of *isl1*, embryo injected with 8 ng of *kmt2a*-MO1 24 hpf, NC – neural crest, RB – Rohon-Beard cells, MN – motor neurons, A – anterior, P – posterior.

4.5. Effect of *kmt2a* knock-down zebrafish eye development

kmt2a-MO1 injected embryos revealed obvious eye and head defects. In agreement with previously reported data¹¹², these *kmt2a* morphants displayed microphthalmia, holoprosencephaly and smaller head (microcephaly). In addition, we noted for the first time coloboma and “blowout-like” eye phenotypes (Figures 12 and 13). The blowout-like eye could be seen due to defects in retina, decreased pigmentation in the retina pigmented epithelium or morphological defects in optic cup and optic stalk. As along with blowout-like phenotype we observed coloboma, we supposed that most probably *kmt2a* knock-down causes defects in optic cup and optic stalk organogenesis including the closure of the choroid fissure (Figure 9). To check our hypothesis and better characterize the morphology of other eye tissues, we performed cryosectioning on the *kmt2a* morphants. The transverse sections showed that the eye of the *kmt2a* morphants has a number of severe defects. In particular, the optic cup and optic stalk looked like a single structure that was not separated from brain. Rather, rather presumptive retinal tissue and RPE were continuous with midline tissue of the brain. The lenses were much smaller than in control embryo (Figure 28).

The *nodal* and *sonic hedgehog* (*shh*) signaling pathways pattern the eyes and diencephalon. Holoprosencephaly, cyclopia and blowout-like phenotype were reported to be caused by a variety of mutations affecting these pathways^{169,219–221}. Based on the phenotype we observed in *kmt2a* morphants we suppose that *kmt2a* plays important *hox*-independent regulatory role in signaling pathways regulating patterning and/or proliferation along the proximo-distal axis of the developing brain.

Figure 28. Defects in zebrafish eyes in *kmt2a* knock-down embryos

(A, B) control and morpholino injected embryos, the blue line represent the transverse plane for cryosectioning (C) transverse cryosections of head, control (D) transverse cryosections of head, *kmt2a*-MO1 injected embryo, RPE – retinal pigmented epithelium.

4.6. *kmt2a* knock-out by CRISPR-cas9 technology

Morpholino knock-down technology has been widely used to study gene function in the zebrafish model since it was introduced by Nasevicius and Ekker in 2000¹⁷⁹. However taking into consideration the limitations in the technology and to better understand *kmt2a* function we attempted to create a null allele of *kmt2a* using CRISPR-Cas9 technology.

4.6.1. Design of targets and sgRNAs for *kmt2a* mutation

The *kmt2a* transcript comprises up to 35 exons and encodes a protein of more than 4,000 amino acids with multiple functional domains. This complexity raises the risk of inadvertently creating a dominant negative form of the protein by targeted mutation. In fact, a dominant negative comprising the N-terminal 84 amino acids has already been described¹¹². Moreover, KMT2A binds a critical functional partner, MENIN, through the two motifs MBM1 and MBM2 located at the extreme N-terminus of KMT2A^{117,222}. To exclude the possibility of creating a dominant negative protein, we selected targets as close as possible to the translational start site. One target localized to a region encoding one of the MENIN binding sites in exon 1 (TgE1) (Figure 29 A). The second target was designed to target exon 3 (TgE3) (Figure 29 B).

To screen the injected embryos, we used the T7 endonuclease assay, PCR with primers specific to the donor cassette and sequencing (Figure 29 E, F).

Figure 29. Target sites, donor DNA constructs and primer's for screening for mutations in *kmt2a* CRISPR-Cas9 knock-down embryos.

(A) alignment of human and zebrafish Kmt2a N-terminus amino acids, red – MENIN binding site, blue – region targeted by SgRNA_TgE1. (B) selected regions targeted in exon1 and exon 3 (highlighted in red), PAM underlined. (C, D) donor DNA constructs for TgE1 and TgE3, repetitive stop codons are highlighted in blue, homology arms are highlighted in green. (E) primers for screening for mutations for TgE1, blue arrows – primers set flanking the stop-codon TgE1_1kb, red arrows – primers sets for nested PCR TgE1N, (F) primers for screening for mutations TgE3, blue arrows - TgE3_1kb, red arrows – TgE3N.

4.6.2. F₀ generation of *kmt2a* knock-out. Screening and phenotype.

To make the F₀ generation of fish, we injected 50 ng of SgRNA_TgE1 and SgRNA_TgE3 together with 300 ng of cas9 RNA. Even at this stage, for both targets we observed a phenotype comparable to the one induced by morpholino-mediated knock-down, including reduced pigmentation, blood pooling, delayed blood circulation, microcephaly, holoprosencephaly, coloboma and cyclopia (Figure 30 A). To genetically screen the embryos, we extracted genomic DNA from pools of 20 embryos as well as from 20 individual embryos and amplified the targeted region using TgE1_1kb and TgE3_1kb primers sets and sequenced the PCR products. Surprisingly, the sequencing did not reveal any successful mutations even in individual embryos with a severe phenotype.

Following this confusing result, we hypothesised that the mutation frequency at the loci we were targeting might be low, with the observed phenotype resulting not from DNA mutation but from interaction of sgRNA and Cas9 with maternally deposited *kmt2a* mRNA. To test whether other loci might be targeted at a higher frequency, we designed and injected sgRNA against two other regions of *kmt2a* in exon3 (Tg2E3) and exon 5 (TgE5). Embryos co-injected with sgRNA and Cas9 RNA demonstrated the same phenotype as for TgE1, TgE3 and morpholino injections (data not shown). However, the sequencing of the amplified targeted regions again did not reveal any mutations.

Since mutation frequency appeared to be low, we decided to modify our approach so as to detect mutational events with greater sensitivity. We therefore tried to exploit the endogenous HEJ repair pathway to tag the site of insertion with a known DNA fragment; the incorporation of the known DNA fragment at the site of mutation would then provide a unique site for primer binding and target amplification by PCR. SgRNA_TgE1 and SgRNA_TgE3 were coinjected with donor DNA and Cas9 protein. The phenotype of the injected embryos reproduced our previous observations (Figure 30 B-D).

To eliminate the possibility of a non-specific effect of sgRNAs or Cas9 protein, we used individual injections of sgRNAs or Cas9 as a control. Individual injections of sgRNAs and Cas9 RNA did not result in any similar phenotype (Figure 30 E-F).

To screen for the incorporation of donor DNA at the targeted loci, we first used genomic DNA of single embryos to amplify the region flanking the presumptive insertion site with primers TgE1_1kb and TgE3_1kb. We then carried out nested PCR for each product. For nested PCR we designed forward primer complementary to the insert (Figure 31 A, H). We also used the T7 endonuclease assay as an additional screening method.

T7 endonuclease assay and PCR with insert-specific primers confirmed the presence of the inserted donor DNA in the targeted region (Figure 31 B, C, J). Surprisingly, sequencing of the 1 kb PCR products (TgE1_1kb, Figure 31 E), which were shown to have the insertion by T7 endonuclease assay and by insert-specific PCR, did not reveal any mutations. At the same time the sequences of nested PCR products showed that the donor DNA is inserted into expected place (Figure 31 J).

Thus, nested PCR clearly revealed the presence of the mutated locus, and yet the sequencing of the first PCR product yielded only wild-type sequence. The first PCR product would be expected to contain a mixture of wild-type and mutant DNA fragments, since the mutation frequency in the F_0 generation would be unlikely to reach 100%. Given that the F_0 generation is mosaic, we would expect sequencing results which deteriorate into noise at the target site due to the overlap in reads from wild-type and mutant loci. A second feature of the targeted region might also contribute to noise, namely the presence of a long repetitive homopolymer T or A region (Figure 31 A, yellow box). During amplification, the homopolymeric tract would provoke enzyme "slippage" giving rise to homopolymeric tracts of varying lengths. Indeed, we observe an increase in noise after these homopolymer regions (Figure 31 D, F), an effect which may further obscure the presence of a mutation in a background of wild-type sequence. We therefore wondered whether the company performing the DNA sequencing might be employing software that "corrects" for such noise, thereby inadvertently "erasing" signs of mutant sequence. In fact, consultation with a company representative confirmed that this was case. The company routinely uses the PeakTrace base caller algorithm which effectively eliminated the mutant sequence from the readout (Figure 29 E). When the same sequence was processed with the KB base caller algorithm, the presence of mutations was obvious (Figure 31 G). We conclude that (a) the T7 endonuclease assay and (b) PCR with insert-specific primers and subsequent sequencing of the product are the most sensitive and stable methods for the screening of selected regions of *kmt2a*. We used these three methods for the further screening of F_1 and F_0 generations.

Figure 30. Phenotype of F0 generation of CRISPR-Cas9 mutated embryos.

(A) severe phenotype of embryos co-injected with SgRNA_TgE1 and CRISPR-Cas9 mRNA, (B) severe phenotype of embryos co-injected with SgRNA_TgE3 and CRISPR-Cas9 protein, (C) mild phenotype of embryos co-injected with SgRNA_TgE1 and CRISPR-Cas9 protein, (D) mild phenotype of embryos co-injected with SgRNA_TgE3 and CRISPR-Cas9 protein, red arrows point to blowout-like phenotype, blue arrow points to blood pooling in the head region, (E) control embryo injected with Cas9 protein, (F) control embryo injected with SgRNA_TgE3.

Figure 31. Screening for the mutations.

(A) primers set flanking the stop-codon TgE3_1kb (B) T7 endonuclease assay of TgE3_1kb PCR product (C) PCR product of insert-specific primers sets TgE3_rev_insert (D) enzyme "slippage" after A homopolymer in the sequence of TgE3_1kb product using PeakTrace basecaller algorithm (E) increase of noise signal after DSB in the sequence of TgE3_1kb product using PeakTrace basecaller algorithm (F) enzyme "slippage" after A homopolymer in the sequence of TgE3_1kb product using KB basecaller algorithm (G) increase of noise signal after DSB in the sequence of TgE3_1kb product using KB basecaller algorithm (H) Insert-specific set of primers TgE1_fw_insert (I) PCR product using insert-specific primers sets TgE1_fw_insert (J) sequencing results of TgE1_fw_insert PCR product using reverse primer for the sequencing, the insert is outlined in black.

4.6.3. Screening and phenotype of the F₁ generation of *kmt2a* null mutants

The positive F₀ fish were raised to sexual maturity and crossed with wild-type. Interestingly that F₁ generation also demonstrated an initial blowout-like phenotype which subsequently recovered to a normal looking eye (Figure 32 C). *kmt2a* mRNA and its product are maternally deposited into the embryo, so deficiency of the *kmt2a* RNA message or Kmt2a protein in the egg might be an explanation for the observed transient defects in F₁ generation.

F₁ progeny heterozygous for *kmt2a* mutation were raised to sexual maturity and mated. Of the F₂ progeny, 75% presented defects at the same morphological structures as *kmt2a* morphants, including microphthalmia, blowout-like phenotype and slow blood flow, however the defects were not so severe as for *kmt2a* morphants (Figure 32 B, D). For the final confirmation of blowout-like defect crysectioning of *kmt2a*^{-/-} embryos should be done.

Figure 32. Phenotype of F₁ and F₂ generations of *kmt2a* mutants.

(A) phenotype of F₁ generation of TgE3 mutated embryos, (B) phenotype of F₂ generation of TgE1 mutated embryos, (C) transient blowout-like phenotype of F₁ generation of TgE3 mutated embryos, (D) blowout-like phenotype of F₂ generation of TgE1, (E) control.

5. DISCUSSION

5.6.1. *kmt2a* knock-down results in defective blood circulation, pigmentation and eye development

Our results are consistent with previously published data¹¹² demonstrating that *kmt2a* knock-down causes slower and delayed blood flow, blood pooling, heart edema, curved and shorter body and microphthalmia. Beyond such observations, we registered that downregulation of *kmt2a* results in reduced pigmentation and a blowout-like eye phenotype with coloboma sometimes accompanied by holoprosencephaly and cyclopia (Figure 11, Figure 12, Figure 13).

We excluded the possible non-specific involvement of the p53 apoptotic pathway in these pathologies and demonstrated the specificity of the *kmt2a* morpholino-induced phenotype. Acridine-orange staining did not reveal any increase in apoptosis in *kmt2a* morphants and coinjection of *kmt2a* morpholino with *p53* morpholino resulted in the same phenotype (Figure 15).

Based on the observed phenotype and available data on *Kmt2a* role in mouse and zebrafish development^{106,112,223,224} we conclude that *kmt2a* may play a role in embryonic hematopoiesis, vasculogenesis, development of NPB derivatives and eye morphogenesis. Further research was done to better characterize the revealed pathologies and to understand the possible role of *kmt2a* in their development at the molecular level.

5.7. The impaired blood circulation in *kmt2a* knock-down embryos caused by both defects in haematopoiesis and angiogenesis

Defective blood flow and blood pooling can result from either impaired hematopoietic function or defective vasculogenesis/angiogenesis. In accordance with the previous observations¹¹², our results with o-dianisidine staining revealed a significant decrease in the number of haemoglobin expressing mature erythrocytes in *kmt2a* knock-down embryos (Figure 16)

Similar to the observation of this same group, our results from vasculature staining revealed that the vessels are broadly formed; however, contrary to the published data¹¹², we registered severe defects in the shaping of the finer vasculature. The SIV and ISV were defective in *kmt2a* morphants as shown by alkaline-phosphatase staining (Figure 16). The use of light-sheet microscopy allowed us to confirm the vasculature defects on *fli1*-EGFP transgenic fish line (Figure 17). The visualization of *fli1*-EGFP *kmt2a*-MO injected embryos

showed that the larger vessels like DA, PCV, PHBC are formed and have an appropriate size, while smaller vessels like SIV, ISV and microvasculature are severely defective. The most obvious defect in the microvasculature was observed in the head region. Moreover, whether for posterior or anterior regions we needed to increase either exposure time or laser power to capture the EGFP signal in morphants relative to controls, providing additional evidence for either decreased *fli1* expression in endothelial tissues or an anomaly in the tissue itself.

The results were confirmed by *in situ* hybridisation to detect the endothelial marker *fli1*, the arterial marker *efnb2a* and the venous marker *ephB4a* at 24 hpf. The trunk vessels like DA, PCV and ISV were formed, but the levels of expression of the markers were significantly decreased (Figure 19).

5.8. *kmt2a* knock-down causes defects in the specification of hematopoietic and vascular progenitors in lateral mesoderm

Angiogenic progenitors are specified together with hematopoietic progenitors in ALM and PLM of the developing embryo^{144,126}. Recent reports question the existence in zebrafish of the hemangioblast, a single hematopoietic and angiogenic progenitor^{127,132,134,154}. Instead, it is proposed that there are two separate populations in both the ALM and PLM giving rise to hematopoietic and angiogenic lineages. Our results demonstrate that *kmt2a* knock-down results in the development of phenotypic defects in both vasculature and blood lineages. We therefore proposed that *kmt2a* was required for the early specification or maintenance of hematopoietic and angiogenic progenitors whether arising from independent lineages or through a common hemangioblast. To test the hypothesis, we used *in situ* hybridization and qRT-PCR to assess early markers of hematopoietic and angiogenic progenitors, *scl* and *fli-1*, and a specific marker of erythrocytes, *gata-1*, which is not expressed in vascular tissue.

For the hematopoietic lineage, our results are consistent with the data reported by the Wan group, as *gata1* is severely reduced in *kmt2a* morphants. Expression of *scl1*, which marks both hematopoietic and angiogenic lineages²²⁵, is also reduced in *kmt2a* knock-down embryos but not to the extent of *gata1*. *fli1* expression, which also marks both lineages²²⁵, is slightly reduced in *kmt2a* morphants (Figure 21).

It is difficult to distinguish hematopoietic and angiogenic progenitors at this stage of development as their expression pattern is very similar. I will refer to the *gata1* marker as the one which exclusively marks the erythropoietic lineage. Despite the reduction in all three markers, *gata1* expression is decreased more significantly relative to *scl* and *fli1*. Moreover,

the reduction of expression of *fli-1* as revealed by *in situ* hybridization is rather due to the decreased number of *fli-1* expressing cells rather than the level of expression. That might show that *kmt2a* plays distinct roles in hematopoietic and angiogenic populations. In hematopoietic progenitors, it acts above *fli1* in the genetic hierarchy and its knock-down results in decreased expression of *fli1* and downstream targets *scl* and *gata1*. On the other hand, in angiogenic progenitors, *kmt2a* might not play any direct regulatory role at the stage of angioblasts specification in the ALM and PLM and rather acts later in development during angiogenesis. This could explain the partial reduction in *fli1* and *scl* expression in *kmt2a* knock-down embryos, as the expression is decreased only in hematopoietic population.

The other possible explanation is that *kmt2a* does not directly regulate angiogenic tissue specification, but influences it quantitatively by regulating proliferation of angioblasts. Despite the fact that larger vessels are formed in *kmt2a* morphants, results of our experiments reveal a reduction in the endothelial tissue in these vessels. Expression of *fli1* and *efnb2* in the larger vessels formed by vasculogenesis is still decreased in *kmt2a* morphants as was shown by *in situ* hybridization and light-sheet microscopy of *fli1*-EGFP transgenic line. The defects in angiogenesis process in *kmt2a* morphants may also be due to defects in proliferation of tip-cells of the sprouts. The recently discovered regulatory role of *kmt2a* in cell proliferation²²⁶ gives additional support for the hypothesis. However, to clearly understand the role of *kmt2a* in angioblasts specification more detailed studies focused on this particular tissue must be done.

5.9. *kmt2a* plays a role in the maturation of primitive erythrocytes

Our results confirm a role for *kmt2a* in the early specification of hematopoietic progenitors and later in primitive erythropoiesis. However, the intriguing observation of the recovery of blood flow between 24 and 72 hpf led us to further investigate primitive erythropoiesis through this developmental window.

We checked the expression of hematopoietic markers *gata1*, *scl* and *hbbe1* by *in situ* hybridization and qRT-PCR at 24 hpf (Figure 20, Figure 21). Although the Wan group reported a reduction of *gata1* and *hbbe1* expression at 24 hpf, in our hands the *in situ* hybridization signals for both *gata1* and *hbbe1* are not reduced in *kmt2a* morphants. The amount of the expressing tissue is less, but may simply be due to the fact that the morphants have a smaller size in general. Moreover, we observed a different pattern of *gata-1* expression in *kmt2a* morphants compared to controls at 24 hpf. While in control embryos *gata-1* expressing cells demonstrated a “patchy” pattern corresponding to individual

erythrocytic progenitors entering the blood flow, in *kmt2a* morphants the *gata-1* expressing cells were distributed evenly along the ICM. *In situ* hybridization of *scl* expression also did not show decreased expression at 24 hpf and resembled the pattern observed for *gata-1*. The marker of later stages of erythropoiesis, *hbbe1*, was significantly increased at 24 hpf and showed the same altered expression pattern as *gata-1* and *scl* (Figure 20). Quantitative evaluation of *gata1* and *scl* expression by qRT-PCR confirmed the data obtained by *in situ* hybridisation. Expression of *gata1* was slightly increased, while expression of *scl* was the same in control and *kmt2a*-MO injected embryos (Figure 21).

To quantify *gata-1* expressing immature RBC at later developmental stage at 56 hpf, we used flow-cytometry of cell suspensions from *gata-1*-dsRed transgenic fish. Unexpectedly, the results revealed a dramatic increase in *gata-1* expressing cells in *kmt2a*-MO-injected embryos comparative to the control (Figure 21). Interestingly, at the same time we observed a decrease in the background cell population with low intensity of emitting light. The gating was done using the back-gating approach which gates the cells first based on fluorescent signal and then on size. So the background population falls in the same gate as *gata-1* expressing cells meaning they have the similar size. We propose that this background population is composed of mature erythrocytes. As *gata1* is a marker of immature RBC^{227,228} we can suppose that the results of flow-cytometry revealed an increase in immature RBC at 56 hpf with a simultaneous decrease in presumptive mature RBC.

To check the development of primitive erythropoiesis at later stages, we used *gata1*-dsRed transgenic embryos at 72 hpf (Figure 17). Consistent with our previously noted vascular defects, light sheet microscopy of *gata1*-dsRed transgenic embryos injected with *kmt2a*-MO revealed that while blood flow is detected along the major vessels, it is completely absent in the head and trunk microvasculature. Nonetheless, there appeared to be a similar number of *gata1* expressing cells at this time in control and *kmt2a*-MO injected embryos. We also did not observe any obvious decrease in the dsRed fluorescent signal in *kmt2a* knock-down embryos compared with controls. This goes along with our own and the Wan group's results with o-dianisidine staining. As mentioned above, we observed a dramatic decrease in haemoglobin-expressing mature erythrocytes at 72 hpf as did Wan's group at 48 hpf.

Taken together, the data demonstrate that at 24 hpf *kmt2a* morphants have the same level of *gata-1*- and *scl*-expressing immature RBC as control embryos. Interestingly, the population of more mature RBC expressing *hbbe1* have expanded in *kmt2a* morphants at 24 hpf. At 56 hpf the population of primitive RBC in *kmt2a* morphants also accumulates

immature *gata-1* expressing cells. So the recovery of primitive erythropoiesis starts before 24 hpf and cells commit to the stage when they express *hbbe1* and accumulate in the ICM, however there is no accumulation of *gata-1* and *scl* expressing cells at 24 hpf yet, which can be observed at 56 hpf. The one possible explanation is that decay of the morpholino after 24 hpf might result in enhanced specification of premature erythrocytes. The other possibility is that *kmt2a* is important for molecular events regulating *hbbe1* before 24 hpf, while its role in regulation of *gata-1* starts after 24 hpf.

The defect in RBC maturation can also explain why circulating cells do not enter the microvasculature which is present, though malformed: immature RBC are bigger than mature cells and may simply be physically excluded from the lumen of smaller vessels.

Unfortunately there is no data on genetic markers or antibodies which would allow to distinguish zebrafish proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts, orthochromatophilic erythroblasts, reticulocytes and mature erythrocytes, which would have allowed to detect exact stages of RBC maturation affected by *kmt2a* knock-down.

5.10. *kmt2a* collaborates with and probably acts upstream of *meis1* in a genetic hierarchy regulating angiogenesis and haematopoiesis

To assess the role of *kmt2a* in molecular pathways regulating the development of hematopoietic progenitors, we checked the expression of transcriptional regulators lying upstream of *fli1* in the genetic hierarchy. In particular, we focused on *meis1*, *hoxd4a* and *cdx4*. *MEIS1* together with a subset of *HOX* genes are among the main targets of KMT2A-fusion proteins in KMT2A-fusion-induced leukaemia's^{19,100,111}. *MEIS1* also plays important roles in vertebrate embryonic haematopoiesis and vasculogenesis as was shown by experiments on zebrafish^{93,112} and mouse²²⁹. We therefore checked the expression of *meis1.1* in *kmt2a* morphants by *in situ* hybridization and qRT-PCR at 12 hpf. Interestingly, our results revealed a significant decrease in *meis1.1* expression in embryos injected with *kmt2a*-MO1 but not *kmt2a*-MO2 (Figure 18, data not shown). As *kmt2a*-MO2 blocks splicing leaving the maternally deposited message intact this may indicate a very early role for *kmt2a* in *meis1.1* regulation carried out by the protein of maternal but not zygotic origin. Alternatively, meeting a threshold level of effective Kmt2a levels may require translation of both maternally and zygotically derived mRNA.

Analogous to our results on *kmt2a* knock-down, a previous study of *meis1.1* knock-down effect revealed a decrease in the number of circulating mature RBC after 30 hpf.

meis1.1 morphants also showed a delay in maturation of erythrocytes progenitors. Both *kmt2a* and *meis1.1* knock-down zebrafish embryos demonstrated delay in hematopoietic development. At 24 hpf *gata-1* expression was increased in *meis-1* knock-down embryos and at 48 hpf ICM accumulated *gata1* expressing cells was still present in both *kmt2a* as *meis1.1* knock-down embryos, while in control embryos this site of primitive hematopoiesis is already not functioning at 48 hpf and primitive erythrocytes are circulating in the formed vasculature^{112,113}. As mentioned above, our results also revealed severe defects in vasculogenesis and haematopoiesis together with decreased *meis1.1* expression in *kmt2a* knock-down embryos. This leads us to propose that both *kmt2a* and *meis1.1* regulate primitive erythropoiesis, RBC maturation and angiogenesis and *kmt2a* probably acts upstream of *meis1.1* in these processes

Published data on *meis1.1* also report severe defects in vasculogenesis in *meis1.1* morphants. Comparable to the phenotype we saw in *kmt2a* morpholino-injected embryos, larger vessels were formed and displayed only mild malformations, while the microvasculature was severely defective. The expression of the endothelial marker *fli1*, the arterial marker *ephb2* and the venous marker *flt4* was also severely reduced in *meis1.1* morphants¹¹³.

A study of *Meis1* loss of function in mice²²⁹, demonstrated severe haemorrhaging in *Meis1*^{-/-} embryos coincident with death at E13. *Meis1* null embryos were found to have a severe defect in angiogenesis. The large blood vessels were formed properly, but the lumen of capillaries was too thin and capillaries were curved with accumulated RBC in their sinuses. Primitive haematopoiesis was normal in *Meis1*^{-/-} mice, while definitive haematopoiesis was severely defective.

The preliminary results of our attempts to rescue the blood and vasculature phenotype of *kmt2a* morphants with either *meis1*, *fli1* or *scl* mRNA demonstrated that *meis1* and *fli1* can rescue both endothelial and hematopoietic lineages, pointing to a possible upstream position of *kmt2a* in this genetic hierarchy. However, the rescue experiments were done only once and need to be repeated to confirm this preliminary data.

We also checked the expression of *hoxd4a* which was recently shown to regulate *meis1.1* in the developing vasculature and blood lineages at early times of development⁹³. *hoxd4a* was not decreased in *kmt2a* morphants, possibly indicating a role for *kmt2a* upstream of *meis1.1* but downstream or parallel to *hoxd4a*.

cdx4 plays an important role in embryonic hematopoietic progenitor specification through regulation of *Hox* genes¹⁴⁰. *cdx4* expression was not disturbed in *kmt2a* morphants, putting *kmt2a* downstream of *cdx4* in the regulative network or in a parallel pathway.

5.11. *kmt2a* knock-down regulates neurogenesis early in development taking part in NPB specification

kmt2a knock-down caused decreased pigmentation, which led us to ask whether *kmt2a* might be important in NCC specification or migration, since melanocytes are NCC-derived. NC cells are specified at the NPB together with RB cells, which give rise to sensory neurons²⁰⁹. Preplacodal tissue, which gives rise to the eye lens, inner ear, olfactory epithelium and some sensory tissues (e.g. some components of the trigeminal ganglia), arises in close association with the neural plate border (NPB)²³⁰. To check whether the NPB-derived lineages NC and RB cells are defective, we visualized RB cells with an antibody against acetylated gamma tubulin and examined development of gills which has NC origin. The gills were stained by alcian-blue and demonstrated revealed a dosage-dependent defect in the development of Meckel's cartilage, ceratohyal cartilage, palatoquadrate and ceratobranchials in embryos injected with *kmt2a*-MO1 at 4 ng and 8 ng per embryo (Figure 24). The immunohistochemical staining of RB cells also revealed defect in the lineage (Figure 25).

kmt2a is a well-known regulator of *hox* gene expression and the role of *hox* in the development of NC cells into different lineages is well described⁸⁶. Cranial NC cells form separate streams from hindbrain rhombomers (r2,4,6,7,8) which move in lateral direction and populate PA. PA further give rise to craniofacial structures. The defects observed in *kmt2a* knock-down embryos might be due to the failure to maintain proper *hox* gene expression patterns during hindbrain patterning, which influence development of hindbrain derived NC. However, the Meckel's cartilage develops from NC stream derived from forebrain and its development is known to be *hox*-independent. In our experiments we see severe malformations of Meckel's cartilage resulting from impaired *kmt2a* function. So the other possibility is that *kmt2a* also regulates the specification of NC cells from the NPB, or the NPB itself. Moreover recent research reveals that *hox* genes not only regulate NC patterning during its migration but are involved in the induction of NC cell fate early in development²³¹. *kmt2a* might also regulate development of NC cells directly by deregulating *hox* pattern inside the NC cells population itself.

Together these data allow to conclude that *kmt2a* is important for NC cell induction and might regulate the specification of the NC cells in NPB either through its *hox* regulative function or through *hox* independent pathways. Moreover, when *kmt2a* function was suppressed by injecting splice-blocking morpholino, we did not observe any obvious defects in cartilage (data not shown). Meaning that maternally deposited protein and RNA message of *kmt2a* which is not influenced by splice blocking morpholino are enough for proper development of the cartilage. This strengthens the hypothesis that *kmt2a* acts very early in development regulating induction of NC progenitors from the NPB.

To check whether *kmt2a* play a role at the specification of NPB by itself we checked expression of *prdm1a* in *kmt2a*-MO1 injected embryos. The *prdm1a* gene is one the earliest marker of the NPB in zebrafish embryos. It encodes a zinc finger transcriptional repressor known to be important for specification of dorso-anterior and ventro-posterior structures very early in embryonic development²³². It regulates development of a variety of tissues including NPB derivatives. *prdm1a* is expressed in the presumptive NPB starting from 50% epiboly. At later stages it is expressed in NC and the developing pharyngeal arches^{232,216}. To confirm that the specification of the NPB is disturbed in *kmt2a* morphants, we checked the expression of *prdm1a* at 70% epiboly, 10 and 12 hpf by qRT-PCR. *prdm1a* expression was significantly decreased at all developmental stages examined (Figure 26)

The expression of *prdm1a* targets *tfap2a*, *zic2a*, *sox10* and *islet1* was dramatically reduced at 70% epiboly, 10 and 12 hpf as was shown by qRT-PCR (Figure 27). *zic2a*, marks both neural ectoderm and NPB and also plays an important role in forebrain development, NCC induction and migration and formation of craniofacial cartilage²¹¹. Interestingly, mutations in *zic2a*, along with defects in NC cells derivatives, result in holoprocerephaly which is comparable to the phenotype we observe in *kmt2a* mutants (Figure 30)¹⁶⁹. At the later stage of development NC is cells are marked by the expression of *tfap2a* and its target *sox10*. Both markers are also significantly reduced in *kmt2a* knock-down. Expression of the RB marker *islet1* is also reduced in *kmt2a* morphants. Thus, qRT-PCR data confirm phenotypic observations and point to the role of *kmt2a* in the specification of the NPB and the further development of its derivatives, the NC and RB cells.

To better characterize the role of *kmt2a* in the development of NC and RB lineages, we did *in situ* hybridization of the corresponding markers at 12 and 24 hpf (Figure 27). As mentioned above, we used *sox10* as a marker of NC cells and *islet1* as a marker of the RB population. Interestingly, the intensity of *sox10* staining was the same in control and *kmt2a* morphants. However the *sox10* expressing area was smaller in *kmt2a*-MO1 injected embryos.

Other aspects pattern of the *sox10* expression pattern also looked different in *kmt2a* morphants. The anterior *sox10* expressing cells were not condensed in two sharp migrating lines, but rather gave a smeared appearance in a medio-lateral orientation. The region encompassing trunk NC cells was much shorter along the anterior-posterior axis. Based on the observed pattern, we can infer that *kmt2a* is involved in the regulation of NC cell migration. The shortening of the AP domain of NC cells in alignment with the observed defect in gills (Figure 24) allows us to assume that *kmt2a* plays a role in the development of NC cells located along the hindbrain. The known function of *kmt2a* in hindbrain morphogenesis through the maintenance of *hox* genes expression additionally supports the hypothesis.

The other possibility is that NC cells in *kmt2a* knock-down embryos does not proliferate properly. This hypothesis is analogous to the one which might explain the defective vasculogenesis by the ability of *kmt2a* to regulate cells proliferation. Moreover, *kmt2a* might regulate the development of the anterior and posterior NC cells derivatives by different mechanisms. At 24 hpf, the anterior expression of *sox10* does not appear to be decreased in *kmt2a* morphants, while in the trunk the sprouts of NC cells show significantly reduced expression of *sox10*.

islet1 is expressed in a variety of neural tissues including NPB derivatives RB cells and developing motor neurons. We found *islet1* to be significantly downregulated in *kmt2a* morphants at 12 hpf. Expression was reduced not only in the RB population, but in all *islet1* expressing tissues. The global downregulation of *islet1* expression suggests a tight functional coupling with *kmt2a*, direct regulation of *islet1* by *kmt2a*, or an earlier role for *kmt2a* in the induction of progenitors which subsequently give rise to *islet1* expressing tissues.

Taken together, the data demonstrates that *kmt2a* plays an important role in the specification of NPB and the further development of its derivatives. Whether the role of *kmt2a* in NPB development is limited by its function at early stages or also plays tissue-specific roles in the RB and NC lineages requires further research. The question as to whether *kmt2a* plays a role in NPB induction and later development through regulation of *hox* genes is also not answered in my study, providing a novel direction for further study.

Taking into consideration the known collaborative function of *kmt2a*, *meis1* and *hox* genes in a variety of biological processes, together with data on their individual roles in the development of neural tissues including our results, we can conclude that *kmt2a*, *meis1* and

hox might play unknown roles in the induction and development of the neural system at early stages of development during gastrulation.

5.12. *kmt2a* is required for bilateral development of the eyes and their proper morphogenesis

Along with the impaired angiogenesis, hematopoiesis and development of NPB derivatives, *kmt2a* morphants demonstrated specific defects in eye development (Figure 12, Figure 13). The observed blowout phenotype is likely due to defects in eye patterning along proximo-distal (midline to lateral) axis. As we saw from the results of cryosectioning, it appears that there is no optic stalk separating the optic cup from the brain, with RPE extending directly from the optic cup to the midline of the embryo (Figure 1328). To distinguish which molecular mechanisms regulating eye development are impaired by *kmt2a* loss of function, more studies must be done. Interestingly, the reports of other groups^{233,234} and recent findings from the Featherstone lab (data not published), reveal a role for *crtc1* and *meis1* in eye development. Based on preliminary data, we propose that *kmt2a*, together with *meis1* and *crtc1*, may play important *hox*-independent regulatory roles in signaling pathways regulating patterning and/or proliferation along the proximo-distal axis of the developing brain. Moreover, based on the observed phenotype and preliminary results of *in situ* hybridization of *shh* and *patched* markers (data not shown as experiment was done only once), we believe that *kmt2a* might participate in or regulate *shh* and *nodal* signaling pathways during eye patterning. This would be consistent with the fact that *notch* and *shh* signaling pathways are known to regulate angiogenesis^{235–238} and neural development including NC specification where they modulate *hox* gene function^{231,239}.

5.13. *kmt2a* CRISPR-Cas9 knock-out embryos resemble phenotype of morpholino knock-down

While my studies using morpholino approach were in progress, serious doubts as to the specificity and reliability of morphant phenotypes were raised in the literature^{188–190,240}. To confirm the specificity of phenotype induced by *kmt2a* morpholino action in my project, I generated two *kmt2a*^{-/-} mutant fish lines by CRISPR-Cas9 technology. In the first line, we mutated exon1 of *kmt2a* targeting the sequence encoding the MENIN binding site (TgE1). The second line harbours a mutation in exon 3 of *kmt2a* (TgE3). The approach we used create a stop codon at the targeted site to prevent translation of the rest of the protein.

Following the injection of one-celled embryos with Cas9 protein along with corresponding sgRNAs, the F₀ generation of both TgE1 and TgE3 demonstrated a phenotype

highly reminiscent of the *kmt2a*-MO1 morphants with slower blood flow, blood pooling, heart edema, coloboma, microphthalmia and reduced pigmentation (Figure 30). Some fish demonstrated a more severe phenotype with severely shortened and deformed body shape, complete absence of blood flow and cyclopia. The resemblance between the key phenotypic characteristics of the morpholino-induced and CRISPR-Cas9-induced phenotypes strongly supports a specific role for *kmt2a* in vaculogenesis, hematopoiesis, NC and eye development. The fact that some of the CRISPR-Cas9/sgRNA-injected embryos had a more severe phenotype may be explained in the next way.

kmt2a-MO1 downregulates *kmt2a* translation by binding to AUG codon, but does not fully eliminate the message, allowing some level of Kmt2a expression. As development proceeds, more *kmt2a* message is synthesized which stays intact as injected at 1-cell stage morpholino is bound to the RNA which was present at earlier stages. The possibility of interaction of Cas9/sgRNA with mRNA message has not been reported in literature yet. However if this interaction takes place injection of Cas9 with sgRNAs might interact with both – maternally deposited RNA message and zygotic DNA, fully eliminating the *kmt2a* from the embryo at early stages. The severe phenotype of F0 generation in *kmt2a* mutant embryos points to the possible role of both maternal and zygotic message in the development. The phenotype is specific as separate injection of Cas9 or sgRNA does not phenocopy co-injection.

Interestingly, the heterozygous F1 generation also displayed a transient resemblance to the morpholino-induced phenotype, however the embryos recovered with time. The closure of the choroid fissure, proper development of blood flow and pigmentation were delayed till 72 hpf and further recovered (Figure 32). This might be due to a result of haploinsufficiency at the earlier stages which is compensated later by the accumulation of the zygotic product.

The F2 generation resembled the phenotype of *kmt2a* knock-down (Figure 32). Approximately 75% of embryos had defects in blood flow, blowout phenotype, decreased pigmentation and eye coloboma. The phenotype was not as severe as for some fish from the F0 generation, which further supports our hypothesis that residual maternal message and protein may allow the relevant tissues to develop at least partially.

According to the Mendelian ratios 75% of embryos from F2 generation should have some form of deficiency in *kmt2a* function (25% null + 50% heterozygous). We observed defects in 75% of F2 generation which points again to the possible role of haploinsufficiency

in the developing phenotype. *KMT2A* haploinsufficiency in humans results in severe neurological and skeletal defects Wiedemann–Steiner¹²⁵.

6. CONCLUSIONS AND FUTURE DIRECTIONS

6.1. *kmt2a* role in specification of hematopoietic and angiogenic lineages

Our work confirmed involvement of *kmt2a* in specification of hematopoietic and angiogenic progenitors in zebrafish lateral mesoderm previously shown by Wan's group¹¹² and gave additional data on the position of *kmt2a* in the genetic hierarchy regulating specification of the tissue, putting *kmt2a* downstream or in a parallel pathway with of *cdx4*, but upstream of *meis1*, *fli1*, *scl* and *gata1*. However the precise position of *kmt2a* in the genetic hierarchy regulating early specification of hematopoietic and vascular progenitors is still unknown and requires further investigations. To determine the epistatic relationship between *kmt2a* and *meis1.1* we will do rescue experiments on *kmt2a* morphants and generated *kmt2a*^{-/-} fish line using mRNA for *meis1.1* following by qRT-PCR and *in situ* experiments on the expression level of *scl*, *gata1*, *fli1* at the stage of specification of ALM and PLM. To check whether *kmt2a* and *meis1.1* or *kmt2a* and *hox* make synergistic contribution to the developmental programmes highlighted in this study, we will do double knock-down using minimal (sub-threshold) concentrations of morpholino for each gene. Comparison of phenotypes of double and single morphants will allow us to infer whether the genes act in the same or parallel pathways. We will do relevant *in situ* hybridization and qRT-PCR experiments for single and double morphants as well as rescue of double morphants by *meis1.1* or *hox* mRNA.

6.2. *kmt2a* role in vasculogenesis

For the first time we demonstrated that *kmt2a* plays crucial role for zebrafish vessels development, in particular affecting angiogenesis. We demonstrated the severe defects in the development of microvasculature in embryos with downregulated *kmt2a* as well as downregulation of expression of endothelial marker *fli1*, the arterial *efnb2* and the venous marker *ephb4*, however whether *kmt2a* plays its role on the stage of specification of angiogenic progenitors and affects both vasculogenesis and angiogenesis or it acts only by regulating process of sprouting of small vessels is still unclear. Better characterization of vessels morphology in *kmt2a* morphants and *kmt2a*^{-/-} fish by cryosectioning and evaluating of vessels lumen, wall thickness and expression of *fli1* in the walls of bigger vessels can help to answer this question. The *kmt2a* regulative mechanism in angiogenesis is also unclear yet. RNA-seq of *fli1*-expressing endothelial-lineage cells from the *fli1*-EGFP transgenic line

injected with *kmt2a* morpholino can detect *kmt2a* targets in this developmental process. As *hox* and *meis1.1* are known to be involved in a number of processes under regulation of *kmt2a* and their downregulation was also shown to affect vessels development^{93,113}, the further research on the collaborative role of *hox*, *meis1.1* and *kmt2a* in angiogenesis can shed more light on the role and mechanism of their action in the developing vessels. *hox* and *meis1.1* expression level in endothelial lineage can be checked by qRT-PCR in endothelial cells collected from *fli1*-EGFP transgenic line injected with *kmt2a* morpholino at different developmental stages. Double knock-down of *meis1.1* and *kmt2a* and *hox* and *kmt2a* followed by phenotypic observations, *in situ* hybridization and qRT-PCR experiments for endothelial lineage specific genes would help to better understand the role of *kmt2a*, *hox* and *meis1.1* in vasculogenesis and mechanism of their action.

6.3. *kmt2a* role in RBC maturation

As shown by our work *kmt2a* along with its known role in zebrafish embryonic primitive and definitive hematopoiesis plays a distinct role in erythrocyte maturation. As a continuation of this research direction more detailed characterization of changes in RBC morphology as well as quantification of RBC at different maturation stages in embryos with downregulated and knocked-out *kmt2a* should be done. May-Grünwald/Giemsa staining at 24, 56 and 72 hpf will allow to quantify RBC at different stages of maturation based on the difference in their morphological characteristics²⁴¹. To check whether *kmt2a* affects RBC maturation through regulation of *hox* genes or in an independent manner and whether *meis1.1* is involved in the process, we will collect RBC by FACS from *gata1*-dsRED transgenic line injected with *kmt2a* morpholino and check the expression of *meis1* and *hox* genes in the purified RBC population. RNA-seq experiment for RNA from RBC of *kmt2a*-MO injected *gata1*-dsRED transgenic line can identify *kmt2a* targets in the development of erythroid lineage.

6.4. The role of *kmt2a* in the specification of the NPB, specification and migration of NCC, specification of RB cells, preplacodal tissue and neural ectoderm

We observed number of defects in the development of NPB derivatives in zebrafish embryos with downregulated *kmt2a*. Expression of the markers of NC and RB cells *prdm1a*, *tfap2a*, *zic2a*, *sox10* and *islet1* were also shown to be downregulated in *kmt2a* morphants. However it is still unclear whether *kmt2a* regulates the NPB derivatives at the stage of NPB specification in ectoderm or at the later stage of NC and RB lineages development. As *kmt2a* is ubiquitously expressed at the time of NPB specification its early function has high

probability. *In situ* hybridization of *prdm1* and assessing of expression of the early neural ectodermal marker *mdkb* in *kmt2a* knock-down embryos can help to better understand the role of *kmt2a* in specification of neural ectoderm and NPB. Checking of expression of *cvl2* (marks the region of NCC with high BMP activity) and *dlx3b* or *bampi-b* (marks PPC with low BMP signaling) will help to check the role of *kmt2a* in regionalization of NPB NCC and preplacodal tissue, thus giving more information on the role of *kmt2a* in early specification of NPB and neural ectoderm. We also did not confirm whether *kmt2a* regulates NC lineage development in *hox*-dependent or *hox*-independent way. Additional experiments checking corresponding *hox* genes expression in NC in *kmt2a* morphants as well as rescue experiments with *hox* mRNA would help to resolve this question.

6.5. The role of *kmt2a* in eye morphogenesis

In this work for the first time we demonstrated the defect in separation of the eye field into two lateral structures as a result of mosaic mutations in *kmt2a*. We also showed the role of *kmt2a* in the development of optic stalk and optic cup by both downregulation and loss-of function approaches. There are no studies describing the molecular targets of *kmt2a* in eye development available. Bilateral patterning of eye field is known to be regulated by Shh and Nodal signalling pathways, thus making the players of this pathways being a possible targets of *kmt2a* regulation in the developing eyefield. *In situ* hybridisation and qRT-PCR experiments checking the expression of *shh* and *patched* in *kmt2a* morphants and *kmt2a*^{-/-} embryos at 12 and 24 hpf will help to test the hypothesis. The patterning of lateral eyefields into optic cup and optic stalk is known to be under regulation of *pax2* and *pax6*. Thus *kmt2a* may also regulate *pax2* and *pax6* in the developing optic stalk and optic cup and the experiments checking the expression of these two markers will also help to better understand the role of *kmt2a* in eye morphogenesis.

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