Chapter 4

Embryonal precursors of Wilms tumour

4.1 Introduction

The development of the human through the stages of zygote, embryo, fetus and adult organism constitutes myriad precisely orchestrated cascades of division, differentiation and migration. The analyses in the previous chapter leveraged somatic mutations to trace the journey of a normal cell from the fertilised egg to its eventual adult destination, exploring the developmental relationships between normal cells and tissues. The somatic mutations I used to infer cellular ancestries represented passive marks of cellular division with little or no impact on cellular phenotype. However, somatic mutations have the potential to profoundly alter the programming of individual cells if they occur in certain locations in the genome. Most notably, the sequential acquisition of such driver mutations can transform normal, well-functioning cells into tumour cells.

The introduction of errors into the genome happens continuously, but at a low rate such that the accumulation of the required set of driver mutations usually requires many decades. Childhood cancers, however, lack the long period of mutation accumulation prior to their emergence. In some childhood tumours an inherited germline mutation constitutes the founding driver mutation. A classic example of this is retinoblastoma and the gene that carries its name (*RB1*) (Knudson, 1971). Both copies of this tumour suppressor need to be inactivated to enable cancer formation. Frequently in these patients, the first hit is a disrupting mutation in this gene supplied by one of their parents, either acquired during their own lifetime or inherited in turn. This is effectively the first driver mutation in these children and severely increases the probability of malignancy through inactivation of the second copy. In fact, between 60 and 75% of children with a hereditary form of retinoblastoma develop tumours bilaterally (Knudson, 1971).

In addition to inherited drivers, somatic mutations arising early in development have the potential to act as a first hit in many cancers (Narod and Lenoir, 1991). Because such genomic alterations are automatically present in many, but not all cells of the individual, they can establish a mosaic predisposition throughout large parts of the body. Unlike inherited events, post-zygotic mutations have the ability to create a differential fitness landscape across different embryonic progenitors if the mutation in question has an oncogenic effect. In other words, if all cells carry a driver mutation, none of them have an advantage over one another. However, if only some cells harbour a genomic alteration that increase their fitness, they have the opportunity to outcompete their unmutated neighbours. In such a scenario, the imbalance of potency can potentially disrupt the physiological course of development and create large precursors lesions spreading through different organs.

Many paediatric cancers exhibit a close link to developing cells. While adult cancers might hijack mechanisms of early development, in the form of dedifferentiation and replicative immortality, paediatric tumours appear to be a consequence of cells in arrested development, which are unable to differentiate. This notion is mainly derived from the histologically undifferentiated appearance of paediatric tumour cells. More recently, this has been fortified by transcriptomic studies, characterising childhood cancer cells as having a fetal potential akin to developing cells (Young et al., 2018). In addition, paediatric malignancies have a distinct underlying suite of potential driver genes, many of which have functions intricately linked to development and differ from those found in adult cancers (Grobner et al., 2018).

In a similar vein to clonal haematopoiesis, paediatric cancers might arise from precursor clones that have their origin in aberrant homeostasis or development. As with normal development, somatic mutations can be used to retrace the shared ancestry of tumour and surrounding normal cells. In addition, the possibility of an early driver might be confirmed by such a lineage tracing exercise.

Although a deep phylogeny needs many single cell-derived readouts of somatic mutations, the question of the relationship between tumour and normal tissue can be answered without the need to experimentally obtain such samples. The cancer itself represents a single clonal lineage, from zygote to the founder cell of the tumour. If the mutations that delineate the developmental trajectory of the cancer are also found in normal cells, they will have shared that part of development with the tumour. The proportion of such normal cells harbouring this shared ancestry is naturally encoded in the VAF of these mutations. Therefore, large clonal expansions can be picked up in traditional bulk DNA sequencing experiments with relative ease.

Among the childhood cancers conventionally considered embryonal, i.e. morphologically resembling fetal tissue, is Wilms tumour, also known as nephroblastoma. It is the most common renal cancer in children (Breslow et al., 1993). Most cases of Wilms tumour will manifest as sporadic, unilateral tumours. Early studies assumed that bilateral Wilms tumours originate in much the same fashion as bilateral retinoblastoma, i.e. as a consequence of an inherited predisposing first hit in a tumour suppressor (Treger et al., 2019). Intriguingly, a large French epidemiological study found that a family history of Wilms tumour was no more common in bilateral than in unilateral cases (Bonaiti-Pellie et al., 1992), raising the possibility that an early, post-zygotic event rather than an inherited driver mutation might play a prominent role in carcinogenesis. In order to investigate this hypothesis, I used somatic mutations derived from multi-site biopsies to determine the phylogenetic relationships between Wilms tumour and normal tissues (kidney and blood).

4.2 Detecting early clones in normal kidneys

The study of somatic mutations in early embryonic development from the previous chapter has revealed that large, polyclonal aggregates of cells, such as bulk biopsies, generally obey the same early asymmetries as the whole body. No organ or tissue is derived from a single precursor later than the most recent common ancestor of the entire body, which I presume to be the zygote. Therefore, if the presence of certain mutations and their VAFs differ between different bulk biopsies, the possibility arises that these represent large, aberrant clonal expansions.

To investigate patterns of mutation sharing, the Wilms tumour(s) of 23 patients in total were subjected to whole-genome sequencing, along with at least one sample of normal kidney and blood. For eight of the patients, blood samples from both parents were sequenced as well in order to evaluate and identify any deleterious *de novo* germline mutations. In one case, it was possible to sample a nephrogenic rest, a benign lesion sharing some morphological features with Wilms tumour. Calling and filtering of single nucleotide variants (SNVs) was performed in an unmatched fashion, as described in Chapter 2, section 2.2.

The initial discovery cohort consisted of three children that had unilateral Wilms tumour. The unmatched variant calling revealed a subset of mutations found in tumour that were present across all normal samples as well. These SNVs are presumed to represent the post-zygotic mutations that are acquired during the first few cell divisions of life (early embryonic mutations). However, in two out of three cases, I also identified SNVs that were shared between one or more renal samples and the Wilms tumour, but absent from the blood. This is best illustrated by PD37272, from whom renal pelvis, medulla and cortex were sampled (**Fig.**

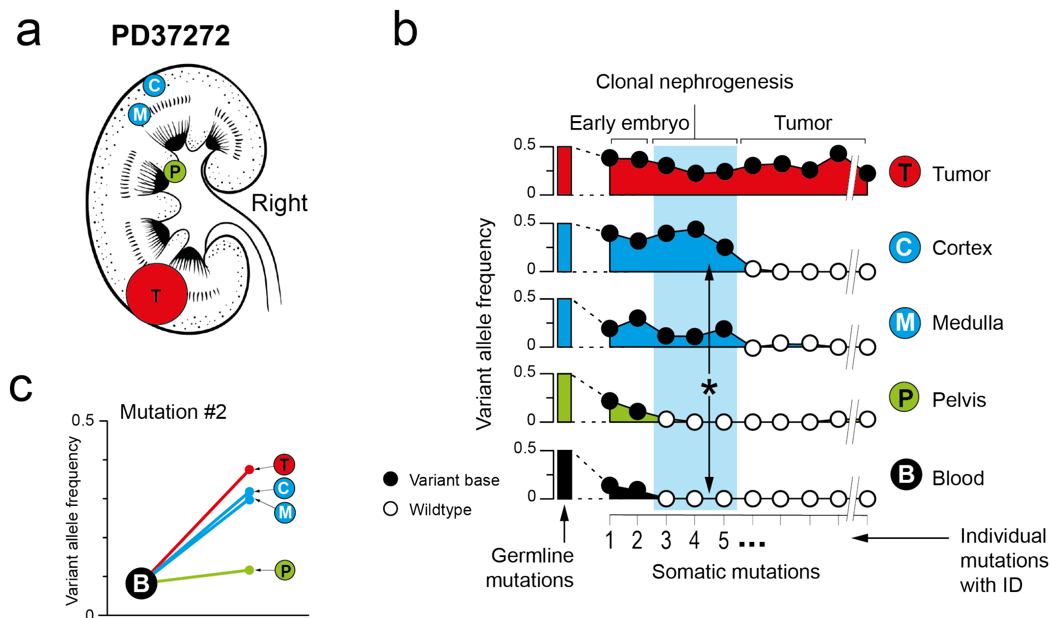

Figure 4.1 (a) Overview of tissue sampling in the kidney of PD37272. (b) Classification and overview of early somatic mutations. If the mutation is present in tumour, kidney, and blood, it is classified as early embryonic (mutation #1-#2). If it is present in kidney samples and tumour only, it is clonal nephrogenic (mutations #3-#5, marked by an asterisk). If it is only in the tumour, it is labelled as such. White and black circles indicate whether the observed VAF is insignificant (white) or significant (black), $p < 0.001$ (test of presence using beta-binomial overdispersion, Methods) (c) The VAF for the last embryonic mutation in kidney samples and tumour compared with blood. From Coorens et al. (2019). Reprinted with permission from AAAS.

4.1a). Intriguingly, three SNVs were shared between the cortex, medulla and tumour that were absent from pelvis and blood (**Fig. 4.1b**). In this case, blood was deeply sequenced, to a genome-wide coverage of 106x, thus minimising the probability of missing the variant by chance. The VAF of one of these mutations was as high as 0.44 in renal cortex, suggesting that a remarkable 88% of cells in the biopsy were derived from the progenitor cell that acquired that SNV. I use the term 'clonal nephrogenesis' to describe this phenomenon in which a substantial proportion of normal kidney cells derives from a single cell that existed later than the zygote. Beyond the pattern of sharing between different tissues, the VAF of embryonic mutations is higher in cortex and medulla, which contain the clonal nephrogenesis, further confirming a more pronounced shared ancestry between these biopsies and the tumour (**Fig. 4.1c**).

It is conceivable that these variants shared between normal samples and the cancer could represent contaminating tumour cells in the normal kidney that are not visible on histology sections or due to cross-contamination of DNA during extraction, library preparation or

sequencing. This explanation is implausible, as contamination would manifest as a sharing of all tumour mutations at a VAF consistent with the contamination rate. Given the absence of any morphological evidence of tumour contamination on histology, the contamination rate would have had to be low, inconsistent with the observed high VAF of shared mutations. Moreover, the VAF of these clonal nephrogenesis mutations gradually decreased along an anatomical gradient. This pattern is much more consistent with a developmental history of clonal nephrogenesis. I also further statistically excluded the possibility of infiltration or contamination by tumour DNA using a binomial mixture model applied to all normal samples. This approach is outlined in the Chapter 2, section 2.3.3.

To evaluate whether clonal nephrogenesis is a frequent antecedent of Wilms tumour, another 20 cases were studies. Four of these cases were bilateral Wilms tumours, for which bilateral biopsies of normal kidney tissue were also obtained. For one of the 16 cases with unilateral Wilms tumour, it was possible to sequence ten biopsies of five normal renal tissues **Fig. 4.5**). As before, I identified SNVs constituting the tumour lineage and evaluated their VAF in the normal biopsies. If there was at least one mutation present in a renal sample and tumour, but absent from blood, I counted the case as one harbouring clonal nephrogenesis.

Within the entire group of 23 patients (both the discovery and validation cohort), I identified mutations signifying clonal nephrogenesis in 10 of 19 children with unilateral disease (53%) and in all four children with bilateral cancers (**Fig. 4.2a**). The presence of such nephrogenic clones was confirmed by a significant ($p < 0.01$, Wilcoxon signed-rank test) inflation of VAFs of early post-zygotic mutations (**Fig. 4.2b**). Importantly, none of the normal renal samples harboured any copy number variants, which might alter the VAFs of such early embryonic mutations.

Collectively, these observations hint that the renal cortex and medulla are derived from a pool of progenitors more closely related to the tumour than renal pelvis and blood. The developmental segregation between the corticomedullary lineage and the blood and renal pelvis is known. Both blood and kidney cells are mesodermal in origin, but segregate from one another soon after gastrulation (Barresi and Gilbert, 2020). The renal pelvis is derived from the ureteric bud, unlike the renal corticomedullary lineages, which have their origin in the nephrogenic cords. This course of development provides an opportunity for a genomic alteration in the corticomedullary lineage to cause a localised clonal expansion without affecting the blood and renal pelvis. However, it is unclear whether this pattern of early SNVs genuinely represent a *bona fide* aberrant nephrogenic clone or is a consequence of natural bottlenecks during the development of the urinary tract. In other words, is clonal nephrogenesis a ubiquitous phenomenon also prevalent amongst patients without Wilms tumours or other renal malignancies?

4.3 Nephrogenic clones are exclusive to individuals with Wilms tumour

Whether clonal nephrogenesis is an aberrant feature of kidneys harbouring Wilms tumours or whether it represents the normal clonal architecture of human nephrons is unclear at this point. This was investigated further using three approaches: by looking at the clonal architecture of normal human kidneys by laser capture microdissection (LCM), whether patients with other paediatric or adult kidney cancer share this phenomenon, and whether the distribution of non-tumour variants in Wilms tumour kidneys is distinct from those derived from other kidneys.

First, to assess whether the normal human kidney has clonal units, glomeruli (n = 7) and proximal and distal tubules (n = 15) were excised from the kidneys of three individuals (**Fig. 4.2c**): the warm autopsy patient described in the previous chapter (PD28690) and two patients who underwent nephrectomies for clear cell renal carcinoma (ccRCC). Neither tubules nor glomeruli had a VAF distribution centred above 0.25 (**Fig. 4.2d,e**), signifying that they do not generally represent monoclonal units as seen in, for example, endometrial glands or colonic crypts. This fact also becomes apparent when taking into account the experiment outlined in the previous chapter, where the majority of renal samples were of an insufficient level of monoclonality to be included in the phylogeny reconstruction.

Second, I assessed whether mutations were commonly shared between other renal tumours and surrounding normal kidney tissue. I studied childhood congenital mesoblastic nephroma (CMN; two tumours and six normal kidney samples), childhood renal malignant rhabdoid tumour (MRT; one cancer and one normal kidney sample), and adult ccRCC (eight cancers, including one bilateral case, and 15 normal tissue samples).

Applying the same unmatched variant calling strategy as before, I sought early post-zygotic mutations shared between tumour, normal kidney tissues, and blood. However, none of these cases harboured mutations that were shared only between neoplastic and normal renal samples but absent from blood (**Fig. 4.2f**). This shows that this pattern of mutation sharing, i.e. clonal nephrogenesis, is specific for Wilms tumours and highly prevalent in this group ($p<0.001$, Fisher's exact test). The absence of clonal nephrogenesis in CMN and MRT may be attributable to their origins in different developmental lineages within the kidney. However, ccRCC and Wilms tumour are both thought to arise from proximal tubular cells (Hohenstein et al., 2015; Kovacs et al., 1997; Treger et al., 2019). If normal embryological clonal dynamics typically generated large clonal expansions, I would have expected to find clonal nephrogenesis in ccRCC cases as well, since the emergence of the tumour in adulthood would not obliterate the developmental history of surrounding normal cells.

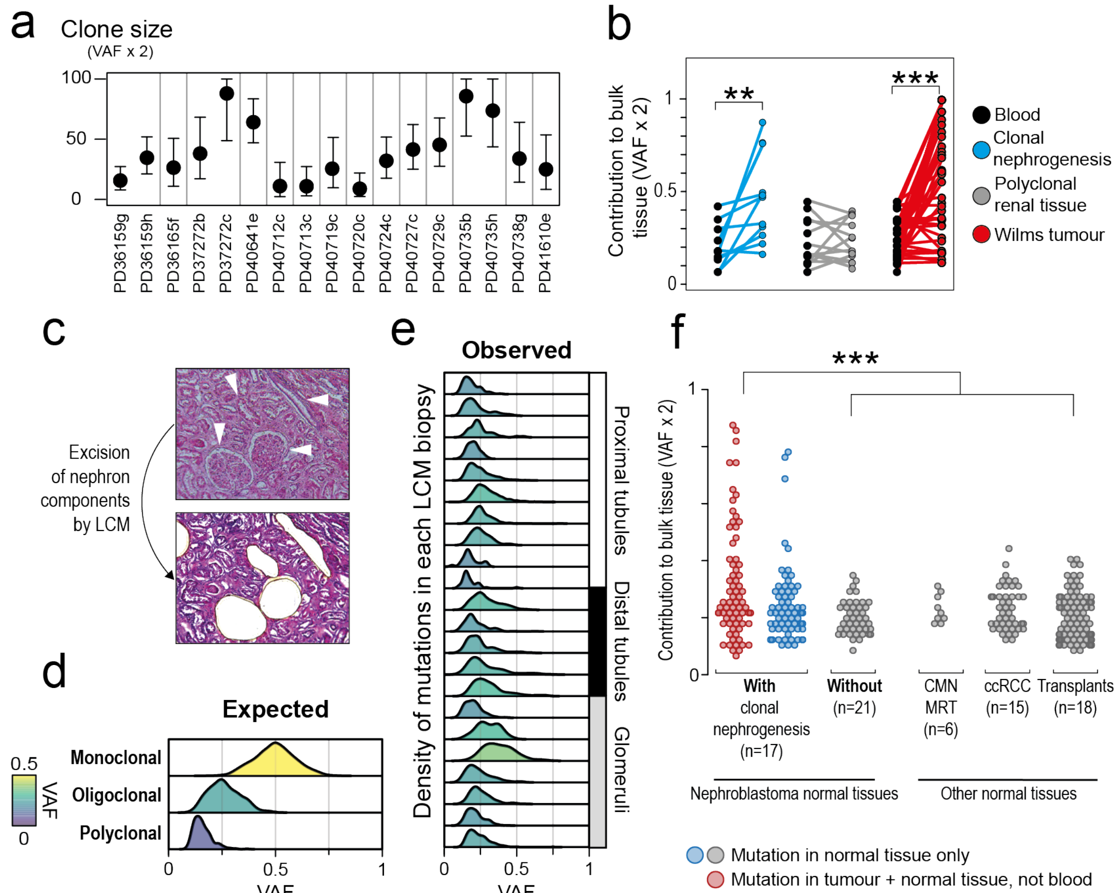

Figure 4.2 (a) Sizes of nephrogenic clones in normal renal samples as predicted by twice the VAF of the most prominent nephrogenic mutation. (b) Plot showing the contribution of the last embryonic mutation in tumour (red) and in samples with (blue) and without clonal nephrogenesis (grey), alongside the contribution to blood (black). The increase was significant in clonal nephrogenesis and tumour samples, but not in renal tissues without clonal nephrogenesis (**$p<0.01$;***$p<0.001$, Wilcoxon signed-rank test). (c) Histology images showing components (arrow heads) of the human nephron excised by LCM. (d) VAF simulations to derive expected distributions depending on clonality of a tissue; monoclonal origin (peak VAF 0.5), oligoclonal origin (peak VAF 0.3), or polyclonal origin (peak VAF 0.1). (e) VAF distributions for 22 microdissected samples (10 proximal tubules, 5 distal tubules, and 7 glomeruli) from 3 patients, 1 rapid autopsy donor and 2 ccRCC patients. Colour indicates the underlying maximum likelihood peak VAF as predicted by a truncated binomial mixture model (see Methods). (f) Mutations present in samples obtained from normal kidneys but absent in matched blood. Only in Wilms tumour were some of these mutations shared with the corresponding tumour. In the presence of clonal nephrogenesis, the VAF distribution of these mutations was significantly elevated (***$p<0.001$, Wilcoxon rank-sum test). From Coorens et al. (2019). Reprinted with permission from AAAS.

As a third approach, I interrogated all mutations of normal kidney tissues listed thus far, supplemented by an additional 18 biopsies obtained from bilateral kidneys that had been declined for transplantation. I analysed somatic mutations present in kidney tissue and absent from non-renal tissue, irrespective of whether they were shared with tumours. Collectively, these analyses of 77 normal kidney biopsies revealed that variants of tissues without clonal nephrogenesis have a significantly lower VAF distribution than clonal nephrogenesis mutations ($p<0.001$, Wilcoxon rank-sum test, **Fig. 4.2f**). Many of the SNVs absent in tumour but present in kidneys containing clonal nephrogenesis will have been generated by alternative lineages after the initial clonal expansions. These are cells that are descended from the initial founder cell but have split from the lineage that will have generated the eventual tumour founder.

Taken together, these results indicate that these nephrogenic clones represent an abnormal state of kidney development, intimately associated with Wilms tumour pathogenesis.

4.4 The driver of clonal nephrogenesis

So far, I have identified the peculiar phenomenon of early embryonal clonal expansions that frequently precede Wilms tumour development. An explanation of genetic drift passively causing such early expansions is unlikely given the specificity to Wilms tumour patients and the short time frame in which genetic drift would need to act to manifest in childhood. In this instance, a more likely explanation for a cell generating a clonal expansion is the acquisition of a selective advantage. In other words, the founder cell must obtain a gain of fitness in order to have a relative advantage over unmutated cells. Therefore, to understand the mechanism of clonal nephrogenesis, it is essential to identify the potential driver event causing this abnormal feature of development.

Surprisingly, almost all SNVs identified as being part of nephrogenic clones fell in non-coding regions (64 out of 66).The remaining two mutations did fall in gene regions and generated a missense change, but in genes (*REXO1* and *R3HDM2*) that have not been implicated in carcinogenesis. These missense mutations were predicted to be benign according to the Variant Effect Predictor algorithm (McLaren et al., 2016). Moreover, none of these SNVs recurred in a similar site or region in the genome, strongly hinting that either a diverse range of genomic changes could generate clonal nephrogenesis, or, perhaps more plausibly, that all the identified genetic alterations were passenger mutations. In the case of the latter, the driver event at the root of clonal nephrogenesis may not be a genetic change.

Whole-genome sequences of Wilms tumour and normal samples were further supplemented by methylation data from arrays and by RNA sequencing to assess the transcriptomic

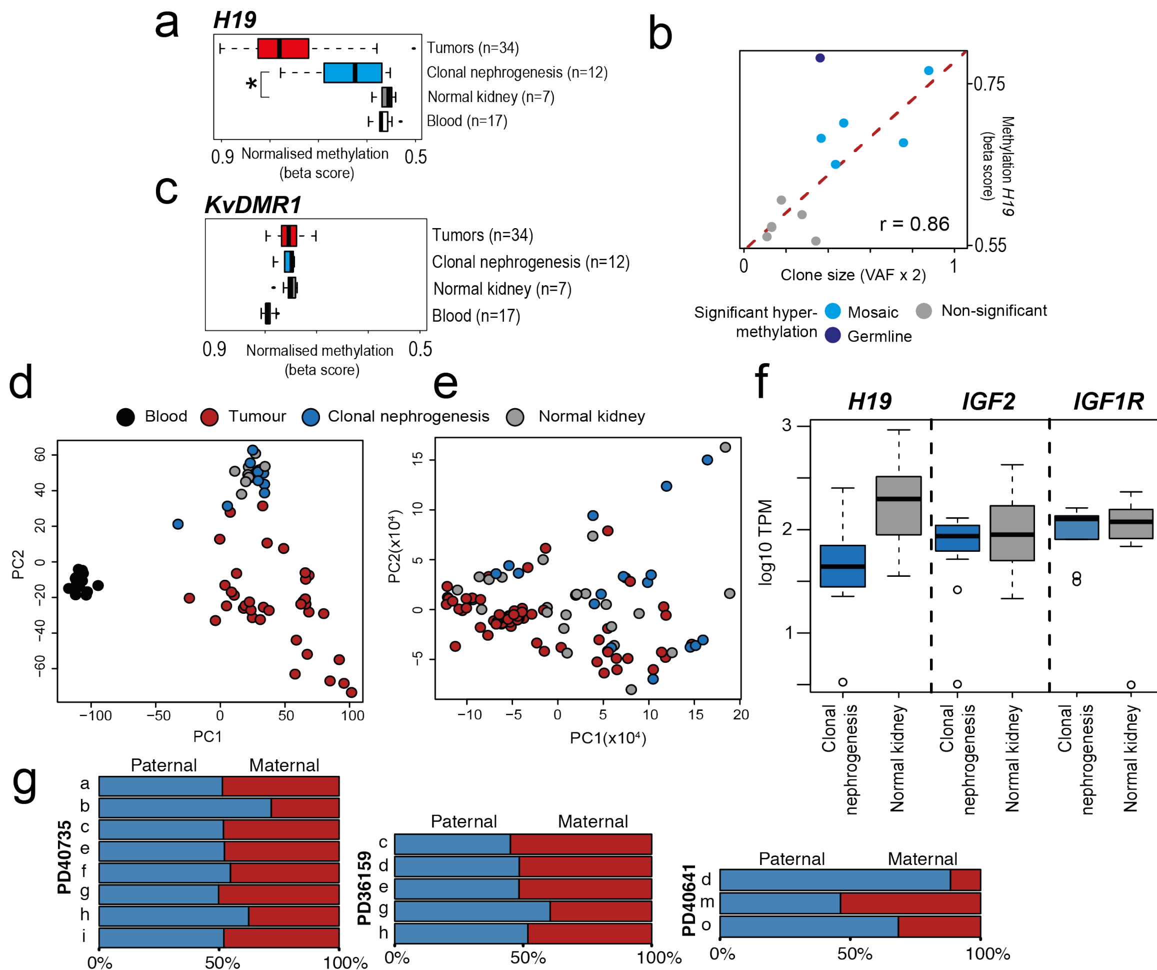

Figure 4.3 (a) Group-level methylation beta values of *H19* (*p<0.05, Wilcoxon rank-sum test). (b) Relationship between predicted clone sizes from nephrogenic mutation and the methylation level of *H19*. The dark blue dot represents PD40738g, which is affected by germline H19 hypermethylation (omitted from correlation and linear regression). The light blue dots indicate samples with clonal nephrogenesis and significant deviation from the background methylation distribution of *H19* as obtained from normal kidney samples without clonal nephrogenesis. Grey dots indicate significant deviation from the background. (c) Group-level methylation levels of KvDMR1 (non-significant, Wilcoxon rank-sum test). Plots of principal component analysis of methylation data from arrays (d) and transcriptome data from RNA sequencing (e). (f) Expression levels of *H19*, *IGF2* and *IGF1R* in kidney samples with and without clonal nephrogenesis. (g) Parental allele-specific expression pattern of *IGF2* in patients with *H19* hypermethylation. From Coorens et al. (2019). Reprinted with permission from AAAS.

profiles of these samples (see Chapter 2, section 2.5.2). Strikingly, I found significant hypermethylation of the *H19* locus in seven out of twelve normal kidney samples containing

nephrogenic clones (**Fig. 4.3a,b**). *H19* hypermethylation was absent from blood and other non-clonal renal tissues, bar the blood of a child with Beckwith-Wiedemann syndrome (PD40738), often caused by this hypermethylation body-wide (Weksberg et al., 2010). Hypermethylation of *H19* is a well-established driver event in Wilms tumour pathogenesis (Charlton et al., 2015; Moulton et al., 1994; Okamoto et al., 1997). The *H19* locus itself encodes a long non-coding RNA that suppresses the expression of growth-promoting genes, such as *IGF2*, that all reside in close proximity to the locus itself, on chromosome 11p15. Furthermore, the *H19* locus falls within one of the few strongly imprinted regions in the human genome (Giannoukakis et al., 1993; Zhang and Tycko, 1992). Normally, the maternal copy of this region is unmethylated, while the paternal one is always methylated. Hence, this hypermethylation event is more appropriately described as loss of imprinting (LOI).

The degree of hypermethylation of *H19* strongly correlated with the VAF delineating the nephrogenic clones (**Fig. 4.3b**), indicating that hypermethylation was present in the founding cell of the clone and pervaded the clone in its entirety. In the five samples with clonal nephrogenesis, but without significant *H19* hypermethylation, it is likely that the size of the clone was small enough to preclude detection of such focal LOI by methylation arrays. Alternatively, I cannot exclude the possibility that these clones harbour distinct, unrecognised genetic or epigenetic driver events.

In addition to *H19*, hypermethylation of the *KvDMR1* locus (chromosome 11p15.5) is also able to cause Beckwith-Wiedemann (Weksberg et al., 2010). However, in those cases, the predisposition to Wilms tumours is only minimal (Treger et al., 2019). Interrogating the methylation status of the *KvDMR1* locus, I determined that its imprinting remained intact in clonal nephrogenesis (**Fig. 4.3c**). This further substantiates the specificity of the LOI as an epigenetic mutation to generate Wilms tumours, effectively by a mosaic version of the Beckwith-Wiedemann syndrome.

Besides LOI of *H19*, I was unable to identify any further driver events accounting for clonal nephrogenesis, despite using whole-exome sequencing to re-interrogate coding mutations in 15 of 17 tissues containing nephrogenic clones. Global gene expression profiles, including the prevalence of fetal transcripts, did not differ between normal renal tissues that did or did not display clonal nephrogenesis (**Fig. 4.3d**). Similarly, global methylation patterns did not differ between these two groups (**Fig. 4.3e**). However, while the expression of *H19* differed between renal samples with and without clonal nephrogenesis, the level of expression of *IGF2* and *IGF1R* remained unchanged between these groups (**Fig. 4.3f**). By assessing the prevalence of parent-specific SNPs in the *IGF2* locus, we could determine that both parental alleles of *IGF2* were expressed in samples with hypermethylation of *H19* (**Fig. 4.3g**). This is in contrast to cells with proper imprinting of this locus, which should show

monoallelic expression. This hints at the ability of the cells with LOI of *H19* to eventually neutralise the up-regulation in *IGF2*-signalling and effectively overcome the overgrowth syndrome.

4.5 Timing the early expansion

From the large contribution of nephrogenic clones to normal renal tissues, we can deduce that the hypermethylation of *H19* must have happened during the development of these organs. The exact timing of this event, however, is much more challenging to pinpoint. In two cases of bilateral Wilms tumour (PD36159 and PD40735) it is noteworthy that the nephrogenic clones span both kidneys, as left and right tumour and normal samples all share clonal nephrogenesis as a common ancestor (**Fig. 4.4a,c**). Therefore it stands to reason that in these cases the LOI of *H19* must have happened prior to the segregation of both kidney primordia, soon after gastrulation (Short and Smyth, 2016).

The observation that clonal nephrogenesis must have been an early event is further reinforced by the size of the clone in the normal kidney samples. In PD40745, two mutations delineating clonal nephrogenesis are found in both kidneys, accounting for 63% of cells on the left and 86% of cells on the right (**Fig. 4.4b**). Strikingly, the mutations identified next separate into branches specific to the right and left kidney, raising the possibility that this lateral split was established a few cell divisions after the original expansion. The size of these clones signifies a remarkable deviation from the early embryonic asymmetry seen in blood, where the first mutation giving rise to kidney and Wilms tumour only accounts for 20%-25% of cells in blood. The remaining 75%-80% of blood cells is delineated by an early embryonic mutation only accounting for 10-20% in normal kidney samples. This reversal of early embryonic asymmetry underscores how aberrant these expansions truly are, particularly, when compared to patterns found the individual in Chapter 3, in whom the early embryonic asymmetry is maintained across tissues throughout a 78 year lifespan.

In another patient with bilateral Wilms tumour, PD40378, all five left tumour samples, but not the right tumour, were related to a clonal expansion in the left kidney (**Fig. 4.4e**). However, it is of note that this patient was diagnosed with a germline Beckwith-Wiedemann syndrome. Notably, the tumour on the right did not appear to arise from an early clone. It is possible that the precursor clone for this cancer only resided in the right of the kidney, from which no normal tissue was available for sampling. The finding that a germline LOI of *H19* grants the potential imbalance to generate precursor clones is surprising, since presumably all cells would carry the hypermethylation and thus have an equal fitness. The mechanism underpinning these clonal expansion therefore remains unclear in this instance.

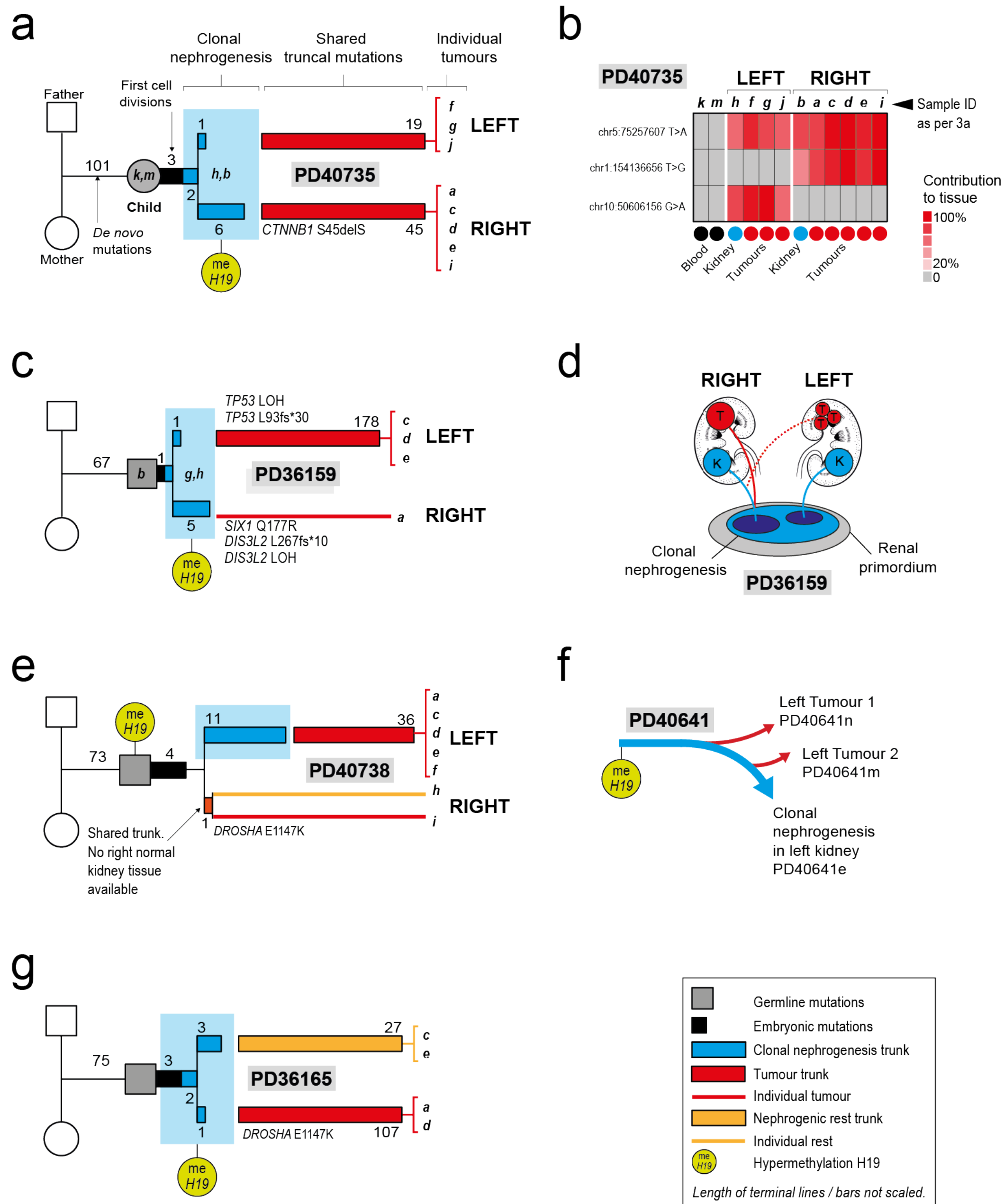

Figure 4.4 (a,c,e,g) For each tumour, the phylogeny is shown including *de novo* germline mutations, embryonic mutations, mutations demarcating clonal nephrogenesis, and tumour mutations. Numbers refer to the number of substitutions defining each developmental branch. Truncal driver events are detailed. (b) Heatmap showing the contribution of a mutation to a sample (as per legend). The pattern of shared mutations reveals a split between left and right kidney, in both tumour and normal samples. (d) As revealed by the shared mutations, the left tumour is more closely related to the right branch of clonal nephrogenesis than to the left in PD36159. (f) Two mutations indicate the independent emergence of tumours at different time points from the nephrogenic clone in PD40641. (g) Tumour and nephrogenic rest in PD36165 both originated from clonal nephrogenesis despite being situated at opposing kidney poles. From Coorens et al. (2019). Reprinted with permission from AAAS.

In the case of unilateral tumours, the timing of the initiation of clonal nephrogenesis remains unclear and is more difficult to establish. It may have evolved before the kidney was formed or thereafter, followed by a "clonal sweep" of clonal nephrogenesis replacing kidney tissue. However, it is probable that the size of the nephrogenic clone hints at the original timing of the *H19* LOI. In PD37272, the SNV demarcating the expansion accounts for 88% of the cortex sample, while also being pervasive in the medullary biopsy. Of course, whether this clone is also present in the contralateral kidney is unknown. Nevertheless, this is in clear contrast with PD41750, where the nephrogenic clone was only found in one cortical sample, out of the ten normal renal samples in total (**Fig. 4.5**). In this cortical sample, the clone accounted for only 25% of cells. It is conceivable that this means the hypermethylation of *H19* occurred at a later point in development and therefore the clonal expansion is only prevalent locally.

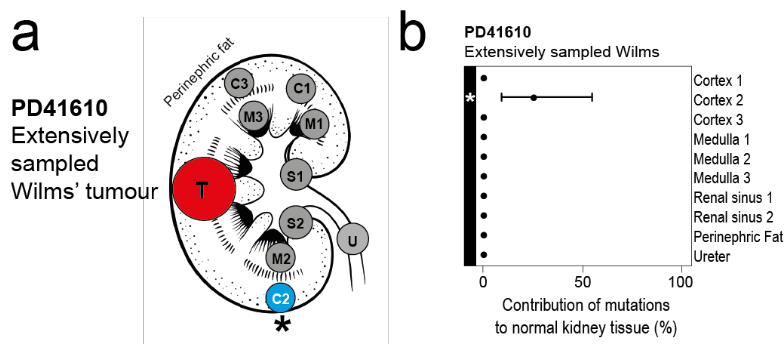

Figure 4.5 (a) Sampling overview for PD41610, the extensively sampled nephroblastoma kidney. This patient shows localised clonal nephrogenesis as depicted by the estimated nephrogenic clone size (twice the VAF) only contributing to a single renal cortical sample (b), highlighted in blue and marked by an asterisk in (a) and (b). From Coorens et al. (2019). Reprinted with permission from AAAS.

The time at which imprinting of *H19* was lost will have a direct impact not only on the predisposition to Wilms tumours, but to other embryonal cancers as well. Hepatoblastoma is the second most common cancer associated with germline Beckwith-Wiedemann, and it appears probable that it can arise as a consequence of mosaic overgrowth as well. More rarely, rhabdomyosarcoma, neuroblastoma and adrenocortical carcinoma are associated with this overgrowth syndrome as well. In a subset of cases, the timing of the emergence of the nephrogenic clone might very well be prior to the commitment of the cell to renal development. In fact, if *H19* imprinting is lost in a cell prior to gastrulation, it is conceivable

that the predisposing effect might be mosaic across germ layers, generating a field effect of cancer risk in more than one organ.

4.6 Sufficiency of *H19* hypermethylation

I have now established that a significant proportion of clonal nephrogenesis is most likely driven by an early cell losing the imprinting pattern of *H19*, which causes expansions of these cells through lost suppression of *IGF2*-mediated signalling. This results in large sections of renal tissue effectively harbouring a first driver in the transformation of normal kidney cell into Wilms tumour. However, the sequencing data from tumours reveals that in the majority of cases, the Wilms tumour acquired more drivers prior to the formation of the malignancy, while no additional drivers were identified in the normal tissue. A driver mutation in *CTNNB1* was found in three tumours, while the *DROSHA* E1147K mutation was found in two cases. Other identified drivers include hits in *TP53* (followed by loss of heterozygosity), *WHSC1* and *SIX1*. In five cases with clonal nephrogenesis, I did not identify any additional driver events beyond the LOI of *H19*, raising the possibility of cryptic genetic driver events (Martincorena et al., 2017) or other epigenomic alterations that might have triggered the formation of the cancer.

Insight into the necessity of additional drivers is perhaps gained best by comparing the genome of the benign nephrogenic rest to the Wilms tumours in PD36165 and PD40738 (**Fig. 4.4e,g**). In these cases, both nephrogenic rest and tumour originate from a common nephrogenic clone. Neither of the nephrogenic rests carry any plausible driver mutation in addition to LOI of *H19*. However, in both cases, the cancer on the ipsilateral side of the nephrogenic rest only differs from the benign lesion by the acquisition of the *DROSHA* E1147K. This suggests that this additional driver is necessary to transform a nephrogenic rest into a full-blown Wilms tumour.

Two tumours found on the left side of PD40641 were revealed to have originated independently from two related but different cells belonging to the same nephrogenic clone (**Fig. 4.4f**). This difference was established by two SNVs only, indicating the transformation events must have occurred soon after one another. Nevertheless, this recurrence points at a sustained potential of the nephrogenic clone to spawn Wilms tumours.

A particularly intriguing aspect of clonal nephrogenesis is the large proportion of morphologically and functionally normal cells carrying the hypermethylation of *H19*. Since there appears to be no genomic difference between these cells and nephrogenic rests, there might be a mechanism that ensures the normal and regular differentiation of these cells into function units of the kidney. After all, nephrogenic rests are essentially clusters of undifferentiated

renal cells residing in the kidney. The question arises whether differentiated renal cells with *H19* hypermethylation retain the potential to transform into Wilms tumours or whether only nephrogenic rests have such a capability.

Of note, the incidence of Wilms tumour plummets after age 6 and is essentially zero beyond age 10 (Breslow et al., 1988). If clonal nephrogenesis represented a lifelong predisposition to nephroblastoma, such tumours would continue to appear throughout the entirety of childhood and long after. However, the absence of such prolonged increase in risk suggests that the predisposing effect of mosaic *H19* LOI is transient. In other words, the epigenetically primed "neoplasia-ready" (Feinberg et al., 2006) cells of the nephrogenic clone lose their malignant potential over time. This is further reinforced by the lack of a clear distinction of the global transcriptome and methylome between normal renal samples with and without clonal nephrogenesis, as mentioned before. This suggests that over time even cells carrying this overgrowth driver somehow repress its effect and become indistinguishable, both in terms of their methylation and expression landscape, from renal cells that never lost this imprinting to begin with. The exact mechanism by which these precursors differentiate over time remains unknown. It is conceivable that this occurs via the conventional pathways operating in renal development, but at a lower rate due to the imprinting loss. In such a scenario, over years, one would only be left with pockets of undifferentiated cells, in essence nephrogenic rests, which slowly disappear with age. With differentiation and specialisation, the carcinogenic potential of these cells is lost, perhaps since these cells no longer rely on the H19-IGF2 pathway.

4.7 Loss of imprinting versus loss of heterozygosity

LOI of *H19* appears to lie at the heart of clonal nephrogenesis, but loss of heterozygosity (LOH) of the same locus, while having the same genomic effect, does not seem to be the dominant pathway in these early expansions. None of the normal renal samples with detected clones have any copy normal abnormalities, including 11p LOH. Moreover, out of the 18 unique tumours originating from a nephrogenic clone, only one also exhibited 11p15 LOH (**Fig. 4.6**). However, of the nine tumours not preceded by clonal nephrogenesis, five exhibited LOH of 11p15. This difference is significant ($p<0.01$, Fisher's exact test) and indicates that LOH and LOI of 11p15 are generally alternative pathways to generating Wilms tumours. In total, only four of 27 unique tumours had neither LOI nor LOH of *H19*. This high prevalence demonstrates that dysregulating the imprinting pattern of the *H19* locus is a key driver of Wilms tumour.

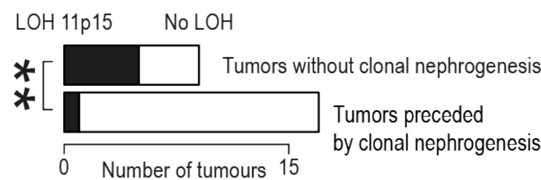

Figure 4.6 Comparison of incidence of copy neutral LOH of 11p15 in this nephroblastoma cohort. Out of the 18 unique tumors that originate from a nephrogenic clone, only one exhibits LOH of 11p15. Out of the nine tumours that did not have such a clone, five have 11p15 LOH (**p<0.01, Fisher's exact test). From Coorens et al. (2019). Reprinted with permission from AAAS.

Recently, we have observed cases of Wilms tumour with paired normal kidney cells that exhibit uniparental disomy of 11p, clearly showing clonal nephrogenesis can be driven by LOH of *H19*. However, the difference in prevalence is striking. Even though both LOI and LOH achieve the same phenotypic result, the underlying rate or tolerance of the different mutational processes, an epimutation versus mitotic recombination, might explain why these mechanisms predominate at different developmental stages. The observation that all bilateral tumours originated through LOI of *H19* and not LOH, and that we can, in a subset of these, time the emergence of the nephrogenic clone to early embryogenesis supports this hypothesis. A higher prevalence of LOI in the early embryo might be expected during its remodelling of the methylation landscape after the first few divisions of life (Eckersley-Maslin et al., 2018). It is possible that an imprinting error at this time happens more frequently than uniparental disomy from mitotic recombination, rendering LOI the commoner pathway to generate embryonal precursors to Wilms tumour.

4.8 Conclusion

The findings presented in this chapter show that early clonal expansions in histologically and functionally normal kidney tissue are an aberrant outcome of renal development that commonly antedates Wilms tumour. These nephrogenic clones are the consequence of hypermethylation of *H19*, effectively producing the phenotype of a mosaic Beckwith-Wiedemann syndrome. Clonal nephrogenesis has the ability to generate histologically and functionally normal kidney cells, which occupied the majority of renal biopsies in the most pronounced cases.

The extent of clonal nephrogenesis might be a marker of malignant potential and inform on the risk of cancer occurrence, at least in childhood, presumably while pockets of undifferentiated precursor cells remain sown in the normal tissue bed. This information

could potentially be used to guide treatment of Wilms tumour patients and surveillance for relapse. Moreover, if it were possible to manipulate the neoplastic potential of clonal nephrogenesis by inducing differentiation, prevention of Wilms tumour in could become feasible. Conceivably, this would also apply to individuals diagnosed with full *H19*-driven Beckwith-Wiedemann syndrome.

Collectively, these findings demonstrate that Wilms tumour frequently represents an insurrection on the background of a premalignant tissue bed, rather than a clearly demarcated neoplasm in an otherwise normal polyclonal kidney. It is highly likely that embryonal clonal expansions, possibly also driven by epigenetic mechanisms rather than genetic changes, may be a common phenomenon in the emergence of childhood cancers.