DEADEND1 GENETICS IN MOUSE MODELS OF TESTICULAR GERM CELL TUMOURS AND THEIR METASTASES

by

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Submitted in partial fulfillment of the requirements

For the degree of Doctor of Philosophy

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CASE WESTERN RESERVE UNIVERSITY

August, 2013

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Dedication

To my boys,

Who reminded me to question everything.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AFP	Alpha-fetoprotein
BEP	Bleomycin, Etoposide and Cisplatin
BMP	Bone Morphogenic Proteins
CGH	Comparative Genomic Hybridization
CIS	Carcinoma *In Situ*
CpG	C—phosphate—G
CSS	Chromosome Substitution Strain
CT	Computed Tomography
DDE	Dichlorodiphenylethylene
DDT	Dichlorodiphenyltrichloroethane
EC	Embryonal Carcinoma
EGCT	Extra-gonadal Germ Cell Tumour
ES cell	Embryonic Stem Cell
ExE	Extra-embryonic Ectoderm
ExM	Extra-Embryonic Mesoderm
hCG	Human Chorionic Gonadotropin
IACUC	Institutional Animal Care and Use Committee
IGCCC	International Germ Cell Consensus Classification

IGCCCG	International Germ Cell Cancer Collaborative Group
IGCNU	Intratubular Germ Cell Neoplasia, Unclassified
IUCC	International Union Against Cancer
IVF	*in vitro* Fertilization
LDH	Lactate Dehydrogenase
LINE-1	Long Interspersed Nuclear Elements-1
MRI	Magnetic Resonance Imaging
OCT4	Octamer-binding transcription factor 4
PCB	Polychlorinated Biphenyls
PET	Positron Emission Tomography
PGC	Primordial Germ Cell
PLAP	Placental Alkaline Phosphatase
pMET	Putative Metastasis
POP	Persistent Organochlorine Pestiticides
RPLND	Retroperitoneal Lymph Node Dissection
RRM	RNA Recognition Motif
SEER	Surveillance Epidemiology and End Results
shRNA	short hairpin RNA
siRNA	short interfering RNA
SNP	Single Nucleotide Polymorphism

TGCT	Testicular Germ Cell Tumour
TNM	Tumour Node Metastases
TPHC	Tissue Procurement and Histology Core
TVDT	Tumour Volume Doubling time
VE	Visceral Endoderm
YST	Yolk Sac Tumour

Mouse Nomenclature:

♀ × ♂ - All crosses are given female × male unless otherwise indicated.

Gene^{+} - Wild-type allele of a gene.

Gene symbol – gene symbols are italicized, first letter upper case all the rest lower case.

PROTEIN - proteins designations are the same as the gene symbol, but not italicized and all upper case.

mRNA - mRNA uses the gene symbol and formatting conventions.

Acknowledgements

–in no particular order

A dissertation, and all the work it that it represents, is never the work of a single individual. There are so many people who have contributed to this body of work and I owe each of them a big thank you.

Many thanks to Dr. Joseph Nadeau, who took me in as a lab manager and peaked my interest in testicular cancer. When I stood at the fork in the road you gave me the opportunity to pursue my passion. As a graduate student you gave me the freedom to follow my own path but provided markers to keep me on track. As a result I've learned how to be independent but still work in a group.

To Dr. Jason Heaney – I can't say thank you enough! You were my mentor in more ways than I can count. Your guidance, helpful suggestions and even our arguments contributed to my growth as a scientist and my rudimentary understanding of statistics. Most importantly I appreciate and value our friendship.

My fellow orphan, Dr. Stephanie Doerner – in the end we were the last ones left standing. Your help, critical evaluations and suggestions made my science better. Your friendship and chats made the lab better. I don't think I could have survived the last few years without you.

I owe a huge debt of gratitude to my fellow lab-mates, particularly the Drs. Soha, Ghunwa, Philip, and Vicki. Your friendship and guidance were invaluable, I enjoyed working with you! Thank you to my committee members, who listened, suggested and provided the help I needed to find my way through. A special thank you to Dr. Helen Salz, your door was always open and you listened. Sometimes that's the best help there is.

I need to thank my family, who made this possible. In particular I need to thank my mom, who always told me that I could be whatever I wanted to me. I owe an eternal debt of gratitude to my husband, Marc, who dragged me to Cleveland. Your support and love have been invaluable to me these last five years. Most importantly, I need to thank my boys, who have been gracious enough to share me with the lab. They've kept my focus clear, and reminded me of what was most important in life. I love you.

Deadend1 Genetics In Mouse Models Of Testicular Germ Cell Tumours And Their Metastases

Abstract

by JENNIFER LYNN ZECHEL

Testicular germ cell tumours (TGCTs), which comprise 95% of all testicular cancers, are a group of neoplasms that affect young men. In the last 40 years, the incidence of this cancer has steadily risen throughout the world. Despite the strong heritability of the disease, few genetic or epigenetic risk factors have been identified. Metastases are a prominent feature of the human disease and are responsible for most of the morbidity and mortality associated with TGCTs. The 129 strain family of mice develop spontaneous TGCTs and are a useful organism to study this disease, but the applicability to human disease is subject to debate. We discovered that the mouse model also develops spontaneous metastases in approximately 11% of affected males, suggesting that TGCTs in mice may be more relevant to human TGCTs than previously thought. Moreover, metastasis rates remain consistent between 129 strains with TGCT modifier genes, even when TGCT rates vary. This suggests that the TGCT genetic susceptibility found in 129 strain mice may include a predisposition to metastasis. In order to further characterize one of these genetic modifiers, $Dnd1^{Ter}$, we created *Dnd1* knockout mice and have found several new functions for DND1. Surprisingly, we found that complete loss of DND1 is embryonically lethal, and that intercrossing animals with $Dnd1^{KO}$ alleles reveals a *Dnd1* segregation bias. We have also

shown that the most powerful TGCT modifier gene in mice, *Dnd1*^{Ter}, is not a loss of function allele. Loss of a copy of *Dnd1* does not affect TGCT rates, but the presence of the *Dnd1*^{Ter} allele significantly increases TGCT rates. This increase is dose-dependent; a second copy of *Dnd1*^{Ter} further increases TGCT rates. We have also found that the pro-oncogenic effects of *Dnd1*^{Ter} are not limited to testicular cancer; it also increases polyp numbers and burden in an *APC*^{MIN} mouse model of intestinal polyposis. These results show that the mouse model of TGCTs may allow for experimental investigation of TGCT metastasis, and that *Dnd1*^{Ter} may identify pathways involved in the oncogenesis of both TGCTs and intestinal polyposis.

Chapter 1: Background and Significance

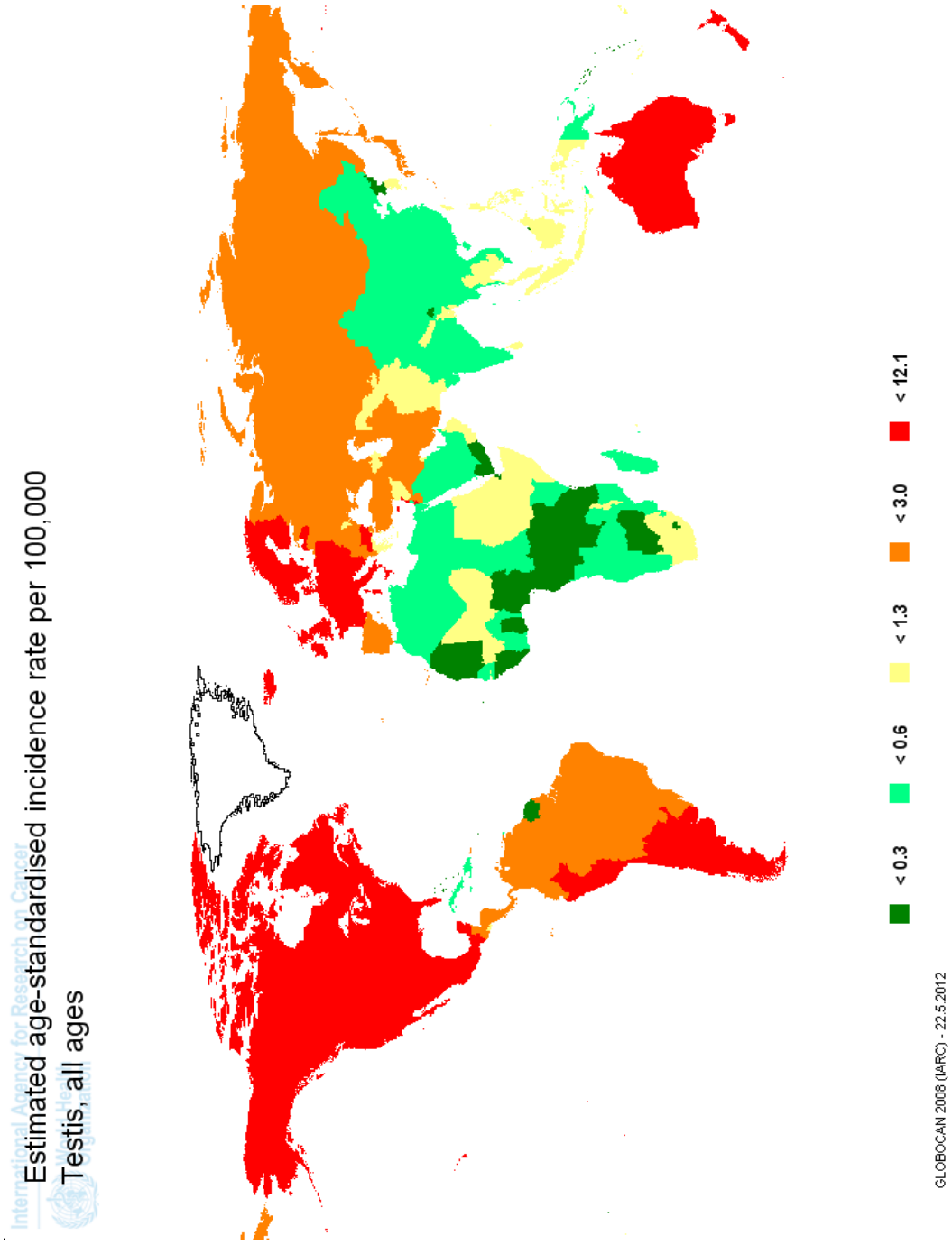
Introduction

Testicular germ cell tumours (TGCTs) are a group of histologically distinct testicular cancers that share a germ cell origin. As a group, they represent the most common cancer in young men between the ages of 15-40 accounting for nearly 60% of all cancers in this age group (DiPietro et al., 2005), but only 1% of all cancers in men (Akbarian et al., 1995; Cancer Res. UK, 2011; Buetow, 1995), The incidence of TGCTs has more than doubled worldwide during the past 40 years (Bosl and Motzer, 1997), but the rate of increase is not uniformly distributed (Figure 1, p.19) (Ferlay et al., 2010). Recent data suggests that the incidence may have peaked for some forms of testicular cancer (Shah et al., 2007) but it is likely that several more years of data collection will be necessary before this is confirmed.

Like most cancers there are many questions that remain unresolved concerning TGCTs; the dearth of answers is due to several experimental difficulties. Because TGCTs are relatively rare it is difficult to obtain enough subjects and samples to perform large studies that have statistical power. Studies are also complicated by the genetic complexity of the disease; even well designed analyses have been unable to identify genetic variants that play a significant role in a majority of TGCT cases. Lastly, because TGCTs (with the exception of spermatocytic seminoma) are a disease that originates *in utero* (Skakkebæk et al.,

Figure 1. Worldwide estimated testis cancer incidence.

Estimates from the World Health Organization International Agency for Research on Cancer (2008) of testicular cancer incidence per 100 000 males standardized by age.



2008; Jørgensen et al., 1995; Rajpert-De Meyts et al., 2003), it is difficult to identify initial transformation and progression. Because of the difficulties mentioned above, the work done for my thesis has taken advantage of a mouse model of TGCTs. There are a number of benefits to using mice: we are able to study any stage of TGCT development; genetically identical inbred strains minimize individual variation; and we are able to compare large numbers of control and affected mice. Using this model, my thesis will focus on two separate aspects of TGCTs that remain unresolved in testicular cancer research. My first study looked at spontaneous metastasis in the mouse model of TGCTs; this had not been previously described and was a novel finding in mice (Zechel et al., 2011). The second study looked at the *Dnd1*^{Ter} allele, the potent modifier of TGCTs in mice which has not been recapitulated in human TGCT cases. This work suggests that *Dnd1*^{Ter} has a novel role in TGCTs, intestinal neoplasia and embryonic viability; and may explain why mutations in *Dnd1* are not necessarily found in human TGCTs.

In this section I will describe the major aspects of human TGCTs with respect to their classification, staging, metastasis, treatment known risk factors. In the second half of this background section I will switch to animal models to outline what is known about primordial germ cells (PGCs), which are the stem cell of TGCTs; and then describe the current mouse models of TGCTs.

Part I - Introduction to the Clinical Aspects of Human TGCTs

Germ cell tumours are unlike most cancers in their etiology, age of onset and treatment outcome, each of which present challenges to tumour research. Understanding the clinical aspects of human TGCTs allows the identification of areas where the mouse model of TGCTs are a useful tool elucidate human disease, and also permits a critical evaluation of the applicability of the mouse TGCTs to human disease.

Histological classification of TGCTs

Here I will describe each of the subtypes and explain the similarities and differences that exist between them. Unlike many other cancer types, TGCTs develop from a single cell type of origin and are able to differentiate into a variety of histologically discrete tumours. Discussion of these general classifications is essential not only to understanding the evolution of TGCTs from a cell of origin, but also facilitates comparison of human and mouse TGCTs.

TGCTs are broadly classified as non-seminomas, seminomas and spermatocytic seminomas. Unlike most cancers, the incidence of TGCTs does not increase with age. Instead incidence peaks in specific age groups according to TGCT subtype. The general frequency, peak age, and immunoreactivity characteristics for each class of TGCT are summarized in Table 1 (p.26).

Intratubular germ cell neoplasia, unclassified

Originally called Carcinoma *In Situ* (CIS) of the testis, intratubular germ cell neoplasia, unclassified (IGCNU) is a lesion believed to be the precursor that leads to all other adult germ cell tumours (Soosay et al., 1991; Guinand and Hedinger, 1981) (Figure 2, p.25). Although the evidence supporting this hypothesis is not based on direct experimental confirmation, the correlation between IGCNU and TGCTs is strong. Approximately 90% of adult TGCTs (not including spermatocytic seminomas) have IGCNU within the surrounding area (Jacobsen et al., 1981; Dieckmann and Skakkebæk, 1999). Without treatment, approximately half of IGCNU cases will go on to become TGCTs within 5 years (von der Maase et al., 1986) and virtually all IGCNU patients were found to develop TGCTs during long-term follow-up (Giwercman et al., 1993).

IGCNUs are termed *unclassified* because they represent an uncommitted cell type that is poised between a primordial germ cell and a germ cell tumour. IGCNU is likely present from infancy (Skakkebæk et al., 2008) but is rarely detected until the neoplastic transformation to a more invasive cell type when patients present with symptoms (Muller et al., 1984). IGCNU cells express Octamer-binding transcription factor 4 (OCT4), Placental Alkaline Phosphatase (PLAP), c-Kit and D2-40 (de Jong et al., 2005; Koide et al., 1987; Rajpert-De Meyts and Skakkebæk, 2008; Sesterhenn et al., 2006; Zhu et al., 2010).

Non-seminomas

Non-seminomas as a group include embryonal carcinomas, teratomas, yolk sac tumours and choriocarcinomas, or a mixed tumour which combines of two or more of these; they account for ~38-40% of all TGCTs (Jemal et al., 2006). Although the incidence of non-seminomas has increased in the last 40-50 years, close examination of the trends shows that this increase has slowed in recent years, particularly in non-Hispanic white men (McGlynn, 2003;(SEER, 2012). In general, non-seminomas grow and spread faster than seminomas but have a better prognostic outcome. Overall, the incidence for non-seminomas peak between the ages of 25-29 years; but each histological subtype within the non-seminoma category has a peak age of incidence (see Table 1, p.26)

Embryonal carcinoma

Embryonal carcinoma is found as a pure germ cell tumour in only 3-4% of TGCT cases, but is found as a cellular component in 40-80% of mixed tumours (Bahrami et al., 2007; Mostofi et al., 1988). The peak age of onset for embryonal carcinomas generally occurs between the ages of 20 and 30, a full decade earlier than most non-seminomas. Despite being a less differentiated cell type, they are seldom restricted to the testis (Ulbright et al., 1999); 10-66% of patients have metastatic disease upon presentation (Tavora, 2011; Rodriguez et al., 1986). The tumour cells are large, with scant cytoplasm and a bulky nucleus and irregular nucleoli. Immunologically, embryonal carcinoma cells stain positively for PLAP,

OCT4, CD30 and low molecular weight cytokeratin (Jones et al., 2004; Latza et al., 1995; Leroy et al., 2002).

Teratoma

A teratoma is a tumour composed of tissues derived from some or all of the germinal layers of endoderm, mesoderm, and ectoderm. Mature teratomas are composed almost entirely of differentiated tissues, whereas immature teratomas have undifferentiated, fetal-like tissues. Between 14-38% of germ cell tumours in infants and children are pure teratomas; the majority of which are the mature form. Even in childhood cases of immature teratomas, the tumours are localized, benign, and present little risk of metastasis (Weissbach et al., 1984). Pure teratomas may also be found in a small number of adult germ cell tumours (2.7%; (Mostofi et al., 1987). In adults, teratomas are mainly immature, and have already metastasized in approximately one third of cases (Leibovitch et al., 2006).

Teratomas are the most diverse germ cell tumours pathologically; in addition to diverse tissue types, they may contain fluid filled cysts, cartilage and bony spicules (Bahrami et al., 2007). Because metastases are often derived from the immature components in adult cases, the tissue profiles from the primary and metastatic tumours differ frequently. They are not commonly immunoreactive with any of the markers that are used to distinguish TGCTs, making histological identification by a pathologist the primary means of diagnosis.

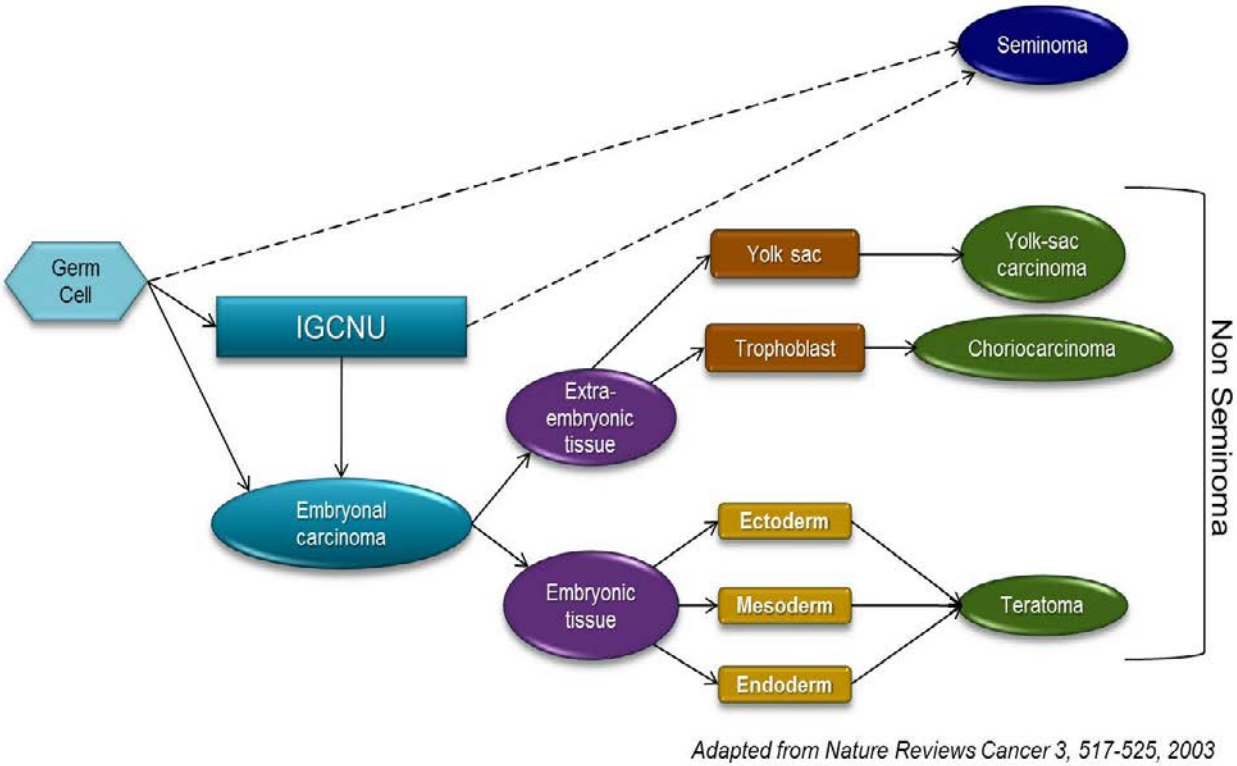


Figure 2. Theoretical histogenesis of testicular germ-cell tumours.

The origin of germ-cell tumours is controversial, and many different schemas have been presented to map the progression from PGC to TGCT. It is generally agreed that most tumours pass through an IGCNU phase before becoming seminomas or non-seminomas. Non-seminomas may contain embryonal carcinoma cells, or a mix of undifferentiated and differentiated tissues. From the embryonal carcinoma cell type, the tumour may go on to form tissue types from embryonic or extra-embronic lineages.

Table 1. Characteristics of TGCTs based on histological group.

Abbreviations: PLAP – placental alkaline phosphatase; CK-LMW – low molecular weight cytokeratin; GPC3 – glypican 3; D2-40 – podoplanin; A1AT - α1-antitrypsin.

TGCT group	**Frequency Peak Age**	**Reactivity**	**Metastasis**	**Reference**
IGCNU	n/a	PLAP c-Kit OCT4 D2-40	none	Koide et al., 1987 Rajpert-de Meyts and Skakkebæk, 2008 de Jong et al., 2005 Sesterhenn et al., 2006
Embryonal carcinoma	3-4% 30-40yrs	PLAP OCT4 CD30 CK-LMW	10-66%	Latza et al., 1995 Leroy et al., 2002 Jones et al., 2004 Tavora, 2011
Teratoma (pure)	2-9% 20 months	none	Rare (infant) 40% (adult)	Simmonds et al., 1996
Yolk Sac Tumour (pure)	1% 1.5yrs	CK-LMW A1AT GPC3	<10%	Biggs and Schwartz, 1988 Ximing, 2012 Niehans et al., 2006
Choriocarcinoma	<1% 20-30yrs	CK-LMW GPC3	>80%	Yokoi et al., 2008 Bahrami et al., 2007
Seminoma	60% 35-39yrs	OCT4 PLAP c-Kit D2-40	12-30%	Biggs and Schwartz, 1988 Bahrami et al., 2007 Miller, 2007
Spermatocytic seminoma	1-2% 55yrs	c-Kit VASA	rare	Bahrami et al., 2007 Decaussin et al., 2004 Zeeman et al., 2002

Yolk Sac Tumour

Yolk sac tumours are the most common testicular neoplasm in young children under the age of 2 (58-82%), although a small number of them (2.4%) affect adults (Mostofi et al., 1987). Unlike many other TGCTs, cryptorchidism and IGCNU are not predisposing factors (Ro et al., 2000). In the pediatric form, yolk sac tumours are almost always stage one (Ross et al., 2002) and found in its pure form; in adults yolk sac tumours are found as a component of mixed germ cell tumours in approximately 40% of cases (Bahrami et al., 2007). Immunoprofiling of yolk sac tumours is positive for cytokeratin, glypican3 and α1-antitrypsin. Glypican3 is a distinguishing marker for yolk sac tumours and choriocarcinomas against seminomas, embryonal carcinomas and teratomas.

Choriocarcinoma

Choriocarcinomas are relatively rare, even as a component of mixed germ cell tumours. They typically affect men aged 20-30. Choriocarcinomas are highly metastatic tumours, with more than 80% of new patients presenting with symptomatic metastases (Yokoi et al., 2008). These metastases commonly involve the neurological or gastrointestinal systems (Bahrami et al., 2007). The propensity for metastases also makes the prognosis for choriocarcinomas worse than for other TGCTs; the pure form has a 46% ten year survival rate (Biggs and Schwartz, 1988). The high metastatic rate may be the result of a high rate of vascular invasion in choriocarcinomas (Bredael et al., 2006). There are no

distinguishing immunoreactive staining patterns in choriocarcinomas; they stain positively for cytokeratin and glypican3, similar to yolk sac tumours.

Seminomas

Seminomas comprise roughly 60% of TGCTs, with a peak incidence between 35-39 years of age (McGlynn et al, 2003), and are rarely found in pre-pubertal boys (Jacobsen et al., 1984). In the United States, the incidence of seminomas in non-Hispanic and black men is increasing faster than non-seminomas (McGlynn, 2003), accounting for the bulk of the TGCT increase. A similar trend has been noted in the UK (Cancer Research UK, 2011).

Seminomas generally present with pain and swelling of the testes, but infertility, and more rarely; metastatic symptoms, may be the chief complaint. Although metastasis is less frequent in seminomas, 15-20% of patients that are diagnosed with isolated disease can have sub-clinical metastases (Sternberg, 1998). The pathological pattern of seminoma is fairly consistent, although there may be some histological variations; foci of necrosis, high mitotic rates, or completely infarcted "ghost" lesions are occasionally seen (Bahrami et al., 2007). Seminomas express OCT4, PLAP, c-Kit and D2-40, all of which may aid in diagnosis of histological variants (Biggs and Schwartz, 1988; Bahrami et al., 2007; Miller, 2007).

Spermatocytic seminomas

Spermatocytic seminomas, which are rare germ cell tumours that affect older men, have a peak incidence in the sixth decade of life. (Carriere et al., 2007; Chung et al., 2004). Although spermatocytic seminomas are classified as germ cell tumours, the cell of origin differs from other TGCTs, it is a more differentiated cell type, capable of spermatogenesis (Stoop et al., 2001). Moreover; spermatocytic seminoma is not associated with IGCNU, cryptorchidism, does not form a component of mixed germ cell tumours, and is rarely metastatic (Steiner et al., 2006).

Identification of TGCT subtype based on histology constitutes the first stage of diagnosis for most patients; the next phase focuses on staging the tumour to determine the extent of tumour growth and if the tumour has spread beyond the confinement of the testicle. The next section deals with two major classification systems and gives a brief description of their staging system.

Classification and staging of TGCTs

The staging and classification of TGCTs is useful in determining therapeutic modalities and prognostic outcomes for clinicians and patients. Therapeutic modalities are generally based upon the TNM staging system developed by Pierre Denoix (Denoix, 1946). In this system, the T describes the primary tumour's size, location and invasiveness; the N refers to the involvement of local lymph nodes; the M denotes any distant metastases. There are also 3 important serum markers

that are used, adding an optional S to TGCT TNM staging. Although the TNM system is fairly straight-forward, it is important to note that there have been several modifications to the classification scheme over the years. Care must be taken when comparing the treatments and outcomes from older studies to more current data. The current TNM staging classification (Edition 7 –see Table 2, p.34) was published in 2009 by the American Joint Committee on Cancer (AJCC) (Edge and Compton, 2010). Notably, several large centers, such as MD Anderson and the Memorial Sloan Kettering Cancer Center, use separate, institutional staging systems.

Separate from the AJCC and International Union Against Cancer (IUCC) TNM staging system, there is a uniform prognostic model that was developed by International Germ Cell Cancer Collaborative Group (IGCCCG) in 1997 (Wilkinson and Read, 1997). This classification is used in addition to the TNM staging; it is does not consider the size or number of primary tumours. Rather, the presence of metastases in organs (other than lung); site of the primary tumour (for non-seminomas); and the elevation of serum markers form the basis for prognosis. Patients are classified into good, intermediate or poor risk groups based on these measures (Table 3, p30). It is important to note that the criteria are different for seminomas and non-seminomas.

Although TNM classification is widely used for most cancers, the IGCCCG staging system is better suited for TGCT prognosis. This is because survival is

not based on tumour size or numbers, but rather the presence and location of TGCT metastases.

Metastasis of TGCTs

As noted in TGCT classification and staging, metastases are a very significant aspect of human TGCTs. With current TGCT treatments, unresponsive or untreatable metastases are responsible for the majority of TGCT-related mortality. Unfortunately, there is very little clinical research regarding metastasis despite its importance in human disease. This highlights the need for a viable animal model that develops TGCT metastases in order to study this aspect of the disease, which I have reported in Chapter 2.

In this section I will describe the mechanisms of metastasis dissemination, clinical detection and how a disseminated metastasis is clinically distinguished from a secondary tumour.

The presence of metastases directly affects treatment modality, tumour surveillance, and survival. A large autopsy study found that TGCTs are highly metastatic tumours, with an average of 5.8 metastases per primary tumour; just slightly more than the metastatic propensity of breast cancer (Disibio and French, 2008). TGCTs are capable of metastasizing via three different mechanisms: (1) direct spreading from the primary tumour to surrounding tissue; (2) lymphatic invasion and spread to lymph nodes that drain the primary tumour; and (3) hematogenous invasion and metastasis of distant tissues and organs.

Direct spreading of TGCTs outside of the male urogenital system is relatively rare. The capsule that surrounds the testis (the tunica albuginea) is thick and prevents direct movement of tumour cells from the testis to the surrounding tissue. However, tumour cells can migrate through the male genital system to colonize the rete testis, tunica albuginea, epididymis, and spermatic cord (Brodsky, 1991). Although this direct spread changes the TNM stage of a TGCT (see Table 2, p.34), it does not affect prognosis.

The most common means of metastatic invasion in TGCTs is via the lymphatic system. Due to embryological development, the lymphatic drainage from the testes does not pass through the inguinal nodes in the groin, but instead drains to the lymph nodes around the aorta and vena cava at the level of the kidneys; these are called the para-aortic or lumbar lymph nodes. As a result, these lymph nodes are typically the first to show signs of metastasis and are often used as sentinel nodes by clinicians to ascertain the presence of metastases in TGCT patients. The pattern of metastasis in the lumbar lymph nodes is further determined by the laterality of the primary TGCT: tumours from the right testis spread to the interaortocaval, precaval, and para-aortic regions and crossover to the contralateral lymph nodes due to the normal drainage of the cisterna chyli and thoracic duct, whereas the left testis drains into the paraaortic and preaortic regions with no crossover (Sesterhenn and Davis Jr, 2004; Donohue et al., 1982). At this point the tumour can spread to the iliac lymph nodes in the pelvis, or

follow the thoracic duct to infiltrate the mediastinal lymph nodes in the chest, the subclavian and supraclavicular lymph nodes in the neck and the subclavian vein.

The last and most serious form of TGCT dissemination is via the vascular system since it facilitates invasion of distant organs. Hematogenous distribution of tumour cells typically occurs in the later stages of the disease; however it is the primary mode of metastasis for choriocarcinomas (Woodward et al., 2002). The main target of choriocarcinoma metastasis is lung (Bredael et al., 2006), though brain metastases are also very common (Ulbright et al., 1999).

Since the advent of current therapies (see Treatment of TGCTs), metastasis poses the greatest challenge to the survival of a TGCT patient. Testicular cancer metastases target a wide variety of tissues, including lymph nodes, lung, liver, spleen and even the contralateral testicle (De Giorgi et al., 2008). These metastases are thought to divide at roughly the same rate as the primary tumour, with a mean tumour volume doubling time (TVDT) of 21 days (Breur, 1966; Demicheli, 1980; Collins et al., 1956) making early treatment of primary tumours and any metastases essential. Because of this rapid growth, it is uncommon for a metastasis to remain clinically undetectable for more than two years after diagnosis (Einhorn and Donohue, 1977) although relapses have been reported as

Table 2. TNM staging of TGCTs.

The TNM staging system is used by clinicians to determine treatment modalities and to help with prognosis of testicular cancers. Adapted from (Edge and Compton, 2010).

Stage	**Description**				
pTx	Unknown status of the testis				
pT0	No apparent primary				
pTis	Intratubular tumour, no invasion				
pT1	Testis and epididymis only no vascular invasion or penetration of tunica albuginea				
pT2	Testis and epididymis with vascular invasion or through tunica albuginea to involve tunica vaginalis				
pT3	Spermatic cord				
pT4	Scrotum				
pNx	Unknown nodal status				
pN0	No regional node involvement				
pN1	Node mass or single nodes ≤ 2cm; ≤ 5 nodes involves; no nodes ≤ 2cm				
pN2	Node mass > 2cm but < 5cm; or > 5 nodes involved				
pN3	Node mass > 5cm				
pMx	Unknown status of distance metastases				
pM0	No distant metastases				
pM1a	Non-regional nodal or lung metastases				
pM1b	Distant metastases other than non-regional nodal or lung metastases				
SX	No marker studies available				
S0	All marker levels normal				
	LDH		**hCG (mIU/mL)**		**AFP (ng/mL)**
S1	<1.5X normal value		Positive < 5000		Positive < 1000
S2	1.5-10X normal value	*or*	5000-50 000	*or*	1000 – 10 000
S3	> 10X normal value	*or*	> 50 000	*or*	> 10 000

Table 3. IGCCCG Staging of TGCTs.

International Germ Cell Cancer Collaborative Group devised a TGCT prognostic staging system with survival rates based on a 1997 study of 5202 patients with nonseminomas and 660 with seminomas* (Wilkinson and Read, 1997). This was further validated with a second study of 1775 nonseminoma patients** (van Dijk et al., 2006).

	Criteria	5-yr survival
Seminomas		
Good risk	• Any primary site and • No metastases to organs other than the lungs and • Normal serum AFP	86%
Intermediate risk	• Any primary site and • Metastases to organs other than the lungs are present and • Normal serum AFP	72%
Non-seminomas		
Good risk	• Testicular or retroperitoneal primary tumour and; • No metastases to organs other than the lungs and • Tumour markers: o Serum AFP <1000 ng/mL o hCG <5000 IU/L o LDH > 1.5X normal value	92*-94**%
Intermediate risk	• Testicular or retroperitoneal primary tumour and; • No metastases to organs other than the lungs and • Tumour markers: o Serum AFP 1000 to 10,000 ng/mL o hCG 5000 to 50,000 IU/L, or, o LDH 1.5 to 10 X normal value	80*-83**%
Poor risk	• Mediastinal primary site with or without metastases or • Metastases to organs other than the lungs or • Tumour markers o Serum AFP >10,000 ng/mL o hCG >50,000 IU/L or o LDH >10 X normal value	48*-71**%

many as 32 years after initial treatment (Kohei et al., 2008; Baniel et al., 1995; Croker et al., 2009; Oldenburg et al., 2006; Ronnen et al., 2005).

Metastases are primarily diagnosed through medical scans. The lung, which is the most common site of metastasis, can be visualized with X-ray. Computed tomography (CT) scans are used to look for systemic metastasis and is useful in staging the primary TGCT. Magnetic resonance imaging (MRI) is particularly beneficial for brain and spinal cord imaging. Positron emission tomography (PET) scans are mainly used in cases of seminoma, to search for small groups of cells.

Occult metastases that remain viable after treatment may also be detectable by measuring the levels of LDH, AFP and HCG in the bloodstream. Of patients who present with stage 1 disease, 30% will relapse within two years (Sagalowsky, 2000); half of those patients will have elevated levels of one or more of these markers in their blood (Krege et al., 2011).

Distinguishing a primary germ cell tumour metastasis from an unrelated secondary cancer that may require separate treatment (Ulbright, 1999) is a difficult task, due to the morphological diversity that arises from the differentiation of GCTs and their metastases. Immunological markers are helpful in identifying true TGCT metastases, including OCT4. The OCT4 pluripotency marker is present in 100% of tested primary and metastatic seminomas and embryonal carcinomas (Table 1, p.26) (Cheng, 2004; Jones et al., 2004; Looijenga

et al., 2003a) and has become an important diagnostic marker for both primary germ cell tumours and their metastases (Cheng et al., 2007).

Treatment of TGCTs

Treatment of TGCTs is highly successful, even in later stages of the disease. Here I will discuss the current recommended treatment paradigms for TGCTs in order to provide an understanding of the physical rigours of this therapy. This will also highlight the need to be able to predict which individuals are likely to develop TGCTs and subsequent metastases.

Treatment options for TGCTs are based primarily on the subtype and stage of the disease. Multiple treatment guidelines have been published, which provide considerable evidence-based options (Motzer et al., 2009; Albers et al., 2011). In general, treatment options include surgery, surveillance, radiotherapy and chemotherapy. Surgery is normally the first step of treatment regardless of TGCT type or stage. A radical inguinal orchiectomy is performed to remove the affected testis. In the absence of metastases, this treatment alone is often curative.

For Stage One seminomas, orchiectomy is combined with radiation therapy, directed to the para-aortic and pelvic lymph nodes. For Stage One non-seminomas retroperitoneal lymph node dissection (RPLND) is used in place of radiation therapy to remove all the lymph nodes around the aorta and vena cava followed with surveillance. Surveillance involves regular follow up with CT

scans, blood work and chest X-rays, and only treating if metastases or recurrent disease becomes apparent.

Stage Two seminomas are treated with orchiectomy, radiation therapy and in the case of bulky tumours, chemotherapy. Stage Two non-seminomas are treated with orchiectomy, RPLND and chemotherapy. Both Stage Three seminomas and non-seminomas are treated identically: orchiectomy and multi-drug chemotherapy.

Most chemotherapy regimens are based on bleomycin, etoposide and cisplatin. Although these drugs provide a high cure rate, they carry the risk of morbidity and mortality. Together with surgery, these treatments carry a mortality risk of 2.3-4.5% (Fossa et al., 1998; Williams et al., 1987). Bleomycin acts in G2 of the cell cycle, inducing both single and double strand breaks, but at high levels this drug can cause a subacute or chronic pneumonitis. Etoposide is a non-intercalating alkaloid that targets topoisomerase II to stabilize the cleavage complex and prevent DNA double strand break repair. Etoposide is widely used in cancer treatment, and carries a range of known toxicities, including neurotoxicity, paresthesias and can increase the risk of leukemia (Baldwin and Osheroff, 2005). Cisplatin is an alkylating agent that results in DNA cross-linking, single and double strand breaks and strand misreading. A wide variety of toxicities are associated with cisplatin use, including acute tubular necrosis and ototoxicity. Resistance to cisplatin occurs in 20% of patients with metastatic

disease, further complicating treatment and worsening prognosis (Piulats et al., 2009). After treatment, patients presenting with metastatic disease have a higher incidence of relapse (Holzik et al., 2008) and lower survival rate (Anant and Davidson, 2001).

Risk factors for TGCTs

Physical risk factors

Several physical characteristics confer an increased risk for developing TGCTs, and it is likely that many of them have a genetic basis. In this discussion I will treat them here on the basis of their physical nature since the genetic links to these characteristics remain unknown.

The most commonly associated risk factor is cryptorchidism or an undescended testicle (Thong et al., 1997; Berkowitz et al., 1993; John Radcliffe Hospital Cryptorchidism Study Group, 1992). It is one of the most common congenital disorders, affecting 3% of all male live births (Banks et al., 2012). A cryptorchidic testis has a significantly higher risk of developing a TGCT (RR=5.4) compared to a descended testis. Interestingly the contralateral normal testis in a patient affected by cryptorchidism also carries a small (RR=1.5) increased relative risk (Banks et al., 2012). Men with an undescended testis also have a higher than expected rate of IGCNU (Dieckmann and Loy, 1996), suggesting that cryptorchidism is a consequence of some other underlying developmental feature rather than a cause of increased TGCT risk. Initial

reports suggested that cryptorchidism predisposes the development of seminomas versus non-seminomas, an association that has also been found in a large meta-analysis (Banks et al., 2012; Swerdlow et al., 1997b).

Other physical risk factors together constitute a generalized testicular dysgenesis syndrome (TDS). These include testicular microlithiasis (Backus et al., 1994; Miller et al., 1998), a previous germ cell tumour (Wanderas et al., 1997; Oÿsterlind et al., 1991), infertility (Petersen et al., 2009; Richiardi et al., 2004), atrophy (Harland et al., 1998), and gonadal dysgenesis (Verp and Simpson, 1987).

Environmental risk factors

The steep rise in TGCTs within the 15-40 year old age group is suggestive of an environmental contribution to the disease incidence, either through *in utero* or direct exposure. The potential effect of endocrine disrupting chemicals on testicular cancer is at the center of the current debate, and research is ongoing in an attempt to understand this relationship. Endocrine disrupting hormones are any exogenous agents that interfere with the natural hormones in the body that regulate developmental processes (Kavlock et al., 1996). There are several classes of isoflavones that mimic estrogen (bisphenol A, phthalates); anti-estrogenic compounds that antagonize the action of estrogen (e.g. dibenzo-*p*-diozin, tribuyltin); anti-androgenic compounds that antagonize the action of androgens (e.g. flutamide, vincolzolin); and anti-progesteronic compounds that

antagonize progesterone activity. Of particular interest are persistent organochlorine pestiticides (POPs) (e.g. dichlorodiphenyltrichloroethane (DDT), dichlorodiphenylethylene (DDE), and polychlorinated biphenyls (PCBs). Most POPs and PCBs act as either weak estrogenic or anti-androgenics through binding to the estrogen and androgen receptors (Toppari et al., 1996). A number of recent studies have found positive associations between either maternal serum levels of endocrine disruptors and male offspring TGCT incidence (Cohn et al., 2010; Hardell et al., 2006), or the levels of endocrine disruptors in TGCT affected individuals (Giannandrea et al., 2011; Purdue et al., 2009). Some of these risks may be related to polymorphisms found in hormone metabolizing genes such as CYP1A1; (Chia et al., 2010a) and the androgen receptor (Vastermark et al., 2011). Despite the positive association found between endocrine disruptors and TGCTs in these studies, a causal relationship has not yet been proven. This is because endocrine disruptor levels are also positively associated with testicular dysgenesis syndrome (see Physical risk factors (Cook et al., 2011a; Toppari et al., 2010), which is also a known risk factor for TGCTs.

Gene expression risk factors

There are a number of studies that measured gene expression in TGCTs in an attempt to identify genes that may contribute to disease in the absence of somatic mutations (Almstrup et al., 2005; Skotheim et al., 2002; Skotheim et al., 2006; Almstrup et al., 2004; Okada et al., 2003; Biermann et al., 2007). Very few gene

expression changes are consistent across studies, primarily due to differentiation in these tumours. A recent meta-analysis of gene expression studies identified two genes that are consistently upregulated in GCTs regardless of histology including ovarian germ cell tumours (Kraggerud et al., 2013). The first is CD9, which is located on human chromosome 12p and has been previously associated with increased TGCT risk (Biermann et al., 2007; Díez-Torre et al., 2010; Singh et al., 2011; Skotheim et al., 2006). The second is Ras-related C3 botulinum toxin substrate 1 (RAC1), a member of the Rac subfamily of the family Rho family of GTPases. RAC1 has also been previously identified as a potential TGCT risk factor (Skotheim et al., 2002; Skotheim et al., 2006; Looijenga et al., 2003b; Santarius et al., 2010; Goddard et al., 2007). Interestingly, RAC1 is has been shown to regulate PGC polarization in zebrafish (Skotheim et al., 2002; Skotheim et al., 2006; Looijenga et al., 2003b; Xu et al., 2012).

Genetic risk factors

TGCT susceptibility is genetically complex. Epidemiological studies point to a strong genetic contribution to TGCTs. Sons and brothers of affected men have a 4- to 15-fold increased risk in the sons and brothers of affected men, respectively (Chia et al., 2008; Neale et al., 2008). This risk is further magnified in twins, with monozygosity and dizygosity increasing the risk 75- and 35-fold, respectively (Swerdlow et al., 1997a). Genetic factors are thought to contribute to 25% of TGCT susceptibility, making it the third most heritable cancer (Czene et al.,

2002). Although large-scale linkage studies have identified genes associated with TGCT risk, they have failed to identify single genes that individually account for a significant portion of TGCT susceptibility, illustrating the complex nature of this disease (Crockford et al., 2006).

First identified as a significant risk factor for spermatogenic failure (Repping et al., 2003), the *gr/gr* deletion removes a portion of the AZFc region of the Y chromosome and is a risk factor for TGCTs (Hemminki and Li, 2004). The *gr/gr* deletion increases TGCT risk, but its contribution is minimal, with the deletion found in 3% of TGCT cases with a family history and 1% of unaffected individuals (Nathanson et al., 2005).

Few somatic mutations have been described in TGCTs. The best characterized is overexpression of KIT, which is found in the stem cells of many TGCTs, more so in seminomas than non-seminomas (Strohmeyer et al., 1995; Bokemeyer et al., 1996; McIntyre et al., 2005), The overall somatic mutation rate in *KIT* is estimated to be of 9% for all TGCT, 20% in seminomas (Rapley and Nathanson, 2010). This mutation is thought to occur early in embryogenesis, since bilateral TGCTs have been found carrying the same mutation (Kemmer et al., 2004; Looijenga et al., 2003b; McIntyre et al., 2005).

Recent linkage studies have identified several other candidate genes or loci SNPs that may play a role in TGCT risk. One such linkage, found on Xq27, has failed to replicate (Crockford et al., 2006; Rapley et al., 2000). Two other recent studies

have found associations within the *KITLG* gene (human chromosome 12), and in the region of the *SPRY4* gene on human chromosome 5 (Kanetsky et al., 2009; Rapley et al., 2009). An additional linkage within the *BAK1* gene (human chromosome 6), was found to be significant in an association study (Rapley et al., 2009). Two separate variants of *DMRT1* (human chromosome 9) have been found to be positively associated with TGCT risk (Kanetsky et al., 2011; Turnbull et al., 2010). Although encouraging, it is important to note that these three linkages account only for approximately 7-10% of TGCT risk susceptibility to sons and individuals (Rapley et al., 2009). It should be noted that sequence differences identified in these linkage studies have no accompanying mechanistic or functional consequences and are yet to be proven causal.

A new meta-analysis of GWAS data has identified four new SNPs that are significantly associated with TGCTs (Figure 3 (Chung et al., 2013). The first is found in the intron of the hematopoietic prostaglandin D synthase gene (*HPGDS*, human chromosome 4). This SNP is of particular interest since *Hpgds* is expressed in the embryonic male gonad (Moniot et al., 2011). The second new SNP is found within intron 17 of the mitotic arrest deficient like 1 gene (*MAD1L1*) on human chromosome 7. The third SNP causes a synonymous codon change in the encoding ring finger WD domain 3 gene (*RFWD3*); within this

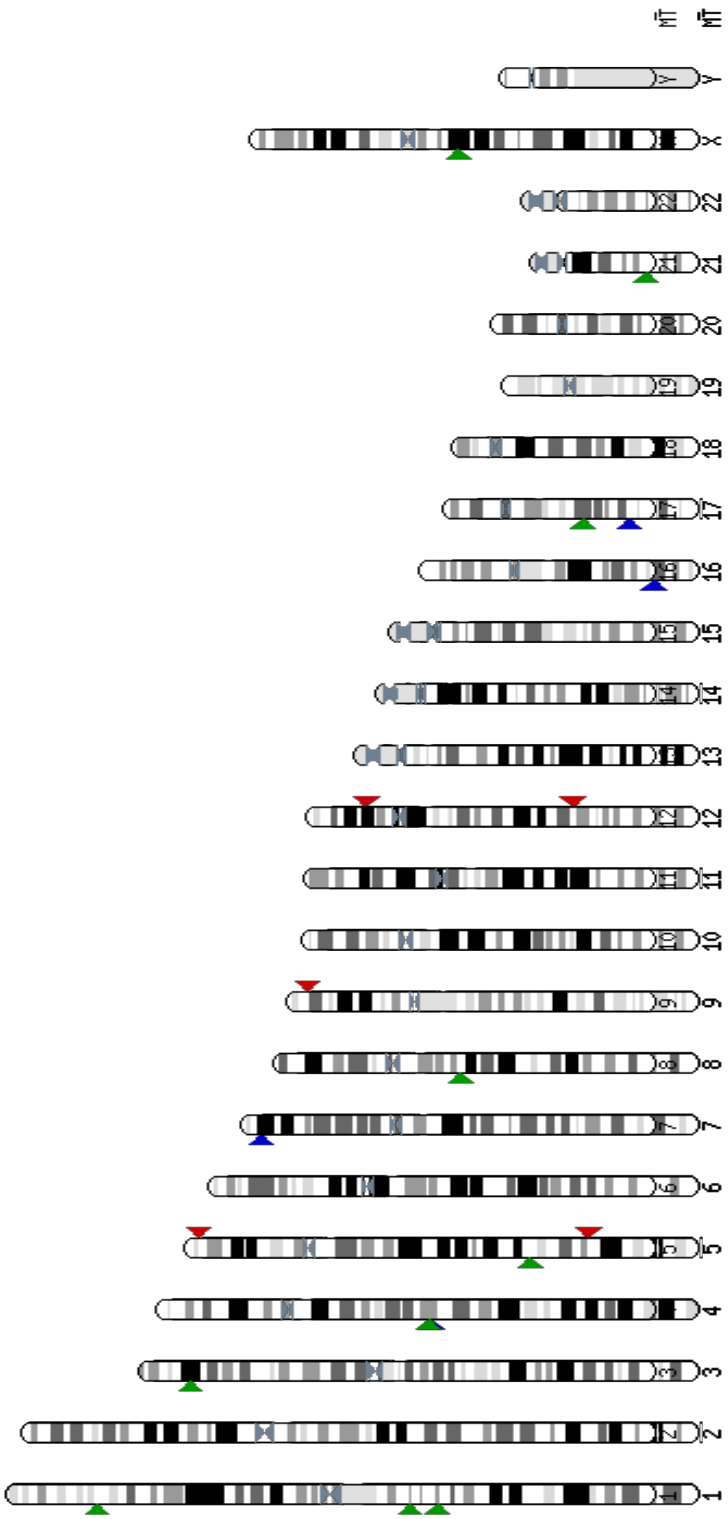


Figure 3. Location of identified SNPs associated with TGCT susceptibility.

Red triangles represent the 6 SNPs previously identified in *KITLG*, *SPRY4*, *BAK1*, *DMRT1* (Kanetsky et al., 2009; Rapley et al., 2009; Kanetsky et al., 2011; Turnbull et al., 2010). Four additional SNPs in *HPGDS*, *MAD1L1*, *RFWD3* and block including *RAD51C* and *TEX14* were recently identified (blue arrows) (Chung et al., 2013) and a further nine SNPs were identified in a second study, including in the genes *PRDM14*, *DAZL* and *PITX1* (green arrows) (Chung et al., 2013; Ruark et al., 2013).

linkage disequilibrium block there are also SNPs that are mapped to the encoding Golgi glycoprotein 1 gene (*GLG1*) and the encoding mixed-lineage kinase domain like gene (*MLKL*). The fourth block of SNPs on human chromosome 17 may be associated with a number of genes, including RAD51 homolog C (*RAD51C*), testis expressed 14 (*TEX14*), protein phosphatase, Mg2+/Mn2+ dependent, 1E (*PPM1E*), septin 4 (*SEPT4*), tripartite motif containing 37 (*TRIM37*) and spindle- and kinetochore-associated complex subunit 2 (*SKA2*). Each of these six genes has been associated with spermatogenesis (Ihara et al., 2005; Kissel et al., 2005; Greenbaum et al., 2006; Kuznetsov et al., 2007; Wu et al., 2000; Karlberg et al., 2004).

A second GWAS study recently reported a total of nine SNPs that are associated with TGCT risk (Ruark et al., 2013). One of these (17q22) was also identified in the study by Chung *et al*. (Chung et al., 2013). Two other SNPs are near genes that are known to be important for PGC development, 8q13.3 near the *PRDM14* gene that is important for early germ cell specification and 3p24.3 which is near *DAZL*, necessary for germ cell development and regulation. A third SNP, 5q31.1, is linked to *PITX1*. PITX1 regulates TERT, a telomerase reverse transcriptase, linking TGCTs with telomerase regulation. The remaining SNPs have not been linked to any genes(1q22, 1q24.1, 3p24.3, 4q24, 16q12.1, and 21q22.3).

The identification of the *PITX1*-linked SNP at 5q31.1 (Ruark et al., 2013) is the third TGCT susceptibility SNP that is also associated with telomerase regulation.

The two others are *TERT* (5p15), the telomerase reverse transcriptase and *ATF7IP* (12p13) which associates with heterochromatin (Turnbull et al., 2010). Interestingly, the amount of telomerase activity is inversely correlated with the differentiation status of TGCTs (Schrader et al., 2002), suggesting that telomerase may play a role in TGCT formation.

Epigenetic risk factors

Gene expression is tightly regulated in cells. Part of the process of fine-tuning this expression involves epigenetic modifications that can up- or down-regulate gene activity. As such, disruption of normal epigenetic patterns in cells can therefore promote tumourigenesis. Recent studies have begun to focus on identifying the epigenome of TGCTs (Lind et al., 2007).

Hypo-methylation, characterized by a loss of 5-methylcytosine, is a permissive state for gene expression. First identified in cancer cells in the 1980s (Feinberg and Tycko, 2004; Feinberg and Vogelstein, 1983), its role in cancer biology is uncertain. There is an association between decreased 5-methylcytosine and tumour formation (Gaudet et al., 2003); hypo-methylation can lead to chromosomal instability in tumours (Saito et al., 2002; Lengauer et al., 1997) as well as increased expression of normally silenced genes such as *Bcl2* (Hanada et al., 1993) and *Sncg* (Gupta et al., 2003).

Genome wide scans for methylation status have shown that seminomas are widely hypo-methylated with few hyper-methylated CpG islands compared to normal testicular tissue and compared to non-seminomas (Smiraglia et al., 2002; Cheung et al., 2010). Very few specifically hypo-methylated genes or genomic elements have been identified in TGCTs, with the notable exception of long interspersed nuclear elements-1 (LINE-1), which have been shown to be inherited from affected father to son in familial TGCTs (Mirabello et al., 2010). Some evidence shows that the KITLG promoter may also have reduced methylation in familial TGCT cases (Mirabello et al., 2012).

Hyper-methylation of CpG islands is a far more common finding in most cancers. When found in promoter regions of genes, hyper-methylation frequently results in gene silencing; this is particularly true of tumour suppressor genes (Larsen et al., 1992). Several hyper-methylated gene promoters have been reported in TGCTs, suggesting that this epigenetic aberration represents an appreciable risk factor for TGCT formation. *Methylguanine-DNA methyltransferase (MGMT)* has been reported as being hyper-methylated in more than half of TGCT samples examined (Rapley et al., 2009; Smith-Sorensen et al., 2002; Mirabello et al., 2012), this hyper-methylation was seen more frequently in non-seminomas compared to seminomas. The *MGMT* gene is interesting because it maps to the mouse *Tgct-1* locus that has been previously associated with TGCTs in mice (Matin et al., 1999). There is also evidence that *PDE11A*, *SPRY4* and *BAK1* gene promoters

are hyper-methylated in familial TGCTs; these genes have previously been associated with TGCT risk (Rapley et al., 2009; Mirabello et al., 2012). *RASSF1A* promoter hyper-methylation has also been demonstrated in demonstrated in TGCTs, with a higher percentage on non-seminomas showing this epigenetic alteration (Mirabello et al., 2012). Interestingly, this study also found that *APC* was frequently hyper-methylated in many non-seminomas.

Current status of TGCT sequencing

The relative paucity of genetic risk factors associated with TGCTs may be attributed to a number of causes. The overall genetic complexity of the disease certainly plays a significant role in hindering the identification of TGCT susceptibility factors. A large number of genetic variations, each making a small contribution to susceptibility, are difficult to detect even in large sample sizes. Compounding the difficulty of identifying TGCT genetic risk factors is the dismal lack of TGCT sequencing data. Moreover, the data that is currently available is of low resolution, preventing detailed analysis of DNA alterations in TGCTs.

Whole genome analysis of TGCTs is limited. The seminal work used CGH of 11 TGCT samples on a 5k cDNA microarray which provides spacing of approximately 700kb and identified 14 regions of genomic imbalance (McIntyre et al., 2004). This was quickly followed by a more comprehensive study which used 22 samples, an average spacing of 303kb and identified 134 genes that were genetically imbalanced between normal testis and TGCTs, many of which were

further validated by qRT-PCR (Skotheim et al., 2006). The first SNP mapping of 25 seminomas was published in 2011, querying 262 000 SNPs with an average inter-SNP distance of 5.8kb (LeBron et al., 2011). No studies have reported whole genome deep sequencing of TGCTs; two studies that used deep sequencing looked specifically at the Dcr-1 homolog (DICER1) RNase IIIb domain in TGCTs (de Boer et al., 2012) and the SRY region in disorders of sex development (Hersmus et al., 2012). There is some indication that the lack of coverage is being address, preliminary reports of familial TGCT exome sequencing have identified nine common alterations that may correlate with disease susceptibility (Schrader et al., 2012).

Studies of epigenetic modifications in TGCTs are similarly deficient. The techniques used in epigenetic studies provide a more comprehensive examination of the genome, and yield a significant number of differentially methylated regions. Several studies have limited examination to long interspersed elements (LINEs) and other repetitive DNA (Mirabello et al., 2010; Mirabello et al., 2012; Ushida et al., 2012). One genome-wide study identified a total of 35 208 differentially methylated regions (Cheung et al., 2010), of which very few mapped to known promoters or genes. More informatively, comparisons between seminomas and non-seminomas have identified distinct epigenetic profiles based on TGCT subtype (Smiraglia et al., 2002).

The dearth of deep sequencing of TGCTs may be partially responsible for the lack of identified genetic risk factors associated with TGCTs. With evolving technology and reduced costs, it is likely that these sequences will become available within the next decade. This information will likely identify new risk factors of the disease. Conversely, the identification of epigenetic abnormalities found in TGCTs is contingent not on the completion of the studies, but on understand how the results affect gene regulation. Promoters, repressors and other genetic regulators may affect genes over 100kb away, and may even act across DNA strands making it difficult to understand how abnormal epigenetic modifications influence transcriptional activity in the cell. Until a complete map of these regulators is available, the understanding of epigenetic influences on TGCT susceptibility and formation will be incomplete.

Part II - Introduction to the Primordial Germ Cell origins of TGCTs: Lessons from Animal Models

Due to the inherent difficulties of working with human samples and tissues, the data pertaining to molecular pathways, the effects of specific genetic mutations and embryological development presented in this section has been obtained from several animal models.

Primordial germ cell origin of TGCTs

The first link that suggested PGCs as the stem cell for TGCTs came from the characterization of testicular tumours and teratomas, which can be comprised of tissues from all three germ layers (Mostofi et al., 1987). Experimental evidence demonstrated that mouse E12.5 PGCs transplanted into adult testes formed teratomas in 93% of cases (Regenass et al., 1982). L.C. Stevens noted that in mice, testicular tumours originate as small clusters of highly proliferative cells in the E15.5 gonad (Matin et al., 1998; Stevens, 1967a), after male PGCs normally enter mitotic arrest. More recently, molecular characterization of the male PGCs mitotic arrest revealed that PGCs in TGCT susceptible strains remained mitotically active at E15.5 and express the germ cell pluripotency factor *Nanog* and the female germ cell differentiation factor *Cdnd1* (Heaney et al., 2012).

Specification and migration of PGCs

Primordial germ cells (PGCs) are totipotent stem cells that normally differentiate into gametes, the cells that will give rise to future generations. PGCs are one of the first embryonic cell lineages to be specified, originating from proximal epiblast cells which are primed to follow a somatic cell fate (Saga, 2008). Because of this, germ cell specification involves three main steps. First there needs to be a reversal of the somatic cell programme through gene silencing (eg. *Hox* gene clusters). Next, permissive chromatin marks on genes that are required

for pluripotency (eg. *Oct4* and *Nanog*) need to be maintained. Finally, germ cell specific genes such as *Dnd1* and *Nanos3* need to be activated.

Beginning at E5.5, cells in the extraembryonic ectoderm (ExE) release bone morphogenic proteins (BMP) BMP4 and BMP8b, which are required for PGC specification (Lawson et al., 1999; Ying et al., 2000) (Figure 4, p.55). BMPs then bind to receptor complexes composed of ALK2 and BMP receptor type II on epiblast cells in the visceral endoderm (VE) (de Sousa Lopes et al., 2004). Downstream signalling is mediated by phosphorylation of SMADs 1, 4 and 5 that are ubiquitously expressed within the epiblast (Hayashi et al., 2002; Arnold et al., 2006; Ohinata et al., 2009). Of these, SMAD1 (Tremblay et al., 2001) and SMAD 5 (Chang and Matzuk, 2001) are essential for PGC specification, embryos lacking these proteins form few to no PGCs. SMAD8 appears to be dispensable since embryos lacking SMAD8 are normal and fertile (Arnold et al., 2006). The visceral endoderm (VE) also secretes BMP2 from E5.5 onwards, which amplifies BMP4 signalling in PGC specification (Ying and Zhao, 2001). Loss of BMP2 reduces PGC numbers but does not completely prevent their specification.

The end result of this signalling cascade is the expression of the transcriptional regulators of PRDM1, also known as BLIMP1, (~E6.25) and PRDM14 (~E6.5). Cells expressing these factors then go on to form the PGC founders (de Sousa Lopes et al., 2004; Ohinata et al., 2005; Vincent et al., 2005). PRDM1 plays several roles in PGC specification (Kurimoto et al., 2008; Ohinata et al., 2005;

Vincent et al., 2005) acting through the signalling intermediary AP2γ (Weber et al., 2010; Schafer et al., 2011). PRDM1 acts to reverse the somatic cell fate originally assigned to the cell through the down-regulation of *Hox* gene clusters and genes involved in mesodermal induction (Kurimoto et al., 2008; Yabuta et al., 2006). PRDM1 as well as PRDM14 are involved in the reacquisition of pluripotency through SOX2 (Yabuta et al., 2006), and they stimulate PGC specific genes including NANOS3, DND1 and KIT. Complete loss of PRDM1 results in an early arrest of PGC specification (Ohinata et al., 2005; Yabuta et al., 2006). Both PRDM1 and PRDM14, acting through a KLF2 mediator (Gillich et al., 2012), repress both the maintenance of existing DNA methylation and any *de novo* methylation of DNA through inhibition of the DNA methyltransferases DNMT3a and DNMT3b (Kurimoto et al., 2008; Kouzarides, 2002). In the absence of PRDM14, PGCs fail to form (Yamaji et al., 2008).

At ~E6.75 the newly specified PGCs and the surrounding somatic cells both have genome-wide H3K4me2 and me3 marks; active H3K9ac modifications; and repressive H3K9me1, me2, and me3 marks; as well as H3K27me2 and me3 chromatin marks (See Figure 5, p.58) (Seki et al., 2007).

Between E7.0 and E7.25, 40-100 cells that express both PRDM1 and PRDM14 become identifiable as PGCs in the base of the forming allantois in the extra-embryonic mesoderm (ExM) (Ginsburg et al., 1990; Lawson and Hage, 1994). PGC-specific proteins are now being translated in the PGCs, including OCT4

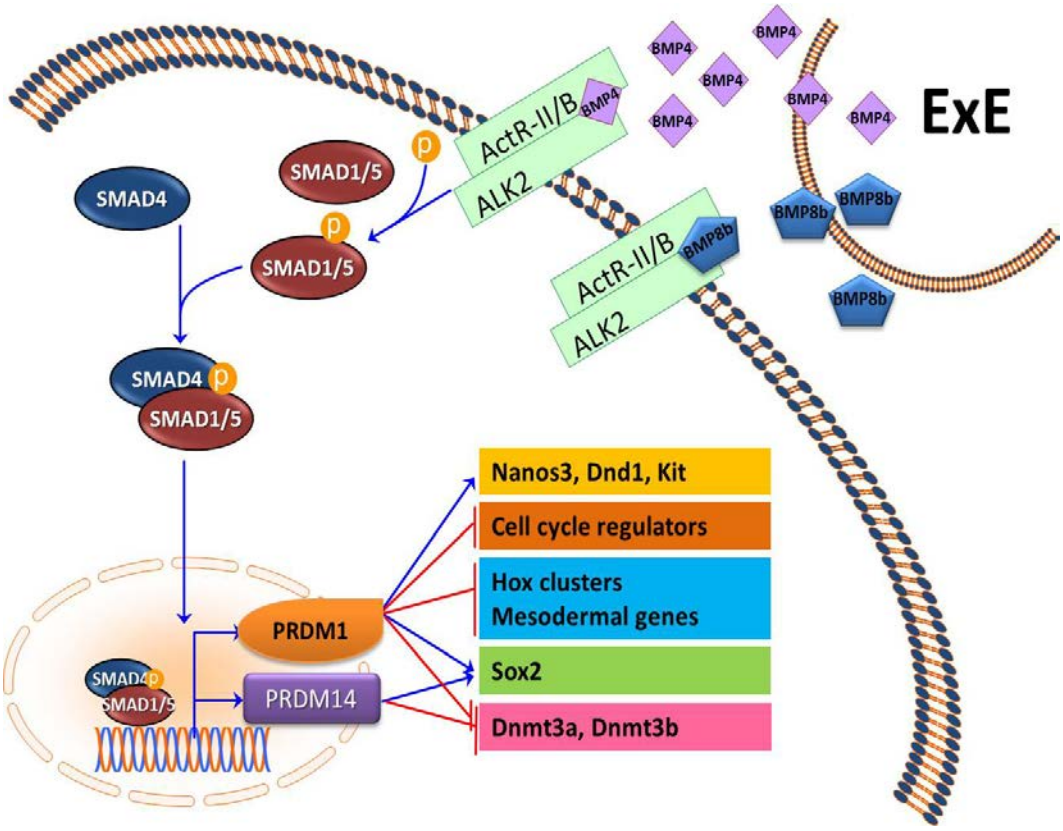


Figure 4. A model of PGC specification signalling.

Beginning at E5.5 cells in the extraembryonic ectoderm (ExE) release bone morphogenic (BMP) factors BMP4 and BMP8b. These bind to receptor complexes composed of ALK2 and BMP receptor type II (ActR-II/B) on epiblast cells in the visceral endoderm. Stimulation of this receptor complex leads to the phosphorylation of SMADs 1/4/5, which then translocate to the nucleus and promote the transcription of PRDM1 and PRDM14. PRDM1 stimulates the expression of pluripotency and PGC-specific proteins, including NANOS3, DND1, KIT and SOX2 and inhibits cell cycle regulatory proteins as well as hox cluster genes and mesodermal genes. Both PRDM1 and PRDM14 prevent *de novo* methylation through inhibition of DNA (cytosine-5)-methyltransferase (DNMT) 3a and DNMT3b and provides additional stimulation to SOX2.

(Kehler et al., 2004; Okamura et al., 2008), NANOS3 (Tsuda et al., 2003; Suzuki et al., 2007), DND1 (Youngren et al., 2005) and KIT (Mintz and Russell, 1957; Buehr et al., 1993). Expression of these proteins appears to mark the end of PGC specification, and the initiation of their migration towards the fetal gonads.

The signals that trigger PGC migration remain unknown (Richardson and Lehmann, 2010). PGCs respond to these unidentified signals at ~E7.5 with changes in morphology: they become polarized and extend cytoplasmic protrusions (Anderson et al., 2000). PGCs then move from the posterior primitive streak to the endoderm and begin their migration. Concomitantly, at ~E7.75, a genome wide reduction in two of the major epigenetic repressive marks occurs: DNA methylation and H3K9me2 (Seki et al., 2007; Seki et al., 2005).

PGCs migrate in response to several attractants, including SDF1 (expressed in the genital ridges and surrounding mesenchyme) and CXCR4 (expressed in the PGCs) (Seki et al., 2007; Ara et al., 2003; Molyneaux et al., 2003). Both of these factors are necessary for successful PGC migration to the genital ridge (Seki et al., 2007; Ara et al., 2003; Molyneaux et al., 2003). KIT, and its ligand KITLG, are also important to PGC migration. Together they are responsible for general PGC motility, but do not provide directional cues (Seki et al., 2007; Gu et al., 2009; Runyan et al., 2006). Loss of KITLG results in reduced PGC motility, but does not prevent PGC migration (Seki et al., 2007; Gu et al., 2009). From E8.0-

9.5 PGCs migrate through the hindgut toward the urogenital ridges, rapidly proliferating as they travel (Anderson 2000; Molyneaux 2001). During this time PGCs show progressively higher levels of the repressive H3K27me3 chromatin marks (Seki et al., 2007; Seki et al., 2005), possibly through the action of enhancer of zeste homologue 1 (*Ezh1*) (Shen et al., 2008). At the same time, the majority of PGCs are arrested in the G2 phase of the cell cycle (Seki et al., 2007). This arrest is accompanied by an up-regulation of pluripotency genes including *Sox2* and *Nanog* (Surani et al., 2007), which may facilitate epigenetic reprogramming of the PGCs (Saitou and Yamaji, 2010).

The PGCs reach the genital ridges between E10.5 and 11.5, where they continue to proliferate. Erasure of DNA methylation continues, initiating chromatin remodeling (Hajkova et al., 2008). Further epigenetic changes include an erasure of parentally imprinted genes and promoter CpG methylation (Hajkova et al., 2002; Maatouk et al., 2006), rendering the PGCs to a basal epigenetic state (Hackett et al., 2012). Any PGCs that fail to complete migration become apoptotic (Anderson 2000; Upadhyay 1982, MacLean 2007, Runyan 2006, Zamboni 1983). By E13.5, there are approximately 25 000 PGCs in the gonads. At this time male PGCs undergo mitotic arrest and will remain quiescent until puberty (Koubova et al., 2006; Mauduit et al., 1999).

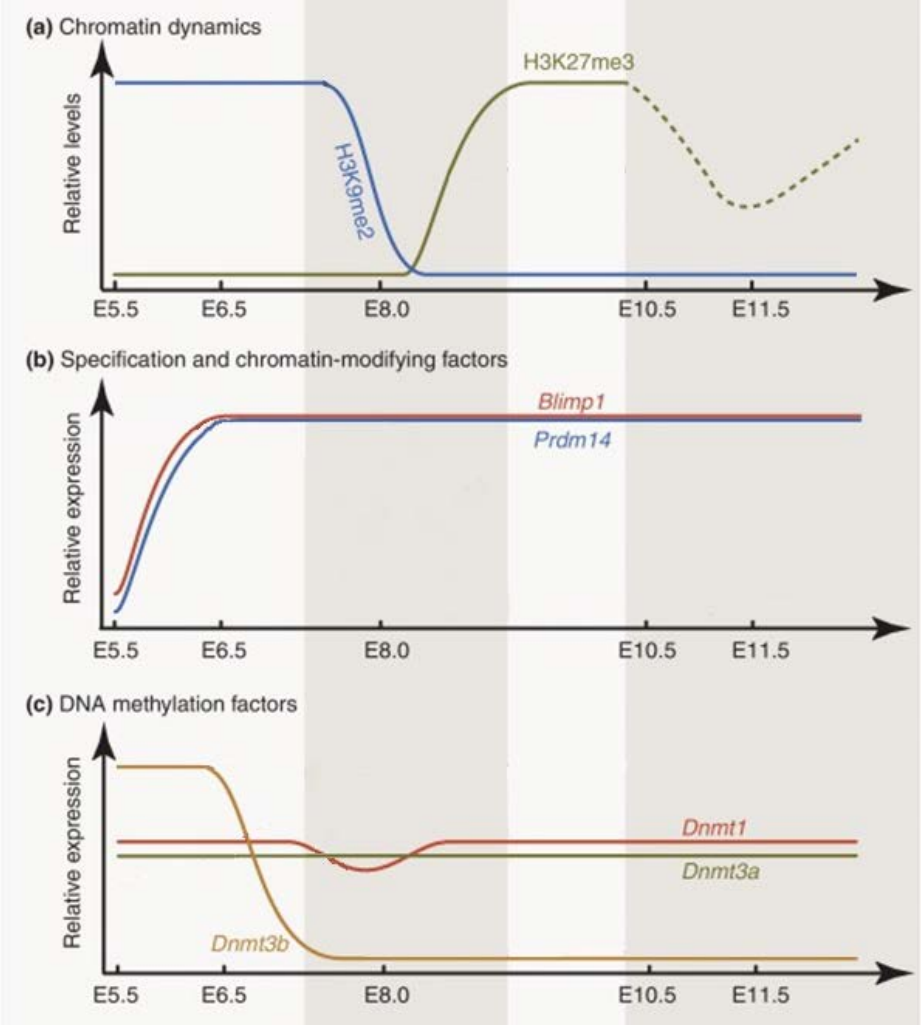


Figure 5. Chromatin and transcriptional changes in primordial germ cells (PGCs).

Following specification, PGCs transit through two sequential phases of reprogramming during migration and subsequent to entry into the genital ridge. (a) Epigenomic reorganisation in PGCs. PGCs exhibit global erasure of histone H3 lysine 9 dimethylation (H3K9me2), upregulation of H3 lysine 27 trimethylation (H3K27me3) and some loss of the DNA methylation signal. After entry into the genital ridge there is a further dramatic loss of DNA methylation, which includes erasure of imprints, correlating with a transient reorganisation of H3K27me3. (b–d) Transcriptional changes during PGC development (b) Specification and chromatin-modifying factors. Upregulation of B-lymphocyte-induced maturation protein 1 (Blimp1) and PR domain containing 14 (Prdm14) is necessary for specification of PGC fate. (c) DNA methylation proteins. Downregulation of DNA (cytosine-5)-methyltransferase 3b (Dnmt3b) may account for DNA demethylation during migration. From: (Hackett et al., 2012). Reproduced under license #3107731256603.

Mouse models of TGCTs

The 129 family of mice are the only inbred strains that develop spontaneous germ cell tumours at an appreciable rate, from 3-10% (Stevens and Hummel, 1957). The precursor lesion in mice is the cluster of embryonal carcinoma cells that are histologically evident at approximately E15.5 (Matin et al., 1999; Noguchi and Noguchi, 1985; Stevens, 1967a). The EC cell cluster may be the mouse equivalent of the human precursor lesion, IGCNU (see Intratubular germ cell neoplasia, unclassified, p.22). This lesion is present in approximately 18% of 129 strain male mice (Rivers and Hamilton, 1986) but only goes on to form TGCTs in approximately 3-10% of mice (Stevens and Hummel, 1957). The difference between the EC cell cluster incidence and published TGCT rates suggest that some clusters fail to form TGCTs. TGCT rates in our 129/SvImJ colony are more closely in line with the EC cluster rates (see Figure 4, page 75). Additionally, several genetic modifiers affect the baseline TGCT susceptibility in 129 strain mice such as *Dnd1*^{Ter}, *Kitlg* and *Eif2s2* (Table 4, p.76); many of these have been found to be relevant in human disease. An in depth examination of many of these models is found in Carouge & Nadeau, 2012.

M19/M19

The 129-Chr 19MOLF (M19) mouse strain is the result of a complete substitution of chromosome 19 from the MOLF strain onto the 129 strain background (Matin et al., 1999). The M19 strain exhibits a very high incidence of TGCTs; up to 85%

of males have TGCTs, most of which are bilateral. The MOLF mouse strain does not develop TGCTs, indicating that the susceptibility loci on MOLF chromosome 19 are germane only in the context of a 129 strain background. Several genes on this chromosome are relevant to human disease: *Dmrt1* which regulates germ cell proliferation and pluripotency (Kratz et al., 2011b; Turnbull et al., 2010), *Pten*, an important component of the KIT/KITL pathway (Kimura et al., 2003; Goddard et al., 2007), *Kit* and *Kitlg*, essential to PGC motility and migration (Vliagoftis et al., 1997), and *Sf1*, an mRNA splicing factor (Zhu et al., 2010).

Kit and Kitlg

KIT and its ligand, KITLG are frequently found to be mutated in both human disease and mouse TGCT models (Heaney et al., 2008; Vliagoftis et al., 1997). Together, KIT and KITLG are essential to PGC survival, migration, proliferation and signaling pathway activation. KIT is expressed on the surface of PGCs during cell migration and differentiation; it also initiates the signaling cascade that leads to mitotic arrest in male germ cells (Bokemeyer et al., 1996; Huang et al., 1992; Strohmeyer et al., 1995). KITLG is expressed by Sertoli cells and their precursors in two forms: soluble and membrane bound. The membrane bound KITLG is expressed primarily during migration and proliferation whereas the soluble form is expressed during quiescent periods (Zsebo et al., 1990; Huang et al., 1992).

In mice, several mutations lead to a loss of KIT, including Kit^{W} and Kit^{Wv} ; they result in high PGC apoptotic rates, beginning at approximately E9, but no changes in TGCT susceptibility (Nocka et al., 1990). Conversely, loss of the KITLG, in either the $Kitlg^{Sl}$, $Kitlg^{SlJ}$ or $Kitlg^{Slgb}$ heterozygous mutants, increases TGCT occurrence by two-fold (Heaney et al., 2008; Stevens, 1967c). In addition, it has been determined that loss of the membrane bound KITLG, but not loss of the soluble KITLG, is sufficient to increase TGCT susceptibility (Heaney et al., 2008).

Pten

PTEN is a phosphatase that acts as a tumour suppressor by regulating signaling cascade activity which forms a component of the KIT/KITLG pathway, and regulates its own expression through TRP53 stability (Tang and Eng, 2006). PTEN plays a role in apoptosis, pluripotency, DNA repair and cell cycle arrest (Kimura et al., 2003; He et al., 2011; Shi et al., 2012). Expressed in PGCs, PTEN null heterozygotes have high TGCT tumour rates (Di Cristofano and Pandolfi, 2000; Di Cristofano et al., 2001), defects in PGC mitotic arrest, and are slow to lose pluripotency (Kimura et al., 2003). Interestingly, *Pten* is the only genetic mutation that increases TGCT rates to 100% on a mixed genetic background (Kimura et al., 2003).

Trp53

TRP53 is a tumour suppressor that regulates the expression of mitotic regulators such as PTEN, the pluripotency factor NANOG, and several key apoptotic proteins (Chen et al., 2012; McCarthy, 2012; McCarthy, 2013). In mice complete loss of TRP53 is developmentally viable, but the propensity to developing spontaneous tumours significantly decrease their lifespan (Donehower and Lozano, 2009; Donehower et al., 1992). On an intact 129 inbred background TGCT rates in *Trp53* null males increase from the baseline 3-10% to 30-50% (Donehower et al., 2006; Rotter et al., 1993), and even on a 129:C57BL/6J mixed background 20% of males are affected by TGCTs, significantly higher than background rates (Jacks et al., 1994).

Dmrt1

DMRT1 is a male-specific transcription factor (Raymond et al., 1999) that regulates the expression of pluripotency factors such as NANOG and SOX2 and several cell cycle regulators (Krentz et al., 2009; Murphy et al., 2010). In undifferentiated spermatogonia, DMRT1 controls the mitotic:meiotic switch (Matson et al., 2010). In the 129 strain, loss of DMRT1 increases by ten-fold the number of PGCs that escape mitotic arrest, which then regain pluripotency and go on to form EC foci (Raymond et al., 1999; Krentz et al., 2009). Complete loss of DMRT1 in the 129 strain leads to infertility in males and 90% of males are affect by TGCTs (Krentz et al., 2009).

Eif2s2

The only genetic modifier known to reduce TGCT susceptibility in 129 mice, *Eif2s2* is one of three genes that are found at the Agouti-yellow (A^y) locus. Until recently *Raly* was believed to be responsible for this decrease in TGCT susceptibility (Heaney et al., 2009). The protective effect has been ascribed to a reduction in proliferating PGCs after E16.5 and a delay in adult germ cell maturation.

Dnd1^{Ter}

Dnd1^{Ter} is one of the most potent TGCT modifier mutations, increasing susceptibility in 129/Sv male mice from the baseline rate of 3-10% in wild-type 129/Sv to 94% in *Dnd1*$^{Ter/Ter}$ homozygotes (Stevens, 1973). Although the ability of the *Ter* mutation to induce germ cell tumours depends on genetic background, both male and female mice homozygous for *Dnd1*^{Ter} show severe germ cell deficiency and are sterile regardless of genetic background. Development, proliferation and migration of homozygous *Dnd1*^{Ter} PGCs is normal until E8.5, when these PGCs cease proliferating (Sakurai et al., 1995). As a result, only 10-20 PGCs are present in the genital ridge of male *Dnd1*$^{Ter/Ter}$ homozygotes at E11.0. At this time point, *Dnd1* expression is normally detectable in the genital ridge and further increases in males between E12.5 and E14.5 in males (Youngren et al., 2005). Despite the very low numbers of PGCs present in *Dnd1*^{Ter}

homozygotes, 94% of these mice will develop at least one TGCT (Noguchi and Stevens, 1982).

The potency of *Dnd1*^{Ter} as a TGCT modifier in mice suggests that DND1 plays an important role in PGCs and that alterations to DND1 may be in part responsible for the transformation of PGCs. In this case, it is necessary to understand the normal biological functions of DND1.

DND1

Introduction

The information regarding normal DND1 function is varied, and comes from a variety of animal models, little is known about the function of DND1 in humans. Based on the evidence, DND1 consistently plays two roles: it is essential for PGC survival and binds to mRNAs. Other functions that have been attributed to DND1 have been described, but it remains unclear if these functions are species specific. Here I shall present an overview of known DND1 functions arranged by species.

D. rerio (zebrafish)

A great deal of knowledge regarding DND1 function has been obtained from work in zebrafish, where the protein was first identified DND1 in zebrafish is 63% identical to mouse and 65% identical to human (Altschul et al., 2005). DND1 has been shown to be involved in restricting miRNA access to mRNA

transcripts, regulating protein levels in a number of mRNAs (Kedde et al., 2007). Some of this work involved zebrafish DND1, although the results were repeated in human cell lines and using human DND1. The consistency of the results indicate that although zebrafish DND1 does not share complete homology with either human or mouse DND1, the results obtained in zebrafish models may be applicable to mouse and human.

In vivo downregulation of DND1 in zebrafish led to a loss of PGCs, but not tumour formation (Weidinger et al., 2003). This suggests that the absence of DND1 in zebrafish does not lead to TGCTs, although the species is capable of developing testicular tumours (Neumann et al., 2011a; Neumann et al., 2011b; Basten et al., 2013). In zebrafish, the function of DND1 in PGC survival may be related to the ATPase activity found in the C-terminal portion of the protein (Liu and Collodi, 2010). Although this ATPase domain is conserved in fish, it only shares 34% homology with mice or humans and no ATPase function has been described in either of these species.

X. laevis (Xenopus)

In Xenopus, DND1 share only 47% homology with mouse and 50% homology with human DND1 protein. The function of DND1 in this amphibians has recently been shown to include axis determination through association with Trim36 leading to the polymerization of microtubules (Mei et al., 2013). This function occurs even before the first cleavage of the Xenopus embryo; loss of

DND1 results in ventralization and improper axis formation. This paper also identified TRIM36 as a novel RNA binding partner of DND1 through interaction with its 3'UTR.

Like zebrafish, DND1 has been shown to be necessary for PGC survival. Inhibition of DND1 mRNA translation in embryos results in the loss of PGCs at the tadpole stages of development (Horvay et al., 2006). The function of DND1 in restricting miRNA access to mRNAs is also seen in Xenopus. DND1 functions with ELRB1 to prevent miR-mediated degradation of germ cell specific mRNAs during gastrulation (Koebernick et al., 2010).

M. musculus (mouse)

These is a high degree of homology between mouse and human DND1, with 86% of the protein sharing identity, including the conserved RNA recognition motif (Altschul et al., 2005). Yet, very little is known about the normal function of DND1 in mouse because no true knockout models have been previously described.

On a molecular level, DND1 is known to bind to multiple pluripotency factor mRNAs (Zhu et al., 2011), and APOBEC3 (Bhattacharya et al., 2008). Like zebrafish and xenopus, DND1 (the α-isoform) is required for germ cell survival (Bhattacharya et al., 2007).

A mouse model has also elucidated the possible role of DND1 in the transformation of keratinocytes through the decreased expression of GRHL3, increased expression of miR-21 and decreased DND1 expression (Bhandari et al., 2012).

Based on sequence comparison, DND1 was originally thought to be a part of the RNA editing machinery, similar to Apobec complementation factor (A1CF), although the data to support this hypothesis is limited (see Appendix B , p.157).

H. sapiens (human)

Most work done on DND1 in humans is limited to tissue culture work or sequencing studies. The work by Kedde *et al*. was repeated in human cells, showing that DND1 prevents mRNA degradation by inhibiting miRNA access (Kedde et al., 2007).

DND1 has been associated with oral squamous cell carcinoma through the up-regulation of miR-24 which subsequently targets DND1 and affects DND1 target mRNA levels (Liu et al., 2010). This is particularly interesting since up-regulation of miR-24 is associated with tumourigenesis, proliferation and cell survival (Du et al., 2013; Gougelet and Colnot, 2013). However, no associations between DND1 (or mutations of *Dnd1*) and human TGCTs have been found (Linger et al., 2008; Sijmons et al., 2010).

Dnd1^{Ter}

With the exception of the human data correlating miR-24, DND1 and squamous cell carcinoma, there is no evidence to suggest that the loss of DND1 contributes to TGCTs. In zebrafish and xenopus the loss of DND1 leads to PGC loss but not germ cell tumours (Mei et al., 2013; Weidinger et al., 2003). In all other cases, it is a *Ter* mutation of *Dnd1*, in mouse (Noguchi and Noguchi, 1985; Stevens, 1973; Youngren et al., 2005) and in rat (Northrup et al., 2011; Northrup et al., 2012) that leads to GCT formation. This suggests that DND1^{Ter} behaves differently than a loss of DND1.

Summary

The inconsistency regarding DND1, DND1^{Ter} and germ cell tumour formation combined with the limited information about normal DND1 function makes it evident that more work is needed to clarify the role of DND1^{Ter} in tumourigenesis. This can be accomplished by generating a *Dnd1*^{KO} mouse to compare and contrast the effects of DND1^{Ter} and a loss of DND1. This fueled the work presented in Chapter 3, where I have redefined DND1^{Ter} has a pro-oncogenic protein.

Mouse TGCTs as models of human disease

Introduction

The mouse models of TGCTs that I used in my thesis have a number of advantages, but there is controversy regarding its applicability to human disease. In general, mouse TGCTs are most like human immature teratomas and yolk sac tumours (Stevens and Hummel, 1957; Walt et al., 1993; Matsui et al., 1992). This has led to the suggestion that mouse TGCTs represent only the pediatric GCTs (Oosterhuis and Looijenga, 2005; Walt et al., 1993) and not the more prevalent adult onset GCTs.

In this section I will compare and contrast mouse and human TGCTs, with particular regard to epigenetic and genetic differences that are most often used as evidence that mouse TGCTs are inappropriate models of adult TGCTs. In the context of this discussion I shall use the nomenclature proposed by Oosterhuis & Looijenga (Oosterhuis and Looijenga, 2005) where the immature teratomas and yolk sac tumours found in infancy are referred to as Type I GCTs while the adult onset seminomas and non-seminomas (including teratomas) are collectively called Type II GCTs.

A great deal of this debate has centered on the differences between protein expression profiles, genetic alterations and epigenetic signatures that are associated with each TGCT class. When available, similar profiles for mouse TGCTs are also analyzed. In many of these comparisons, mouse TGCTs do

appear to cluster with type I GCTs in humans, suggesting that the mouse model is more appropriate for type I GCTs instead of type II GCTs. It is less obvious from these analyses that these results are only accurate for differentiated tumours and may not reflect variances between the tumour stem cells in each group.

Epigenetic Imprinting

It has been proposed that type I GCTs originate from embryonic germ cells (Schneider et al., 2001; Sievers et al., 2005) which are embryonal carcinoma precursors with a partially erased pattern of genomic imprinting. In contrast, type II GCTs are proposed to originate from IGCNU cells (Woodward et al., 2004; Honecker et al., 2006) where the genomic imprinting has been completely erased. Thus, it is these precursor cells that need to be compared in order to assess the validity of mouse TGCTs as a model for both type I and type II GCTs.

It has been argued that differences in imprinting status of type I and type II GCTs acted as a "time-stamp", reflecting the stage at which a PGC diverged from normal development and initiated a neoplasm. Genomic imprinting of maternal and paternally expressed genes is erased from PGCs during their migration and entry into the fetal gonad. Once germ cells enter into meiosis, sex-specific imprinting is re-established (Schneider et al., 2001).

Most GCT studies use the reciprocally imprinted status of *IGF2 (Insulin growth factor 2)* which is expressed from the paternal allele, and *H19*, which is a maternally transcribed long non-coding RNA. Data seems to suggest that type I

GCTs originated from PGC undergoing meiosis I arrest with various stages of epigenetic erasure whereas most type II GCTs have complete erasure of parental imprints in these loci (Schneider et al., 2001; Bussey et al., 2001). More recent evidence has shown that different classes of GCTs displayed the same *IGF2/H19* methylation patterns (Sievers et al., 2005) or that yolk sac tumours have differentially imprinted *IGF2/H19* loci, but not teratomas (Furukawa et al., 2009). No data is available regarding the methylation status of mouse TGCTs or precursors. Limited data on F9 murine teratocarcinoma cell line shows that the *H19* locus is heavily methylated, but is demethylated upon subcutaneous injection into a host animal in a manner that is consistent with embryological development (Yeivin et al., 1996). Thus, while epigenetic differences may exist between type I and type II GCTs, they do not conclusively demonstrate that these tumour types originate from different precursor cells.

Adding ambiguity to the epigenetic evidence of *IGF2/H19* methylation in type I and type II GCTs is a recent paper that demonstrated that Oct4 and Sox2 together bind to specific motifs around *IGF2/H19* sequences to induce demethylation (Hori et al., 2012). Seminomas, in which these methylation marks are completely erased, are strongly positive for Oct4, but negative for Sox2; embryonal carcinomas are strongly positive for both Oct4 and Sox2 and are also hypomethylated (Santagata et al., 2007; Looijenga et al., 1998). In contrast, yolk sac tumours are negative for both Oct4 and Sox2, and retain various states of

methylation in the IGF2/H19 locus (Bussey et al., 2001; Schneider et al., 2001; Sievers et al., 2005). EC cell clusters in mouse TGCTs also express Oct4 (Heaney et al., 2012; Zechel et al., 2011) and Sox2 (Cook et al., 2011b). These results suggest that partial loss of imprinting may be due to the coordinated action of Oct4 and Sox2 instead of representing the epigenetic status of the cancer stem cell in any of the GCT types or in mouse model TGCTs.

Chromosomal differences

There are genetic differences between type I and type II GCTs. In general, type I teratomas are generally diploid (Cunningham et al., 2012; Amps et al., 2011; Mostert et al., 2000), whereas yolk sac tumours have frequent chromosomal alterations, including some frequent losses and gains (Oosterhuis and Looijenga, 2005; Schneider et al., 2001; Mostert et al., 2000). Similarly, type II GCTs show numerous chromosomal gains and losses, the most consistent of which is found on chromosome 12p (Atkin and Baker, 1982; Suijkerbuijk et al., 1993; Rodriguez et al., 1993). While the differences in chromosomal alterations between type I and type II GCTs are consistent, there is no evidence that these alterations are the cause of germ cell tumour divergence instead of being the result of tumour differentiation.

It is more difficult to compare chromosomal compositions between human type I, type II and mouse TGCTs. Grossly, mouse TGCTs are diploid, but little work has been done to date to examine mouse TGCTs using comparative genomic

hybridization or sequencing. In a limited study, copy number variations have been demonstrated in mouse GCTs (Zechel et al., 2011). Interestingly, analyses of mouse EC cell lines has also demonstrated the gain of a region syntenic to human 12p (specifically 12p13) (Blelloch et al., 2004), which is seen in type II GCTs. Because type I GCTs and mouse TGCTs are histologically the most alike, it should not be surprising that genetic alterations are also similar between the two cancer types. Until these chromosomal alterations are shown to be causal, these similarities may only reflect a shared differentiated cell state and not a common cell of origin.

Summary

Given the differences and similarities between type I and type II human GCTs, as well as mouse TGCTs, it is tempting to classify them into different categories, perhaps even to assert that they have a different cell of origin. In general, all GCTs originate from a PGC or gonocyte precursor cell (Stevens, 1967a; Skakkebæk et al., 2008; Jørgensen et al., 1995), and in each case, the neoplasm originates in utero (Skakkebaek et al., 2008; Jørgensen et al., 1995; Rajpert-De Meyts et al., 2003).

Although there is ample data regarding the outcome of GCT differentiation, with regards to epigenetic, genetic and protein expression profiles; very little is known about the transition between the precursor state of the originating cell and growth of the ensuing tumour. In human GCTs, the differences between type I and type

II may be related to the timing of this transition and not fundamental differences between the two classes.

The importance of timing is due to alterations in the testicular environment during growth and maturation. Precursor cells that initiate neoplastic growth in a pre-pubescent testis, similar to the early growth seen in mice, may develop teratomas or yolk sac tumours in response to that particular micro-environment of the testis. The seminomas and non-seminomas seen in type II GCTs develop in a completely different micro-environment from type I GCTs since adult testes are exposed to hormones and proteins that support spermatogenesis. It is tempting to speculate that alterations in the gonadal micro-environment play a large role in TGCT growth initiation, since many of the physical risk factors, including cryptorchidism (Thong et al., 1997; Berkowitz et al., 1993; John Radcliffe Hospital Cryptorchidism Study Group, 1992), microlithiasis (Backus et al., 1994; Miller et al., 1998), atrophy (Harland et al., 1998), and gonadal dysgenesis (Verp and Simpson, 1987) would affect the testicular environment.

The similarities between type I GCTs and mouse TGCTs may reflect a shared timing of tumour growth initiation. The lack of type II GCTs in mice may be due to the absence of an activating event for TGCT growth in mice later in life. This would be particularly true if the activating event was environmental or physical in nature since mice rarely display any of the physical risk factors associated with human TGCTs, and are housed and fed under strict conditions. The lack of type

II GCTs in the 129 families of inbred mice may explain why in genetically identical animals only 5-15% develop TGCTs.

Despite the fact that mouse TGCTs are not completely identical to type II GCTs, evidence exists to suggest that they may share a common precursor cell, and that the observed differences between type I, type II and mouse GCTs are the products of tumour differentiation and not the causal factors. More work using existing mouse models is required to determine the complete genetic, epigenetic and expression profiles of the germ cell origin of mouse TGCTs.

Table 4. Genetic mutations that alter TGCT rates in 129/SvImJ male mice.

Mutations may alter primordial germ cell (PGC) apoptosis, affect mitotic arrest, alter PGC cell numbers at birth (P1)and ultimately reduce fertility. Adapted from Carouge and Nadeau, 2012.

	Affected males	PGC Apoptosis	G1 Mitotic Arrest Defect	P1 germ cells	Fertile
129 strain males	5-10%		✗		✓
***Dnd1*$^{Ter/Ter}$**	94%	↑↑	✓	↓↓↓	✗
M19/M19	85%	↑	✓	↓	✓
***Kitlg*$^{Sl\ -/+}$** *(no KITLG)*	15%	?	?	?	✓
***Kitlg*$^{Sld\ -/-}$** *(no membrane-bound KITLG)*	5%	★ *Proliferation defect*	✗	↓	✗
***Kitlg*$^{Sl17H\ -/-}$** *(no functional membrane-bound KITLG)*	?%		✗		✗
***Pten*$^{-/-}$** *(PGC-specific)*	100%	↑↑	✓	↓↓	✗
***Trp53*$^{-/-}$**	35%		✗		✓
***Dmrt1*$^{-/-}$**	90%		✓	↓↓	✗
***Eif2s2*$^{-/+}$** *(in 129-M19 strain) (compared with M19/M19)*	36%		★ *Rescue*		✓

Chapter 2: Spontaneous Metastasis in Mouse Models of Testicular Cancer

The results from this chapter have been published:

Jennifer L. Zechel, Gregory T. MacLennan, Jason D. Heaney, Joseph H. Nadeau¨ ***Spontaneous metastasis in mouse models of testicular germ cell tumours***. International Journal of Andrology. 2011 Aug;34(4 Pt 2):e278-87.

Abstract

Testicular germ cell tumours (TGCTs) are the most common cancer in young men, the incidence is increasing worldwide, and they show an unusually high rate of metastasis. Despite significant work on TGCTs and their metastases in humans, absence of a mouse model showing spontaneous metastasis has greatly limited our understanding of the mechanisms by which metastatic potential is acquired or their modes of dissemination. We report a new model of spontaneous TGCT metastasis in the 129 family of mice and provide evidence that these are true metastases derived directly from primary testicular cancers rather than independently from ectopic stem cells. These putative metastases (pMETs) occur at similar frequencies among TGCT-affected males among the six genetically distinct strains that were tested, and were largely found in anatomical sites that are consistent with patterns of TGCT metastasis in humans. Various evidence supports their pluripotency and germ cell origin, including presence of multiple endodermal and ectodermal derivatives as well as cells showing OCT4 and SSEA-1 pluripotency markers. In addition, pMETs were never found in males that did not have a TGCT, suggesting that metastases are derived from primary tumours. Finally, shared DNA copy number variants strongly suggest that pMETs are derived from primary TGCTs. Together these results provide the first evidence for spontaneous TGCT metastasis in mice and show that these metastases originate from primary TGCTs rather than independently from ectopic stem cells.

Introduction

Testicular germ cell tumours (TGCTs) are the most common cancer affecting young men between the ages of 15 and 40. Although representing only 1.0-1.5 % of all cancers (Akbarian et al., 1995), incidence has increased steadily in the last 30 years (Bosl and Motzer, 1997) and currently TGCTs account for nearly 60% of all cancers in men aged 20-40 (DiPietro et al., 2005). Although the cure rate is high, presence of metastases reduces survival rates. Interestingly, TGCTs have a disproportionately high number of metastases compared to other tumours, averaging 5.8 metastases per primary tumour (De Giorgi et al., 2008; Anant and Davidson, 2001). Approximately 30% of TGCT patients have metastases at the time of diagnosis (Powles et al., 2005), and 15-20% of patients have subclinical metastases in stage 1 seminoma (Benne et al., 1986), which is the most common presentation (Rodriguez et al., 1992).

Metastasis is an important factor in TGCTs, directly affecting treatment modality, tumour surveillance, and survival. Treatment involves surgery and chemotherapy with BEP (bleomycin, etoposide and cisplatin), which together carries a mortality risk of 2.3-4.5% (Fossa et al., 1998; Williams et al., 1987). Resistance to cisplatin occurs in 20% of patients with metastatic disease, further complicating treatment and worsening prognosis (Piulats et al., 2009). After treatment, patients presenting with metastatic disease have a higher incidence of relapse (Holzik et al., 2008) and lower survival rate (Anant and Davidson, 2001).

Testicular cancer metastases target various tissues, including lymph nodes, lung, liver and spleen (De Giorgi et al., 2008). Differentiation of TGCT metastases leads to morphological diversity, which makes it difficult to distinguish between metastases of primary germ cell tumours and unrelated secondary cancers (Ulbright, 1999). The OCT4 pluripotency marker is present in 100% of tested primary and metastatic seminomas and embryonal carcinomas (Cheng, 2004; Jones et al., 2004; Looijenga et al., 2003a) and has become an important diagnostic marker for both primary germ cell tumours and their metastases (Cheng et al., 2007). OCT4 is a transcription factor associated with the maintenance of pluripotency and is expressed in embryonic cells, germ cells and embryonal carcinoma (EC) cells (Okamoto et al., 1990; Rosner et al., 1990). OCT4 expression is dramatically reduced as both primary tumours and their metastases differentiate (Niwa et al., 2000) and may be absent in cells that possess an EC phenotype (Mueller et al., 2010).

Most mouse models of spontaneous TGCTs are limited to a single genetic background, the 129 family of inbred strains (Stevens and Hummel, 1957), with 5-10% of males affected with a TGCT by 3-4 weeks of age (Maris et al., 2005). Several mutations modify this frequency (DiPietro et al., 2005; Heaney and Nadeau, 2008). For example, the *Ter* mutation in the Dead-end1 gene increases the incidence of TGCTs to over 94% in *Ter/Ter* homozygotes (Clark et al., 2004; Matin et al., 1999). In addition, the 129.MOLF-Chr19 chromosome substitution

strain, which has chromosome 19 from the MOLF/Ei substituted onto the 129/Sv background, has 82-86% of its males affected with a TGCT (Conticello, 2008).

Developmental features of TGCT stem cells have led to alternative hypotheses about whether extra-gonadal germ cell tumours are true metastases of TGCTs in the testis, or whether they originate independently from ectopic stem cells (Fabre et al., 2004; Fine et al., 1962; Hailemariam et al., 1997; Johnson et al., 1973). Primordial germ cells (PGCs), which are the stem cell of many TGCTs, arise early in development; migrate from the base of the allantois to the urogenital ridge where fetal gonads subsequently develop (Molyneaux et al., 2001; Upadhyay and Zamboni, 1982). In the 129 inbred strains, PGCs in the fetal gonad transform to EC cells and during the next several weeks develop into TGCTs (Stevens, 1962a; Stevens, 1967c). In some cases, PGCs migrate to ectopic locations instead of the urogenital ridge (Runyan et al., 2006; Anderson et al., 2000; Upadhyay and Zamboni, 1982; MacLean et al., 2007; Zamboni and Upadhyay, 1983) and these cells have been proposed to be the origin for extra-gonadal germ cell tumours (Dixon and Moore, 1953; Fine et al., 1962; Hailemariam et al., 1997). Absence of metastasis in these mouse models has until now precluded tests of these alternative hypotheses about the origins of metastasis and extra-gonadal germ cell tumours.

During routine necropsies, we discovered putative metastases (pMETs) in several TGCT susceptible mouse strains. We therefore undertook a systematic study to

estimate the rate and anatomical distribution of pMETs in these strains, to test whether they have the same germ cell origin as TGCTs (Stevens, 1962b; Stevens, 1967c) and if they were true metastases from a primary TGCT in the testis, or alternatively from germ cells that migrated to ectopic locations where they then transformed to extra-gonadal germ cell tumours.

Materials and Methods

Mice:

129S1/SvImJ (JR002448, previously known as 129/SvImJ) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). 129T1/Sv (previously known as 129T1/Sv-p^{+} $Tyr^{c\text{-}ch}$ $Dnd1^{Ter}$/J) and 129-Chr 19^{MOLF} CSS (CSS-M19, described previously (Conticello, 2008; Matin et al., 1999) were obtained from our research colony. Mice were maintained in the Case Western Reserve University Animal Resource Center on a 12:12-h light:dark cycle and fed Lab Diet 5010. All protocols were approved by the Institutional Animal Care and Use Committee.

Genotyping:

DNA for PCR genotyping was extracted from tail tissue. The nucleotide substitution in the *Ter* mutation results in the creation of a *Dde1* site that was used for genotyping (Matsumoto et al., 2006).

Tumour Surveys:

Male mice between the ages of three and fifteen months were surveyed for TGCTs and pMETs. pMETs were identified macroscopically and confirmed histologically. Tumour incidence was calculated as the percentage of males with either a unilateral or bilateral TGCT; metastasis incidence was calculated as the percentage of TGCT affected males with at least one metastasis.

Tissue Processing:

Testes and macroscopic pMETs were fixed with 10% formalin for at least 48h. Tissues were then paraffin embedded and sectioned (5µm) at the Case Comprehensive Cancer Center's Tissue Procurement and Histology Core facility (TPHC). Hematoxylin and eosin staining was done in the TPHC facility.

Immunohistochemistry:

Paraffin sections (5µm) were rehydrated and blocked with dilution buffer (1X PBS, 3% BSA, 0.1% NaN_3, 0.3% Triton X-100), unconjugated goat anti-mouse IgM diluted 1:1000 and 5% normal goat serum for 1 hour at room temperature, then rinsed in PBS. Sections were incubated with rabbit anti-OCT4 (ab19857, Abcam) antibody diluted 1:250 and mouse IgM anti-SSEA1 (ab16285, Abcam) diluted 1:50 in dilution buffer with 5% normal goat serum overnight at 4°C. Sections were washed in PBS, and incubated with DyLight™488 anti-mouse IgM and DyLight™594 anti-rabbit IgG (115-486-020 and 711-516-152 respectively, Jackson Immunoresearch) both 1:500 in dilution buffer with 5% normal goat

serum for 2 hours at room temperature. Nuclei were counterstained with DAPI and sections were visualized with a Leica TCS SP2 AOBS filter-free UV/spectral confocal microscope.

Array-based CGH:

DNA was isolated from normal spleen, primary TGCTs and pMETs with Qiagen DNeasy Blood and Tissue kits (Valencia, CA). Samples were sent to Empire Genomics (Buffalo, NY) where hybridizations were done with the Agilent 244k mouse aCGH array, and data analysis was performed in part by Empire Genomics and in part by the authors.

Real-Time PCR:

pMETs were selected for validation with real-time PCR based on the number of DNA copy number changes identified within the pMET and the corresponding primary TGCT. Genes used for DNA copy number validation were chosen on the basis of their central location within the altered genomic segment. Primer sequences are given in Table 5 (p.86). Copy number changes were quantified with the Chromo4 real-time PCR system (MJ Research) and the PerfeCTa SYBR Green Supermix kit (VWR Scientific) using manufacturer-suggested protocols. Copy numbers were normalized to the POMC gene (Hill-Baskin et al., 2009); normal spleen DNA was used to generate standard curves for each primer set. Cut off values of 1.5 and below were used to define a copy number value of 1,

and values of 2.5 and above were used to define copy number values of 3 and above. Changes in genomic DNA were considered significant when the copy number (n) was below 1.5 or above 2.5 (Yang et al., 2010; Braude et al., 2006).

Statistical analysis: Chi-square contingency tests were used to test for significant differences in TGCT and metastatic rates between strains. Spearman's correlation test was used to estimate the correlation between the incidence of primary TGCTs and pMETs among strains.

Results

Frequency of males affected with a primary TGCT

We examined a total of 1,045 males, ranging in age from 3 to 15 months, from six genetically distinct members of the 129 family of inbred strains. These six groups included the 129S1/SvImJ inbred strain, *Dnd1*$^{Ter/+}$ heterozygotes on two backgrounds (129S1 and 129T1), *Dnd1*$^{Ter/Ter}$ homozygotes on the 129T1 background, *Kitl*$^{Sl\text{-}J/+}$ heterozygotes on the 129S1 background, and the 129-Chr19^{MOLF} Chromosome Substitution Strain (CSS). As expected, the frequency of affected males varied widely (Figure 5A), with rates that were consistent with previous reports (Lam et al., 2004; Matin et al., 1999; Noguchi and Stevens, 1982; Stevens and Hummel, 1957; Youngren et al., 2005).

Table 5. Gene symbols, names and primers used for RT-PCR validation of aCGH results

Gene symbol	Gene name	Forward Primer	Reverse Primer
Map2k2	mitogen-activated protein kinase kinase 2	GTGGATCTGGGTCATGGAAC	ATTCAGATTGTGGGCAGGAG
Olfr77	olfactory receptor 77	GCCCATTCATCCAAGAGAAA	GCCCCCTTTACATCCTTGTT
Riken	D230025D16Rik	TATGGTTCCTGGGCCTACAG	ACGAGGATGGGAAGAGTCCT
Igf1r	insulin-like growth factor I receptor	TGAAGAAGGGGAAAGCAGAA	CTTGACCCACAACCTGACCT
Tmem48	transmembrane protein 48	TGGCTCCAGCGTAGTGAAC	ACAGCCTCGGTCCTCTTCTT
Xiap	X-linked inhibitor of apoptosis	GGAACCCTGCCATGTGTAGT	TGATCATCAGCCCCTGTGTA
Nudt3	nudix (nucleotide diphosphate linked moiety X)-type motif 3	CGTGTAGCGGACACACAGAC	CTCGGAGTCCAGGGAAGG
Adam6b	a disintegrin and metallopeptidase domain 6B	CCTGTCAGGGGTCATGAAGT	TCATAGGCCCTGTGTCATCA
Ywhag	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	CGTGAGCTTGGTTCCTTCTC	ACAACACCAGCATGCACAAT
Pou2f2	POU domain, class 2, transcription factor 2	CGCCTCTCTTCTTGAACCAC	GAGGAGAGGCCAGGAGACTT
Slc34a2	solute carrier family 34 (sodium phosphate), member 2	CTCTGTGTTCGTGCTCTTGC	CAGAATGTCTGGGGCATCTT
Skint3	selection and upkeep of intraepithelial T cells 3	CAAATGGAGATTCGCTGGTT	GAGTTCTGTCCGCTCCACAT

Frequency of males with a pMET

We found pMETs in each of the six strains surveyed; with the frequency of pMETs in TGCT-affected males ranging from 4.5% in the 129/S1 strain to 11.6% in the CSSM19 strain (Figure 5B). Occurrence of pMETs did not vary significantly among these strains (X^2=2.7, p=0.7), and there was no correlation between the primary TGCT rate and the metastatic rate (Spearman's r=0.7, p=0.23), suggesting that the probability of a pMET is a function of the frequency of primary TGCTs in each strain and did not depend on the particular genetic attributes of any of the six strains.

Do TGCTs and pMETs arise independently?

An important question concerns whether primary TGCTs and pMETs have independent origins. In particular, if pMETs are derived from primary TGCTs, which would be the case if they are metastases, then pMETs should be found only in males that also have a TGCT. By contrast, independent occurrence would be consistent with separate origins. We tested the hypothesis of independent origins by examining occurrence (numbers) of males with a TGCT or a pMET. Interestingly, pMETs were found only in males that also have a TGCT (X^2 = 61.6, p<0.0001), suggesting that pMETs are derived from the primary tumour (Table 6, p.89).

Distribution of metastases

We categorized pMETs (n=63) from our surveys according to secondary tissue sites (Figure 6C, p.93). Regional lymph nodes (renal, inguinal and lumbar lymph nodes) accounted for 38.1% of the sites. Other sub-diaphragmatic sites were peritoneal masses (14.3%), pancreas (12.7%), liver (7.9%) and spleen (6.3%). Super-diaphragmatic metastases in distal lymph nodes (cervical, mediastinal and axillary/brachial) accounted for 17.5% and lung 3.2% of all metastases. Interestingly, pMETs were not found in adrenal glands or kidneys, which are common sites for metastasis in humans. The wide anatomical distribution of TGCT metastases in our survey is consistent with data for humans (Disibio and French, 2008), suggesting that the source cells for mouse and human TGCT metastases have common biological origins and properties.

Histological composition

To determine whether these pMETs contained cell and tissue types derived from multiple germ layers, which is characteristic of TGCTs and their metastases, we examined their composition histologically. Consistent with the hypothesis that these pMETs originate from germ cells, many contained cells and tissues from multiple germ layers. For example, glandular structures were found in a mediastinal lymph node pMET (Figure 7A and B, p.96) mixed with less differentiated cells and lined with malignant cells. A pMET found in the lung showed both epithelioid and papillary cells (Figure 6C). Although these papillary

Table 6. Chi-square analysis of the co-occurrence of TGCTs and pMETs.

Number of males with or without a primary TGCT and pMET are noted. pMETs were found only in males that also have a TGCT ($X2 = 61.6$, $p<0.0001$).

	No pMET	pMET present	Total
Primary TGCT present	477	56	533
No primary TGCT	556	0	556
Total	1033	56	1089

structures can arise from either ectodermal or endodermal cells, the structures in this pMET suggest an endodermal origin. Epithelioid cells and undifferentiated malignant cells are also found in the pMET from a diaphragmatic nodule (Figure 6D). Other pMETs display tissues consistent with a mesodermal origin (bone and cartilage, Figure 8, p.97). Overall, the composition and histological phenotype of these pMETs are consistent with a pluripotent germ cell origin.

Immunohistochemistry

To test more rigorously the germ cell origin hypothesis for pMETs, immunohistochemistry was used to test for OCT4 expression. In humans, OCT4 positive cells in metastases are diagnostic for germ cell tumours that exhibit pluripotency (Cheng, 2004; Looijenga et al., 2003a; Cheng et al., 2007). As a secondary marker for pluripotent cells, we used SSEA-1, which is a specific marker for EC cells, embryonic stem (ES) cells, and PGCs in the mouse (Fenderson et al., 1987). We evaluated pMETs from three different target tissues (lymph node, peritoneum and pancreas) for the presence of OCT4 and SSEA-1 (Figure 9, p.99). In keeping with its role as a transcription factor, OCT4 is normally found in nuclei (Rosner et al., 1990), whereas antibodies to SSEA-1 react with a lactoseries oligosaccharide antigen on the cell surface (Solter and Knowles, 1978). In this analysis, a small number of cells in a pMET from a diaphragmatic nodule and were positive for nuclear OCT4 staining in the absence of SSEA-1 (Figure 8f. and h, asterisks), and pMETs from a mediastinal lymph

node and the diaphragmatic nodule showed discrete cells that stained positively for SSEA-1 (Figure 8c and g respectively, arrows) without OCT4 (Figure 8d and h respectively, arrows), and a minority of cells from a pancreatic pMET stained positively for both (Figure 8l, arrow). Normal lymph nodes were negative for both OCT4 and SSEA-1 staining (data not shown).

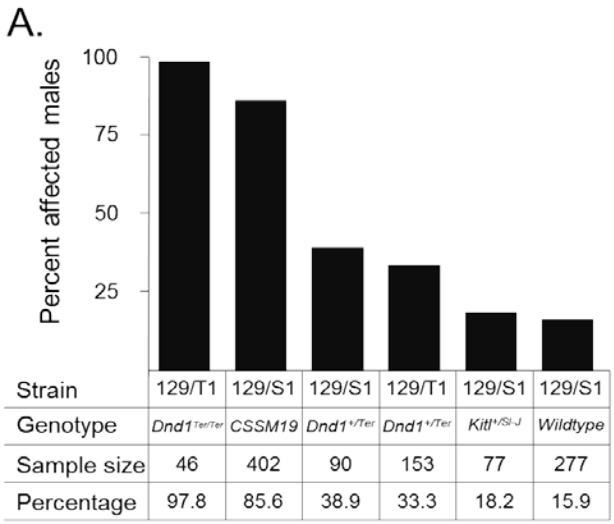
DNA copy number changes in metastases and primary TGCTs

Finally, we sought genetic evidence that pMETs are derived from TGCTs. This evidence was based on tests for shared genomic alterations between pairs of pMETs and primary TGCTs from individual mice. We expected that pMETs that have a primary TGCT origin should share specific genomic alterations with primary tumour cells in testes from the same mouse. Array-CGH (comparative genomic hybridization) was used to identify DNA copy number changes in metastases, with genomic DNA sequence from the same strain as the wild-type reference. We then used real-time PCR to verify these changes in pMETs as well as test for the same changes in the corresponding primary TGCTs. Although numerous small gains and losses were found in all pMETs compared to normal mouse DNA, no DNA copy number changes were common to all metastases (Table 7, p.94). Whenever possible, we examined pMETs from males affected by bilateral TGCTs, because DNA copy number changes in metastases originating from a primary TGCT should match changes found in one, but not both of the primary tumours We found consistent evidence with derivation of pMETs from

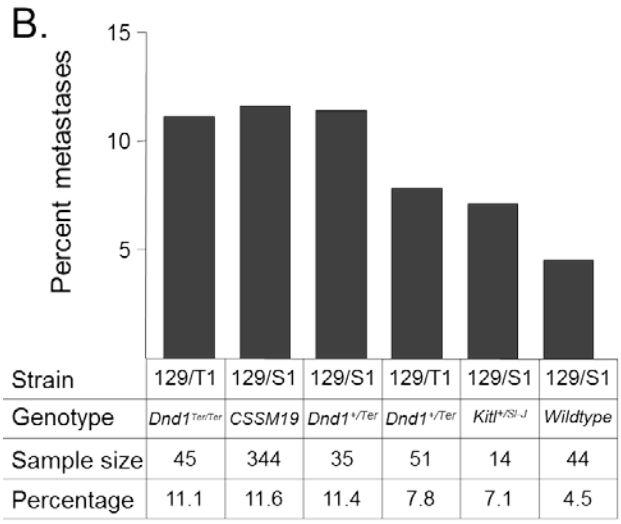
primary TGCTs (Table 7). For example, the peritoneal mass in the CSS-M19 mouse shared 3 of 5 DNA deletions (Chrs. 4, 7 and 8), with the right primary TGCT, all of which were deletions (copy number <1.5), which is consistent with derivation of the pMET from the right primary TGCT. The remaining two DNA copy losses, on Chrs. 9 and 10, were found in the pMET but not in either the right or the left primary TGCT from the same mouse. With the exception of those changes identified in the pMET and the right primary TGCT, the probed genes did not show other detectable copy number variation.

The changes identified in Sample 2 that passed our cut-off threshold were all duplications (copy number >2.5). These DNA changes were most closely shared with the changes found in the left primary TGCT. In particular, the changes on chromosomes 5 and 12 in the pMET were most similar to changes found in the left TGCT because the right TGCT duplications on chromosome 5 were larger, and no copy number changes were found on chromosome 12. The change on chromosome 17 was found in both the pMET and the left TGCT, although a similar change in the right TGCT approached the cut-off threshold. Unlike Sample 1, Sample 2 showed changes in the primary right TGCT that are not found in the contra-lateral TGCT or the pMET itself (Chrs. 7, 10 and X).

Only one DNA copy number change was observed in the pMETs in Samples 3 and 4. Although the bilateral primary TGCTs from the source male in Sample 3



Strain	129/T1	129/S1	129/S1	129/T1	129/S1	129/S1
Genotype	*Dnd1*$^{Ter/Ter}$	*CSSM19*	*Dnd1*$^{+/Ter}$	*Dnd1*$^{+/Ter}$	*Kitl*$^{+/Sl\text{-}J}$	*Wildtype*
Sample size	46	402	90	153	77	277
Percentage	97.8	85.6	38.9	33.3	18.2	15.9



Strain	129/T1	129/S1	129/S1	129/T1	129/S1	129/S1
Genotype	*Dnd1*$^{Ter/Ter}$	*CSSM19*	*Dnd1*$^{+/Ter}$	*Dnd1*$^{+/Ter}$	*Kitl*$^{+/Sl\text{-}J}$	*Wildtype*
Sample size	45	344	35	51	14	44
Percentage	11.1	11.6	11.4	7.8	7.1	4.5

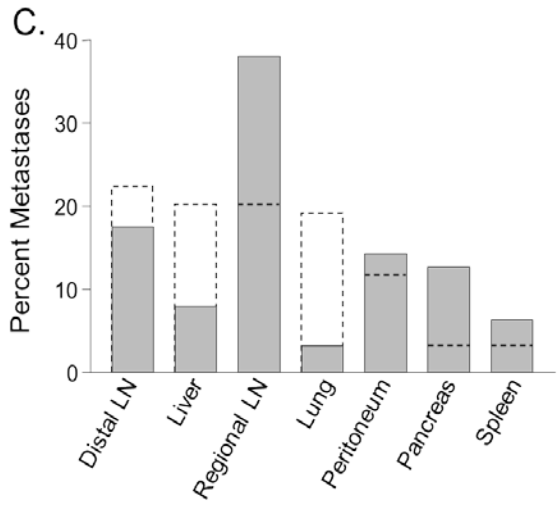


Figure 6. TGCT and pMET incidence.

(A) The percent of males affected with a primary TGCTs varied from the baseline 129/S1 rate depending on genotype, but were in all cases consistent with previous reports (Matin et al., 1999; Noguchi and Stevens, 1982; Stevens and Hummel, 1957; Youngren et al., 2005; Lam et al., 2004). (B) Incidence of pMETs in mouse strains and genotypes. The pMET incidence is calculated as the number of males with a pMET divided by the number of males with a primary TGCT. (C) Anatomical distribution of pMETs. Mouse pMETs incidence for each anatomical location is indicated as a percentage of total pMETs observed (gray bars). Human metastasis incidence adapted from DiSibio and French (Disibio and French, 2008) (dotted bars), and represents the percentage incidence from the noted tissues.

Table 7. DNA copy number variation in pMETs and corresponding primary TGCTs.

Values are given as copy number where n=2 is normal diploid. Probed genes were chosen based on their central location in the altered DNA segment identified with aCGH. Highlighted values represent shared changes between pMETs and primary TGCTs. pMETs in Samples 1 and 2 originated from males with bilateral primary TGCTs, Sample 3 originated from a male with a fused bilateral left and right TGCT (†), and Sample 4 is from a male with a unilateral left TGCT.

Sample	Strain and Metastasis	Gene (Chr)	Left TGCT	Right TGCT	Metastasis
1	CSSM19 Peritoneal mass	Map2k2 (Chr10)	2.44	2.3	1.04
		Olfr77 (Chr9)	2.42	2.28	1.14
		D230025D16Rik (Chr8)	2.14	1.24	1.42
		Igf1r (Chr7)	2.28	1.38	0.84
		Tmem48 (Chr4)	1.84	1.36	1.42
2	CSSM19 Pancreatic mass	Xiap (ChrX)	2.04	1.16	1.62
		Map2k2 (Chr10)	2.52	2.74	1.54
		Nudt3 (Chr17)	2.94	2.46	2.56
		Adam6b (Chr12)	3.86	2.4	3.58
		Ywhag (Chr5)	3.24	4.1	3.1
		Pou2f2 (Chr7)	2.46	3.56	2.26
3	CSSM19 Lumbar lymph node	Slc34a2 (Chr5)	0.97†		1.14
4	129/Sv*Dnd1*$^{+/Ter}$ Peritoneal mass	Skint3 (Chr4)	6.62	n/a	0.4

were fused (a rare occurrence in cases of bilateral TGCTs), the DNA copy number loss on chromosome 5 found in the pMET from Sample 3 was shared with the primary TGCT fusion. In contrast, the loss of genomic DNA on chromosome 4 in Sample 4 found in the pMET was not shared with the unilateral left TGCT, but instead the left primary TGCT showed a significant gain in this chromosomal region.

Discussion

Metastasis is a prevalent feature of human TGCTs, directly affecting treatment and survival. Although many TGCTs and their metastases respond readily to chemotherapy, 10-25% of patients with metastases are resistant to treatment (Piulats et al., 2009) and 5-10% of patients relapse after initial treatment (Loehrer, Sr. et al., 1998). Understanding the mechanisms of metastatic transformation and dissemination is therefore essential to prevent the morbidity and mortality associated with resistant and recurrent TGCT metastases.

Several lines of evidence point to a germ-line origin for pMETs. pMETs are composed of cell and tissue types derived from all three germ lineages with ectodermal and endodermal derivatives predominating (Figure 7, p.96). In addition, undifferentiated embryonal cells were evident histologically and confirmed with two markers of pluripotency, namely OCT4 and SSEA-1 (Figure

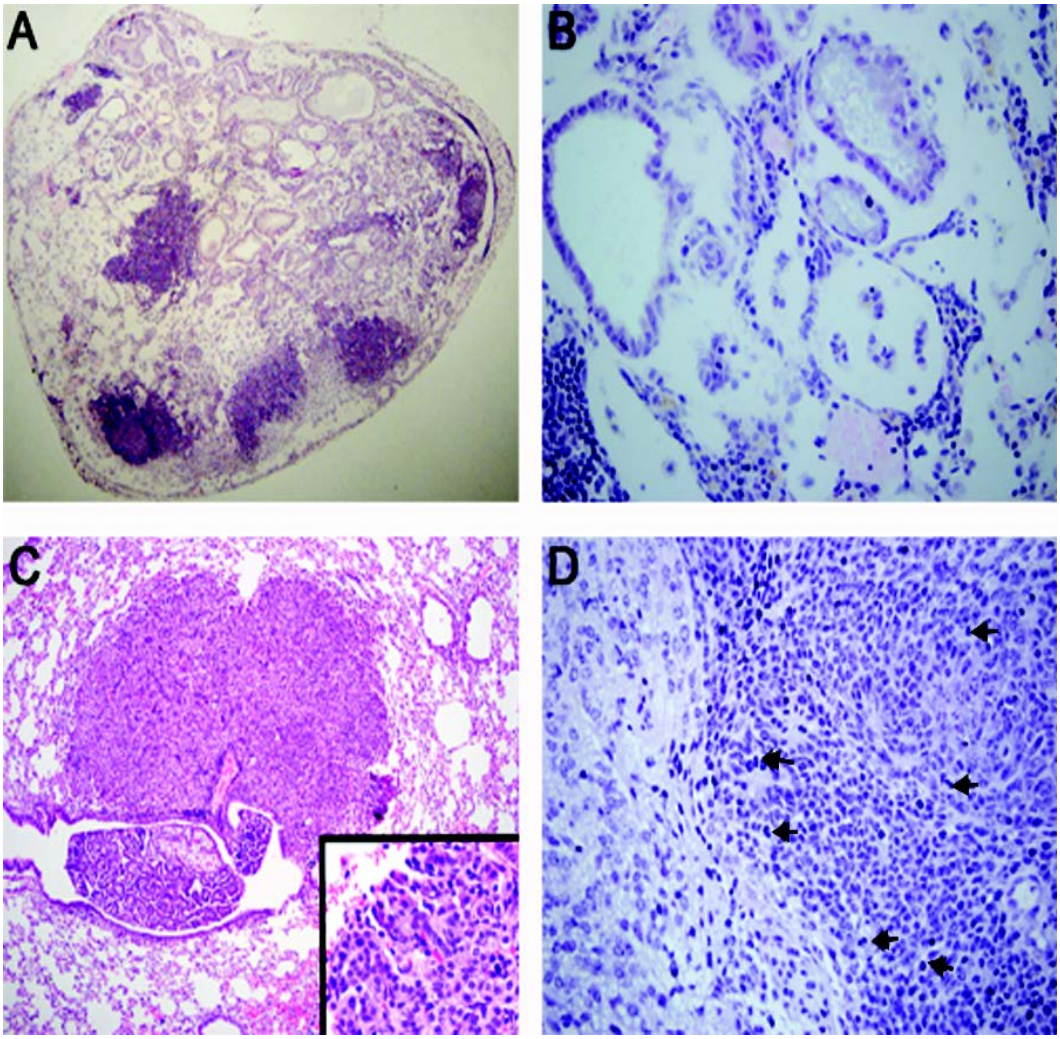


Figure 7. Histology of pMETs.

(A) Lymph node. At low power, much of the node is effaced with a neoplasm composed of glandular structures intermingled with sheets of poorly differentiated malignant cells. (B) High power view of a portion of the lymph node shown in (A). The glands are irregular in shape and size, and are lined by cytologically malignant cells. A mitotic figure is noted in one of the lining cells. (C) Lung involved with metastatic malignant neoplasm, part of which is composed of densely packed epithelioid cells, and part of which shows papillary architecture. The inset shows the neoplasm at high power. The malignant cells have an appearance suggestive of closely packed papillary structures. (D) Section from a diaphragmatic nodule that consists of undifferentiated cells. The larger cells with more abundant cytoplasm (left) have an epithelioid appearance. A large part of the neoplasm (right) consists of small dark immature-appearing cells that are remarkably proliferative. The arrows indicate abundant mitotic figures.

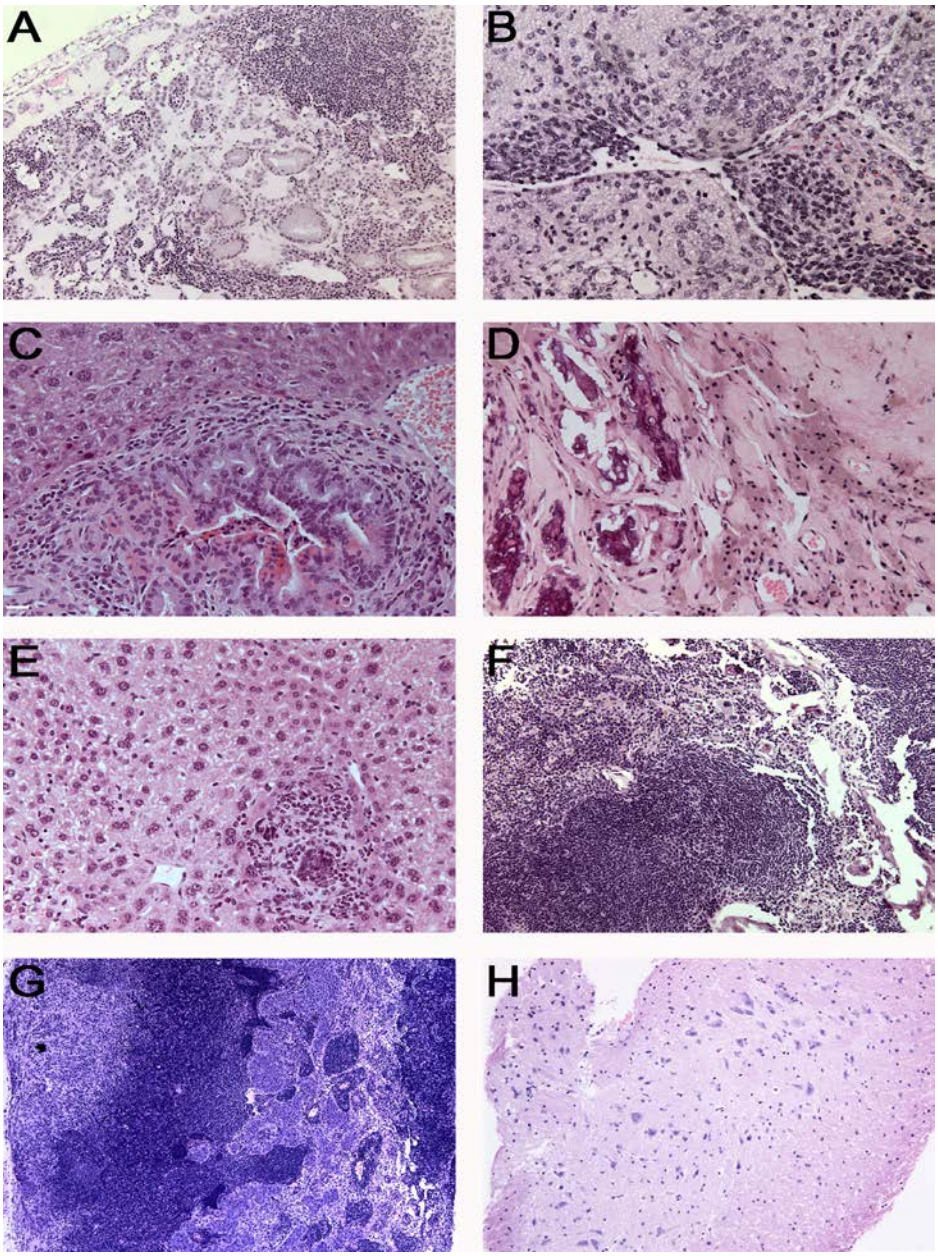


Figure 8. Examples of histological phenotypes in pMETs. (A) Peritoneal mass from a 129/S1 CSSM19 mouse (100X magnification). There is evidence of a number of glandular structures. (B) Liver from a 129/T1*Dnd1*$^{Ter/Ter}$ mouse (200X). Cells exhibit an embryonal carcinoma phenotype. (C) Liver from a 129/S1*Kitl*$^{+/Sl\text{-}J}$ mouse (200X) showing a glandular structure. (D) Peritoneal mass from a 129/S1 CSSM19 mouse (200X). Despite a lack of active neoplasia, the tissue exhibits potential remnants of bone and/or cartilage as well as other necrotic cells. (E) Liver from a 129/S1 mouse (200X) with abundant neutrophils around an undefined cellular center. (F) Liver from a 129/S1 CSSM19 mouse (100X) with the appearance of bone and/or cartilage around a germinal center. (G) A lymph node from a 129/S1 CSSM19 mouse (100X) with large areas of dense lymphocytes and cellular invasion. (H) A peritoneal mass from a 129/S1 CSSM19 mouse (200X) with an undifferentiated cellular phenotype.

9, p.99) and these cells are highly mitotic (Figure 7D, p.96). These results are consistent with a seminoma or EC component in these metastases (Cheng, 2004; Jones et al., 2004). OCT4 is used to diagnose metastatic germ cell tumours in humans (Cheng, 2004) and SSEA-1 is a marker for EC cells in mice (Solter and Knowles, 1978). Although OCT4 and SSEA-1 are both markers of EC cells in mice, co-localization of OCT4 and SSEA1 was uncommon in our sample of metastases. This finding of $OCT4^{+}/SSEA\text{-}1^{-}$ and $OCT4^{-}/SSEA\text{-}1^{+}$ cells is not completely unexpected, since EC cell variants lacking OCT4 (Mueller et al., 2010) and SSEA-1 (Rosenstraus, 1983) have been described.

Assays for DNA copy number changes strongly suggest that pMETs are derived directly from primary TGCTs in the testis rather independently from ectopic stem cells. Among the four pMETs analyzed with CGH, seven significant DNA copy number changes were found, the majority of which were deletions (54%). Three of the 4 samples with validated changes showed shared genomic DNA changes between the primary TGCT and the pMET. Importantly, in the two samples from mice with bilateral primary TGCTs, shared DNA changes were found between the metastasis and one, but not both, of the primary tumours. Together these results suggest that the pMETs are metastases that originate from a specific primary TGCT and do not represent extra-gonadal germ cell tumours (EGCTs).

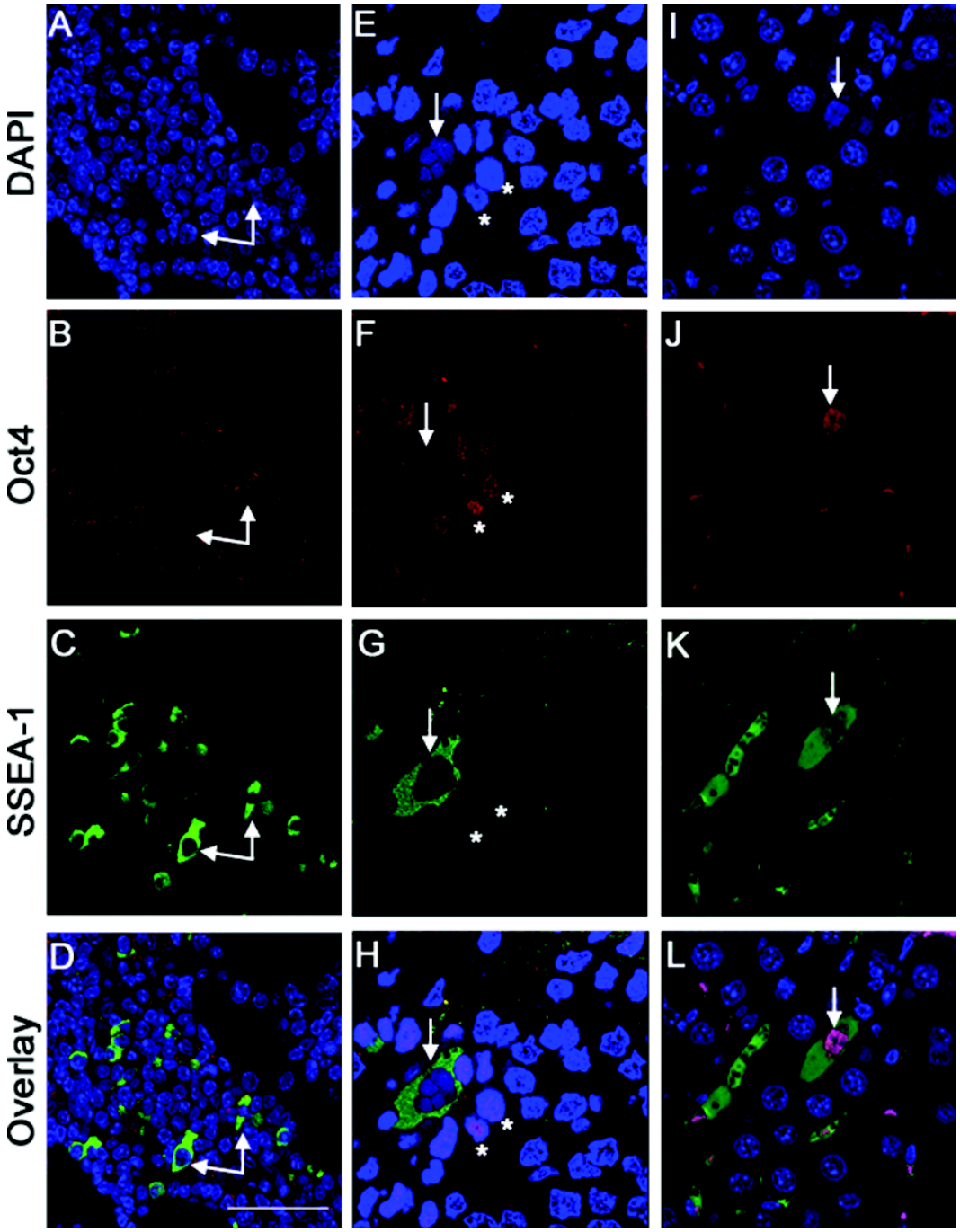


Figure 9. Immunohistochemistry of OCT4 and SSEA-1 in pMETs.

OCT4 positive cells were present in F (asterisks) and J (arrow), but absent in (B). SSEA-1 positive cells (arrows) were observed in each metastasis (C, G, and K) and co-localized with OCT4 in one sample (L, arrow). Nuclei were stained with DAPI (A, E and I). Scale bar: 44µm.

In humans, EGCTs are rare, occurring in 3-5% of all germ cell tumours (Richie and Steele, 2007). These tumours have been proposed to originate either from embryonic germ cells that fail to migrate toward the gonads (Dixon and Moore, 1953; Fine et al., 1962; Hailemariam et al., 1997; Oosterhuis et al., 2005), more primitive totipotent cells (Johnson et al., 1973), or a clinically undetected primary TGCT (Fabre et al., 2004). Recent case reports suggest that all EGCTs can be considered to be silent or "burned out" primary TGCTs (Fabre et al., 2004). Even in cases where a primary diagnosis of an EGCT was made, further investigation found that a primary TGCT was present (Angulo et al., 2009). Our data suggest that this is also the case in the mouse model of TGCTs, because metastases were never observed in the absence of a primary TGCT, even in the 129/S1*Kitl*$^{+/Sl\text{-}J}$ line, which has a known PGC migration defect (Runyan et al., 2006).

Management of metastasis plays a pivotal role in treatment of TGCT patients. In addition to the initial physical effects of chemotherapy and surgical interventions, several late physical effects occur, including an increased risk of cardiovascular disease (Huddart et al., 2003), the development of metabolic syndrome (Huddart and Norman, 2003), secondary cancers (Travis et al., 2005), and reduced fertility (Brydoy et al., 2005). Identification of genetic changes that are associated with TGCT metastasis would enable targeted treatment for at-risk individuals. In our study however, consistent genomic DNA changes were not found, even in the small number of metastases that were examined. The limited human aCGH data available for TGCT metastases has to date not revealed consistent genomic

changes (Korkola et al., 2008). The diversity of incidental chromosomal changes in our metastases reflects the variety of changes found in the primary tumours, and is consistent with results from human aCGH surveys (Korkola et al., 2008; Skotheim et al., 2006). These results suggest that the changes driving metastasis may be unrelated to chromosomal rearrangements, and may instead results from epigenetic or miRNA alterations. Recent evidence suggests that DNA methylation patterns distinguish invasive from non-invasive urothelial cancers (Wolff et al., 2010), and that miRNAs play a role in breast cancer (reviewed in (Shi et al., 2010)). The mouse model reported here may help identify the kinds of genetic and epigenetic changes that are associated with and perhaps responsible for TGCT metastasis.

Identification of a mouse model of spontaneous TGCT metastasis presents a unique opportunity to study the mechanisms involved in the acquisition of metastatic potential and the modes of dissemination. The fixed genetic background in this model helps discriminate between driving and incidental changes in found in metastasis, including miRNA expression and epigenetic states. The nature of this model also allows for genetic manipulation and may serve as a basis for drug discovery in TGCT metastasis treatment. Finding the alterations that lead to metastasis may lead to the identification of metastatic signatures in human TGCTs and the development of treatment strategies based on metastatic risk.

Chapter 3: Contrasting effects of Deadend1 (Dnd1) gain and loss of function mutations on allelic inheritance, testicular cancer and intestinal polyposis.

The results from this chapter have been published:

Jennifer L. Zechel, Stephanie K. Doerner, Angela Lager, Paul Tesar, Jason D. Heaney, Joseph H. Nadeau. ***Contrasting effects of Deadend1 (Dnd1) gain and loss of function mutations on allelic inheritance, testicular cancer, and intestinal polyposis.*** BMC Genetics. 2013 14:54.

Abstract

Background: Certain mutations in the Deadend1 (Dnd1) gene are the most potent modifiers of testicular germ cell tumor (TGCT) susceptibility in mice and rats. In the 129 family of mice, the $Dnd1^{Ter}$ mutation significantly increases occurrence of TGCT-affected males. To test the hypothesis that the $Dnd1^{Ter}$ allele is a loss-of-function mutation; we characterized the consequences of a genetically-engineered loss-of-function mutation in mice, and compared these results with those for $Dnd1^{Ter}$.

Results: We found that intercrossing $Dnd1^{+/KO}$ heterozygotes to generate a complete loss-of-function led to absence of $Dnd1^{KO/KO}$ homozygotes and significantly reduced numbers of $Dnd1^{+/KO}$ heterozygotes. Further crosses showed that $Dnd1^{Ter}$ partially rescues loss of $Dnd1^{KO}$ mice. We also found that loss of a single copy of *Dnd1* in $Dnd1^{+/KO}$ heterozygotes did not affect baseline occurrence of TGCT-affected males and that $Dnd1^{Ter}$ increased TGCT risk regardless whether the alternative allele was loss-of-function ($Dnd1^{KO}$) or wild-type ($Dnd1^{+}$). Finally, we found that the action of $Dnd1^{Ter}$ was not limited to testicular cancer, but also significantly increased polyp number and burden in the $Apc^{+/Min}$ model of intestinal polyposis.

Conclusion: These results show that *Dnd1* is essential for normal allelic inheritance and that $Dnd1^{Ter}$ has a novel combination of functions that significantly increase risk for both testicular and intestinal cancer.

Introduction

Testicular germ cell tumours (TGCTs) are the most common cancer affecting young men. They are disproportionately represented in men aged 20-40, comprising ~60% of all cancers in this age group (DiPietro et al., 2005), while accounting for only 1.0-1.5 % of all cancers (Buetow, 1995). The incidence of TGCTs has increased in the last 30 years (Bosl and Motzer, 1997), rising approximately 3% per year from 1972 to 2002 (Chia et al., 2010b) compared to an overall annual decrease of 0.6% from 1994-2009 for all cancers in men (Jemal et al., 2013).

Testicular cancer is widely considered to be one of the most heritable forms of cancer (Edsgard et al., 2013; Heimdal et al., 1997). Genetic factors contribute significantly to TGCT susceptibility as exemplified by a 4- to 15-fold increased risk in the sons and brothers of affected men, respectively (Chia et al., 2008; Neale et al., 2008). With conventional inheritance however, risk should be similar in sons and brothers. The ~4-fold difference in occurrence of affected sons and brothers implies that other modes of inheritance are involved. Despite strong heritability, known genetic mutations and single nucleotide polymorphisms (SNPs) make only modest contributions to susceptibility, illustrating the genetically complex nature of this disease. The *gr/gr* deletion, which is a common cause of infertility in men, is associated with increased TGCT risk (Hemminki and Li, 2004). But its contribution is modest, occurring in only 3% of TGCT

cases with a family history and 1% of unaffected individuals (Nathanson et al., 2005). *gr/gr* results from a 1.6 Mb deletion at the *AZFc* locus at Yq11 of the human Y chromosome, a region that contains multiple copies of several genes that are involved in male germ cell development including *DAZ* (deleted in azospermia), *BPY2* (basic charge, Y-linked 2) and *CDY1* (chromodomain protein, Y-linked 1). Effects of the g*r/gr* deletion on germ cell development and differentiation are largely unknown.

Several single nucleotide polymorphisms (SNPs) are associated with increased TGCT risk in humans. In particular, studies implicate SNPs that are associated with at least 14 genes: *ATF7IP, BAK1, DMRT1, KITLG, SPRY4, TERT, HPGDS, MAD1L1, RFWD3, TEX14, RAD51C, PPM1E, DAZL and PRDM14* (Rapley et al., 2009; Turnbull et al., 2010; Kratz et al., 2011b; Poynter et al., 2012; Kanetsky et al., 2011; Chung et al., 2013; Ruark et al., 2013). Molecular mechanisms remain unclear, in part because each SNP is located outside the coding region of the associated gene, and in part because the haplotype structure and sequence of these loci have not yet been fully reported. Interestingly, the *TERT* SNP is also associated with adenocarcinoma (Kohno et al., 2010).

Certain mutations in the *Dead End 1* (*Dnd1*) gene are potent modifiers of TGCT susceptibility in both mice and rats. In the mouse, the spontaneous $Dnd1^{Ter}$ mutation significantly increases TGCT susceptibility in the 129 family of inbred

mouse strains. In particular, *Dnd1*^{Ter} increases occurrence of TGCT-affected males from a baseline of ~5% in the 129S1/SvImJ strain to 17% in *Dnd1*$^{+/Ter}$ heterozygotes and 94% in *Dnd1*$^{Ter/Ter}$ homozygotes (Noguchi and Noguchi, 1985; Youngren et al., 2005). The *Ter* mutation results from a single base substitution in exon 3, 3' to its single RNA recognition motif (RRM), that transforms an arginine residue to a premature stop codon (Figure 10, p.114, see also (Youngren et al., 2005). The *Ter* mutation has been proposed to produce an mRNA that is degraded by nonsense-mediated degradation owing to the presence of the premature stop codon. This conclusion was based on northern blots of mRNA isolated from TGCTs in *Dnd1*$^{Ter/Ter}$ males (Youngren et al., 2005). The *Ter* mutation has been proposed to produce an mRNA that is degraded by nonsense-mediated degradation owing to the presence of the premature stop codon. This conclusion was based on northern blots of mRNA isolated from TGCTs in *Dnd1*$^{Ter/Ter}$ males (Youngren et al., 2005). But the tissue type (TGCTs) used in this study may not have been appropriate to assess the fate of *Dnd1* transcripts. In the rat, a spontaneous mutation has been identified where a G to A substitution in exon 4 produces a premature stop codon that is thought to result in a 62 amino acid truncation at the C-terminus of the DND1 protein (Figure 10, p.114, see also (Northrup et al., 2012). This mutation leads to germ cell tumours in males and females as well as to spontaneous metastases. In humans, sequencing of TGCT candidate genes in several large studies failed to detect a significant number of *DND1* mutations (Chia et al., 2010b; Sijmons et al., 2010). Of the two SNPs that

were identified, one (Glu86Ala) is located within the conserved RRM of DND1. The functional consequences of this mutation on *Dnd1* expression and function or on TGCT risk are not known.

Dnd1 has many unique functions. *Dnd1* shares significant sequence similarity with *A1cf*, a gene that encodes the RNA binding subunit of the Apobec1 cytidine deaminase that edits specific sites in specific mRNAs (Youngren et al., 2005). Interestingly, DND1 blocks access of specific miRNAs to their 3' target in mRNAs such as *p27*, *LATS2* and *TDRD7* (Kedde et al., 2007). DND1 also binds several pluripotency factor mRNAs including *Oct4*, *Sox2*, *Nanog* and LIN28 (Zhu et al., 2011), regulators of cell cycle including LATS2, TP53, *p21* and *p27* (Cook et al., 2011b; Zhu et al., 2011), apoptotic factors such as BCLX and BAX (Zhu et al., 2011), and is a positive regulator of geminin mRNA translation through binding to its 3' UTR (Chen et al., 2010). The role of DND1 in these functions and complexes is unknown. DND1 transports mRNA transcripts from germ cell nuclei to germ cell granules (Slanchev et al., 2009). Finally, *Dnd1* is required for primordial germ cell (PGC) survival (Weidinger et al., 2003); PGCs are the stem cell for TGCTs (Stevens, 1967c). Together these observations implicate *Dnd1* in many aspects of RNA translation control.

The *Dnd1* gene in mouse has four exons and encodes an α-isoform, which is 352 amino acids, and a β-isoform, which is 340 amino acids (Figure 10, p.114). These

isoforms are derived through alternative splicing and differ by 12 amino acids at the amino-terminus of the protein. DND1-α is expressed in early embryos, whereas DND1-β is expressed in the germ cells of the adult testis (Bhattacharya et al., 2007). Both isoforms contain a single RNA recognition motif (RRM) and a highly conserved HRAAAMA motif (unpubl). This 7 amino acid motif is found in most orthologues of *Dnd1* and *A1cf* (JHN, unpubl.). In zebrafish, ATPase activity has been attributed to a variant of this motif in *Dnd1* (Liu and Collodi, 2010).

Several considerations suggest that the assumption that $Dnd1^{Ter}$ leads to loss-of-function is erroneous. *In vivo* loss of function using morpholinos to reduce expression in zebrafish showed that *Dnd1* is required for PGC survival (Weidinger et al., 2003), possibly through an ATPase activity (Liu and Collodi, 2010). Although TGCTs have been reported in zebrafish (Neumann et al., 2011a; Neumann et al., 2011b; Gill et al., 2010), reduced *Dnd1* expression was not sufficient for tumourigenesis. However, because genetic background strongly regulates TGCT susceptibility in mice and rats (Northrup et al., 2012; Stevens and Hummel, 1957; Stevens, 1973; Stevens and Mackensen, 1961), absence of TGCTs in the zebrafish study could implicate either the nature of the mutation or genetic background as critical elements determining tumourigenic outcome.
To test whether TGCT susceptibility depends on the nature of *Dnd1* mutations, we generated a line of 129/SvImJ-Dnd^{KO} mice. We found that deficiency of *Dnd1*

leads to highly biased allelic inheritance and that *Dnd1*^{Ter} partially restores normal inheritance. We also found that *Dnd1*^{Ter} acts distinctly from *Dnd1*^{KO} to increase TGCT risk in a dosage-dependent manner in 129S1/SvImJ males, whereas *Dnd1*^{KO} did not significantly affect susceptibility. Finally, recent work suggests that susceptibility to both TGCTs and intestinal polyposis share genetic predisposition (Castillejo et al., 2012) and that DND1 is normally expressed in the intestine (Larsson et al., 2012; Mutch et al., 2006; Thorrez et al., 2011; Saito et al., 2009; Horst et al., 2012). We therefore tested whether *Dnd1*^{Ter} affects intestinal tumourigenesis and found that *Dnd1*^{Ter} significantly increases polyp number and burden in *Apc*$^{+/Min}$ mice.

Materials and Methods

Mice

129S1/SvImJ: This strain (JR002448, previously known as 129/SvImJ) was obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All studies were conducted on this inbred genetic background.

129S1/SvImJ-*Dnd1*$^{+/KO}$: ES cells with a targeted deletion of *Dnd1* were generated from 129S6 mice by the Intrexon Corporation, (Blacksburg, VA). Exons 1-2 and most of exon 3 of *Dnd1* were removed through homologous recombination (Figure 14, p.131), and cells were negatively selected with thymidine kinase (TK) and diphtheria toxin A (DTA), and positively selected with

neomycin. PCR was used to confirm homologous recombination in these cells. ES cells were then injected into blastocysts (Case Transgenic and Targeting Facility) and the resulting chimeras were backcrossed onto the 129S1/SvImJ strain for more than 10 generations.

C57BL/6J (B6; JR000664) and C57BL/6J-*Apc*$^{+/Min}$ (*Apc*$^{+/Min}$; JR002020) mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

All mice were fed an autoclaved standard laboratory diet (Purina 5010 LabDiet (Richmond, IN) and were provided autoclaved water *ad libitum*. All mice were maintained on a 12-h light/dark cycle at the Wolstein Research Facility (CWRU).

Procedures were approved and conducted in compliance with Institutional Animal Care and Use Committee (IACUC) standards at Case Western Reserve University.

Embryonic day 3.5 (E3.5) embryo cultures

Embryos from time-mated *Dnd1*$^{+/KO}$ X *Dnd1*$^{+/KO}$ pregnant females were flushed from the oviducts and uterine horns on embryonic day 3.5 (E3.5) and cultured as previously described (Tesar, 2005). To obtain sufficient material for reproducible genotyping, embryos were cultured for 1 week in individual wells of a 24-well tissue culture plate

Genotyping

DNA for PCR genotyping was extracted from tail tissue (mice) or cell masses (E3.5 embryo cultures). The nucleotide substitution in the *Ter* mutation results in the creation of a *Dde1* site that was used for genotyping (Youngren et al., 2005). The Dnd1KO primers are: CTGCGTGTTCGAATTCGCCAATGA (F), ACAAAGAGAAACCCGGTCTCGGAA (R). The $Dnd1^{KO}$ allele was amplified using primers extending from exon 4 of *Dnd1* into the neomycin gene of the targeting construct. Primers used for genotyping $Apc^{+/Min}$ were previously described (Moser et al., 1990).

Tumour surveys

Male mice between the ages of 4-16 weeks were surveyed for TGCTs. Tumour incidence was calculated as the percentage of males with at least one TGCT. Histological analysis (H&E staining, see below) was used to confirm any TGCTs that were ambiguous at autopsy.

Intestinal polyp survey

Mice were euthanized with cervical dislocation. The small and large intestines were immediately removed, flushed with cold PBS, and cut longitudinally for polyp measurements. Polyps were counted and cross-sectional diameter was measured in the small intestine and colon with a Leica MZ10F Modular Stereomicroscope. Individual polyp size and number were used to calculate a

measure of total polyp mass for each mouse, and this measure was used as a surrogate for polyp burden.

Histology

Testes were fixed with 10% formalin for at least 48h. Tissues were then embedded in paraffin and sectioned (5µm) at the Case Comprehensive Cancer Center Tissue Procurement and Histology Core facility (TPHC). Hematoxylin and eosin staining was done in the TPHC facility.

Quantitative real-time PCR

RNA was extracted from cells and tissues using the RNeasy micro- and mini-kits, respectively (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions and including an on-column DNAse treatment. RNA was reverse-transcribed using the qScript Synthesis Kit (Quanta BioSciences Inc., Gaithersburg, MD). Changes in relative expression were quantified with the Chromo4 real-time PCR system (MJ Research) and TaqMan primers to Dnd1 and normalized to 18S (Invitrogen) using manufacturer protocols and reported as mean ± SEM.

Statistical analysis

Data are presented as mean ± SEM. Two-tailed t-tests were used to evaluate results for quantitative PCR and data for both polyp numbers and burden. Chi-

square contingency tests were used evaluate differences in occurrence of TGCT-affected males. Standard goodness-of-fit tests were used to evaluate differences between observed and Mendelian expectations for backcross and intercross segregation results. Then, to estimate the extent of loss for particular genotypes, the number of wild-type (+/+) mice (or embryos) was taken as the expected number for this genotypic class, and assuming Mendelian segregation (i.e. 1:2:1), the expected number of mutant heterozygotes and homozygotes was calculated. The difference between these observed and expected numbers was used to estimate the extent of loss for each genotypic class.

Results

Loss of *Dnd1* homozygotes and heterozygotes. Unexpected results were found for $Dnd1^{+/KO}$ intercrosses that were intended to produce $Dnd1^{+/KO}$ heterozygotes and $Dnd1^{KO/KO}$ homozygotes as well as $Dnd1^{+/+}$ wild-type controls for TGCT surveys. The genotypic distribution among adult mice differed significantly from 1:2:1 Mendelian expectations (Table 8A; $\chi^2 = 108.4$, $p<0.0001$; two-tailed test, 2 degrees of freedom). In particular, no $Dnd1^{KO/KO}$ homozygotes were observed among 282 offspring (Table 8A, p93). To assess the extent of the deviation from expectations, we assumed that the number of $Dnd1^{+/+}$ mice (N = 120) corresponded to expectations, and then extrapolated from this wild-type number

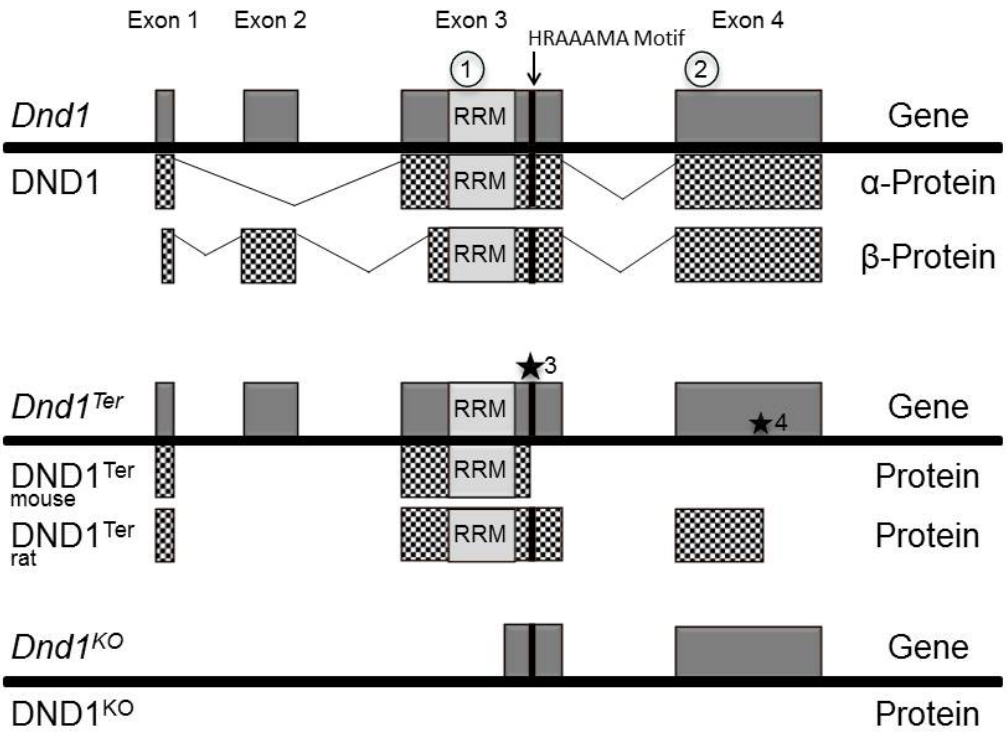


Figure 10. Structure of the *Dnd1*, *Dnd1*^{Ter} and *Dnd1*^{KO} genes and their inferred predicted protein products.

Gene arrangements are displayed above the solid line, and the corresponding protein product is displayed below the solid line. *Dnd1* has two isoforms: *α-Dnd1* and *β-Dnd1* which differ in the amino-terminus of the protein. *Dnd1* has an RNA recognition motif (RRM) in the C-terminal portion of exon 3. The The HRAAAMA motif that is presumably part of the putative ATPase domain, is located between amino acids 181-186 in the mouse genome. SNPs identified in human TGCTs include a (1) Glu86Ala (Linger et al., 2008) and (2) an Asp219Glu (Sijmons et al., 2010). In the mouse, the *Ter* mutation creates a premature stop-codon at amino acid 178 (★3), which is located 37 nucleotides from the 3' most exon-exon junction. The rat *Ter* mutation has the premature stop codon at amino acid 289 (★4) within exon 4. The RRM is intact in both mouse and rat *Dnd1* mutants allowing these proteins to possibly recognize and bind to target RNAs; the putative ATPase domain is lost in the mouse *Dnd1*^{Ter} and truncated in rat *Dnd1*^{Ter}. The *Dnd1*^{KO} allele retains the 3' most portion of exon 3 and exon 4, but does not have a translational start site.

to the expected numbers of *Dnd1*$^{+/KO}$ heterozygotes (N = 240) and *Dnd1*$^{KO/KO}$ homozygotes (N = 120) mice. In addition to a complete deficiency of *Dnd1*$^{KO/KO}$ homozygotes, only 68% (= 162/240) of the expected number of *Dnd1*$^{+/KO}$ heterozygotes was found, suggesting that parental *Dnd1*$^{+/KO}$ heterozygosity either biased segregation against the *Dnd1*^{KO} allele, or alternatively that partial or complete loss of *Dnd1* function leads to lethality for *Dnd1*$^{KO/KO}$ homozygotes and reduced viability for *Dnd1*$^{+/KO}$ heterozygotes.

To examine the developmental timing of genotypic loss, we time-mated heterozygous *Dnd1*$^{+/KO}$ males and females and flushed embryonic day 3.5 (E3.5) embryos from the oviduct. Embryos (N=25) were individually cultured for one week before genotyping. The genotypic distribution differed significantly from 1:2:1 Mendelian expectations (Table 8B; $\chi^2 = 22.4$, $p<0.0001$; two-tailed test, 2 degrees of freedom). No *Dnd1*$^{KO/KO}$ homozygotes were found among 25 cultured embryos, the number of *Dnd1*$^{+/KO}$ heterozygotes was significantly reduced, and no evidence was found for unfertilized oocytes. In addition, the genotypic distributions for E3.5 embryos (Table 8B) and for adult mice (Table 8A) did not differ significantly (not shown). These results confirm observations found among adult mice and suggests that reduced *Dnd1* function either biased segregation, or led to early embryonic lethality. Interestingly, unusual inheritance patterns have

Table 8. ***Dnd1^{KO}*** segregation in intercrosses and backcrosses.

Results are presented after pooling data for both genders (A-D), because occurrence of females and males did not differ significantly (not shown). (**A**) *Dnd1*$^{+/KO}$ intercross – genotype distribution at weaning. (**B**) *Dnd1*$^{+/KO}$ intercross – genotype distribution at embryonic day 3.5 (E3.5). (**C**) *Dnd1*$^{+/KO}$ segregation in backcrosses versus intercrosses. *Mendelian expectations based on *Dnd1*$^{+/+}$; litter size (mean ± SEM, *n* is the number of litters). **(D)** Interaction between *Dnd1*^{Ter} and *Dnd1*^{KO}. Male progeny (N=130) from crosses between *Dnd1*$^{+/Ter}$ and *Dnd1*$^{+/KO}$ mice.

A

Genotype	Observed	Mendelian expectations
Dnd1$^{+/+}$	120	70.5
Dnd1$^{+/KO}$	162	141
Dnd1$^{KO/KO}$	0	70.5

B

Genotype	Observed	Mendelian expectations
Dnd1$^{+/+}$	16	6.25
Dnd1$^{+/KO}$	9	12.5
Dnd1$^{KO/KO}$	0	6.25

C

Cross	No. *Dnd1*$^{+/+}$	No. *Dnd1*$^{+/KO}$ *(expectations)	% expected number	Litter size
Intercross	120	162 (240)	67.5	5.6±0.5 n=18
Backcross: maternal	110	107 (110)	97.2	5.8±0.4 n=30
Backcross: paternal	100	96 (100)	96	5.2±0.4 n=19

D

Genotype	Observed	Mendelian expectations
Dnd1$^{+/+}$	40	32.5
Dnd1$^{+/KO}$	41	32.5
Dnd1$^{+/Ter}$	29	32.5
Dnd1$^{KO/Ter}$	20	32.5

Table 9. *Dnd1* genotype and occurrence of males affected with at least one spontaneous TGCT.

Dnd1$^{+/KO}$ males are offspring of both *Dnd1*$^{+/+}$ × *Dnd1*$^{+/KO}$ and *Dnd1*$^{+/KO}$ × *Dnd1*$^{+/+}$ reciprocal crosses. ǂ Results from Zechel et al. (2012).

Genotype	Affected	Unaffected	Affected males (%)
***Dnd1*$^{+/+}$**	8	121	*6%
***Dnd1*$^{+/KO}$**	7	109	*6%
ǂ*Dnd1*$^{+/Ter}$	35	90	39%

also been reported for *Apobec1*^{KO} – *Dnd1*^{Ter} interactions tests (Nelson et al., 2012) and for Apobec1 complementation factor *A1cf*, the paralog of *Dnd1* (Blanc et al., 2005).

To test whether allelic segregation in *Dnd1*$^{+/KO}$ is inherently biased, we examined the genotypic representation in reciprocal backcrosses of *Dnd1*$^{+/KO}$ heterozygotes or *Dnd1*$^{+/+}$wild-type mice. Segregation did not differ significantly from 1:1 Mendelian expectations for either maternal or paternal heterozygosity (Table 8C). Interestingly, occurrence of *Dnd1*$^{+/KO}$ heterozygous progeny differed in intercrosses versus backcrosses of *Dnd1*$^{+/KO}$ heterozygous parents, where a significant deficiency of *Dnd1*$^{+/KO}$ heterozygotes was found among intercross but not backcross progeny (Table 8C). These results suggest that segregation in each parent is normal and that gametes in each parent are comparably functional in backcrosses but not intercrosses.

Litter size can be used to determine whether unusual inheritance patterns result from embryonic lethality. Litter size should be reduced substantially if deficiency of some genotypes results from lethality. By contrast, if production of gametes or fertilization is biased towards particular alleles, litter sizes should not differ among the various crosses. However, despite significant deviation from the expected genotypic distributions litter sizes did not vary significantly among crosses (Table 8C). These results argue against lethality as the cause of the unusual genotypic distributions.

Based on the premise that DND1^{Ter} protein is translated instead of being lost through nonsense mediated degradation, a single copy of *Dnd1*^{Ter} may be sufficient to at least partially rescue *Dnd1*$^{KO/KO}$ loss. To test this hypothesis, we intercrossed mice heterozygous for the *Dnd1*^{KO} and *Dnd1*^{Ter} mutations and examined occurrence of the four genotypic classes among the resulting offspring (Table 8D). The distribution of *Dnd1*$^{+/+}$, *Dnd1*$^{+/KO}$, *Dnd1*$^{+/Ter}$, *Dnd1*$^{KO/Ter}$ offspring differed significantly from 1:1:1:1 Mendelian expectations (χ^2 = 9.1, p<0.03; two-tailed test, 3 degrees of freedom). The genotype with the largest deviation from expectations was the *Dnd1*$^{KO/Ter}$ heterozygote which supports the hypothesis that the DND1^{Ter} protein is translated and retains sufficient functionality to enable at least partial viability of animals in the absence of wild-type DND1; if *Dnd1*^{Ter} conferred loss of function, then loss of both *Dnd1*$^{KO/KO}$ and *Dnd1*$^{KO/Ter}$ should have been found.

***Dnd1* mRNA levels in *Dnd1*$^{+/KO}$ males.** To determine whether the *Dnd1*^{KO} allele results in reduction of *Dnd1* mRNA, *Dnd1* transcript levels were measured in heart and testis from *Dnd1*$^{+/KO}$ and wild-type littermate control males that were 6-8 weeks old. *Dnd1*$^{+/KO}$ mRNA levels were significantly reduced in heart (0.41 ± 0.12; t = 3.1, p<0.05; two-tailed t-test, n=7) and substantially reduced in testis (0.56 ± 0.19; n=7) compared to their wild-type littermate controls (1.0 ± 0.06 and 1.0 ± 0.26; n=3 and 7, respectively) (Figure 11, p.120). *Dnd1* mRNA levels from

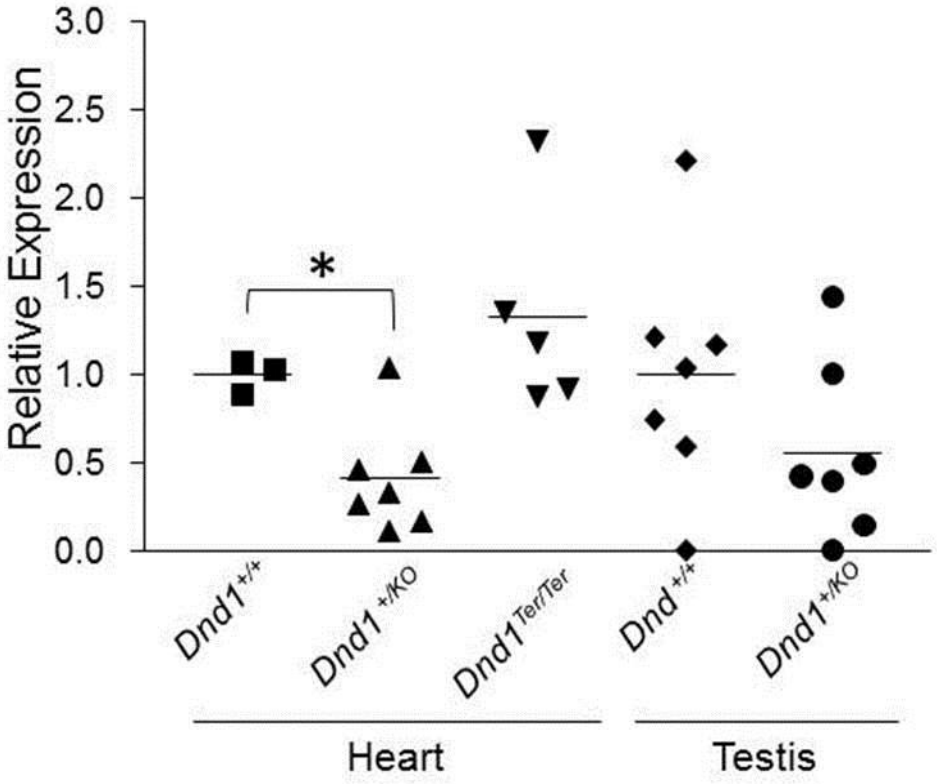


Figure 11. *Dnd1* RNA levels in heart and testes.

Dnd1 expression cannot be measured reliably in *Dnd1*$^{Ter/Ter}$ testes because they deficient in germ cells.

Table 10. Occurrence of TGCTs-affected males of various *Dnd1* genotypes.

Dnd1$^{+/KO}$ and *Dnd1*$^{+/Ter}$ animals were intercrossed to test for occurrence of males with at least one TGCT. ǂ Offspring from *Dnd1*$^{+/KO}$ × *Dnd1*$^{+/Ter}$ crosses; * offspring from *Dnd1*$^{+/Ter}$ × *Dnd1*$^{+/Ter}$. ǂData are from Zechel et al. (2012).

Genotype	Affected	Unaffected	Affected males (
ǂ***Dnd1***$^{+/+}$	6	34	15%
ǂ***Dnd1***$^{+/KO}$	1	28	3%
ǂ***Dnd1***$^{+/Ter}$	14	27	34%
ǂ***Dnd1***$^{KO/Ter}$	7	13	35%
****Dnd1***$^{+/Ter}$	35	55	ǂ39%
****Dnd1***$^{Ter/Ter}$	45	1	ǂ98%

Dnd1$^{Ter/Ter}$ hearts (1.33 ± 0.26) did not differ significantly from wild-type controls. Testis samples were not tested because severe germ cell deficiency in *Dnd1*$^{Ter/Ter}$ males would compromise interpretation of any results (Collin et al., 1996; Noguchi and Noguchi, 1985; Youngren et al., 2005). These results confirm that *Dnd1*^{KO} but not *Dnd1*^{Ter} mice showed reduced *Dnd1* mRNA levels.

Germ cell numbers and fecundity in *Dnd1*-deficient males. We next sought to test whether *Dnd1*^{Ter} and *Dnd1*^{KO} alleles induce similar effects on PGC numbers in testes of heterozygous and wild-type littermates from the *Dnd1*^{KO} line. Testes from *Dnd1*$^{+/Ter}$ males showed modest but significant reductions in germ cell numbers and testis weight (Collin et al., 1996; Youngren et al., 2005). We weighed testes from 6-10 week old Dnd1$^{+/KO}$ males and their wild-type (+/+) littermates to determine if they were phenotypically similar to those from *Dnd1*$^{+/Ter}$. Testes from wild-type males weighed an average of 4.43 ± 0.88g, which was similar to the testes weight in *Dnd1*$^{+/KO}$ heterozygotes, which weighed an average of 4.44 ± 0.69g (Figure 13A, p.127). These results suggest that germ cell numbers were not significantly reduced in *Dnd1*$^{+/KO}$ males. Histological analysis confirmed these results for both *Dnd1*$^{+/Ter}$ and *Dnd1*$^{+/KO}$ males (Figure 13B & C). Finally, both male and female heterozygotes for the *Dnd1*^{KO} allele were fertile as measured by the similar number of offspring produced in backcrosses to 129S1/SvImJ (Table 8C, p.116). Together these results suggest that the *Dnd1*^{KO} allele did not negatively impact germ cell numbers or fertility.

***Dnd1* mutations and TGCT-affected males.** To test whether partial *Dnd1* deficiency affects occurrence of affected males, $Dnd1^{+/KO}$ and their $Dnd1^{+/+}$ wild-type control littermates derived from $Dnd1^{+/KO}$ intercrosses were surveyed for spontaneous TGCTs (Table 9, p.117). Significant differences were not detected for occurrence of TGCT-affected $Dnd1^{+/KO}$ heterozygous and $Dnd1^{+/+}$ wild-type males. By contrast, 39% of $Dnd1^{+/Ter}$ males are affected, which is significantly higher than the occurrence of affected $Dnd1^{+/KO}$ and $Dnd1^{+/+}$ males ($p<0.0001$; Fisher's exact test, two-tailed, 1 degree of freedom). (This rate for $Dnd1^{+/Ter}$ males in our colony is significantly higher than published reports (Lam et al., 2007). The rate has been consistently higher for several years, without obvious explanation. Because of the study design, this increased rate does not affect results or interpretation in the present work.) Thus, the $Dnd1^{Ter}$ but not the $Dnd1^{KO}$ mutation increases TGCT risk.

To test whether the increased occurrence of affected $Dnd1^{Ter/Ter}$ males was a result of increased $Dnd1^{+/Ter}$ or the absence of $Dnd1^{+/KO}$, we crossed $Dnd1^{+/Ter}$ and $Dnd1^{+/KO}$ animals to generate compound heterozygous $Dnd1^{Ter/KO}$ offspring that were surveyed for TGCTs (Table 10, p.121). A single copy of $Dnd1^{Ter}$ increased occurrence of TGCT-affected males from a baseline of 15% for $Dnd1^{+/+}$ wild-type males to 39% for $Dnd1^{+/Ter}$ males. A second copy of $Dnd1^{Ter}$ ($Dnd1^{Ter/Ter}$) further increased occurrence of affected males to 98% (Noguchi and Noguchi,

1985; Youngren et al., 2005). We then asked whether the higher occurrence of affected *Dnd1*$^{Ter/Ter}$ males was due to the absence of wild-type DND1, or a result of an increased dosage of *Dnd1*^{Ter}. Compound heterozygous males (*Dnd1*$^{KO/Ter}$) have a rate that does not recapitulate the rate observed for *Dnd1*$^{Ter/Ter}$ homozygotes (35% versus 97.8%, respectively) (Table 10). Instead, the rate for compound heterozygotes was similar to the *Dnd1*$^{+/Ter}$ heterozygote rate (34% and 39%). These results suggest that DND1^{Ter} protein has dose-dependent effects on TGCT risk.

***Dnd1*^{Ter} affects intestinal polyposis.** Although *Dnd1*^{Ter} is a potent modifier of TGCT susceptibility, we speculated that the tumourigenic properties of *Dnd1*^{Ter} may also be relevant in the intestine where *Dnd1* is also expressed (Detwiller et al., 2005). To test this hypothesis, we used the *Apc*$^{+/Min}$ mouse model of intestinal polyposis. These mice develop numerous intestinal polyps at an early age and are a model of human Familial Adenomatous Polyposis (FAP) (Su et al., 1992). We crossed *Dnd1*$^{+/Ter}$ and *Apc*$^{+/Min}$ mice to generate compound *Apc*$^{+/Min}$ *Dnd1*$^{+/Ter}$ heterozygotes (test) as well as a single-heterozygous *Apc*$^{+/Min}$ *Dnd1*$^{+/+}$ controls. After 100 days of age, a significant 1.5-fold increase in polyp number was observed in *Apc*$^{+/Min}$ *Dnd1*$^{+/Ter}$ double-heterozygous test males compared to *Apc*$^{+/Min}$ *Dnd1*$^{+/+}$ control males (130 ± 1.3 and 96.2 ± 1.4, respectively; $t = 4.4$, $p<0.001$, two-tailed t-test; Figure 12A, p.126). Similarly, total polyp mass was

also significantly elevated from 192.2 ± 11.6 mm^2 in $Apc^{+/Min}$ $Dnd1^{+/+}$ mice to 352.6 ± 11.4 mm^2 in $Apc^{+/Min}$: $Dnd1^{+/Ter}$ males ($t = 4.5$, $p<0.0001$; two-tailed t-test; Figure 12B), suggesting that a single copy of $Dnd1^{Ter}$ exacerbates intestinal polyp initiation and development in mice that are genetically susceptible to intestinal polyposis and that the action of $Dnd1^{Ter}$ is not limited to TGCTs.

Discussion

TGCTs are the third most heritable form of cancer, with approximately 25% of the susceptibility being attributed to underlying genetic factors (Heimdal et al., 1997). The limited success in identifying genetic variants that account for a significant proportion of TGCT cases highlights the complex nature of TGCT susceptibility (Kratz et al., 2011a; Lam et al., 2004; Stevens, 1967b). Discovery *DMRT1*, *KITLG*, *SPRY4*, *TERT*, *HPGDS*, *MAD1L1*, *RFWD3*, *TEX14*, *RAD51C*, *PPM1E*, *DAZL and PRDM14* (Rapley et al., 2009; Turnbull et al., 2010; Kratz et al., 2011b; Poynter et al., 2012; Kanetsky et al., 2011; Chung et al., 2013; Ruark et al., 2013) has provided some of the genetic basis of the disease. Even with these findings however, the majority of inherited TGCT susceptibility remains unknown. In the mouse, several TGCT susceptibility genes have been identified, including *Kit*, *Kitlg*, *Pten*, *Dmrt1*, *Eif2s2* and others (for a review, see (Carouge and Nadeau, 2012)). Together these human and mouse genes implicate pathways and functions relating to germ cell proliferation, PGC differentiation and cell cycle control in TGCT development. But much remains to be learned about the

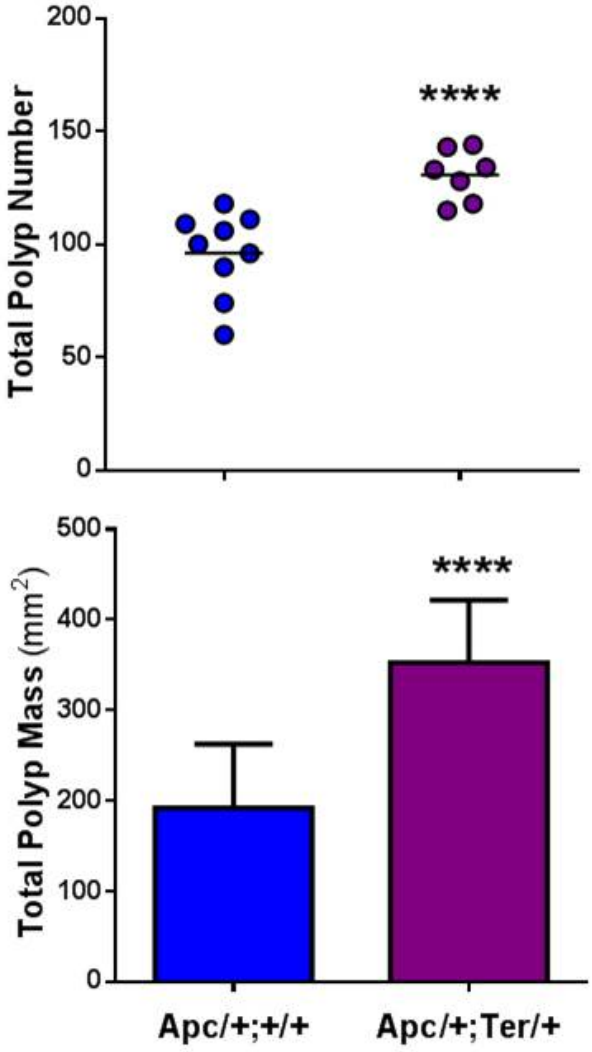


Figure 12. *Dnd1*^{Ter} increases polyp number and mass.

Polyp number (A) and mass (B) in C57BL/6-*Apc*$^{+/Min}$ *Dnd1*$^{+/Ter}$ males. Males were surveyed for polyp number and mass after 100 days on the 5010 diet.

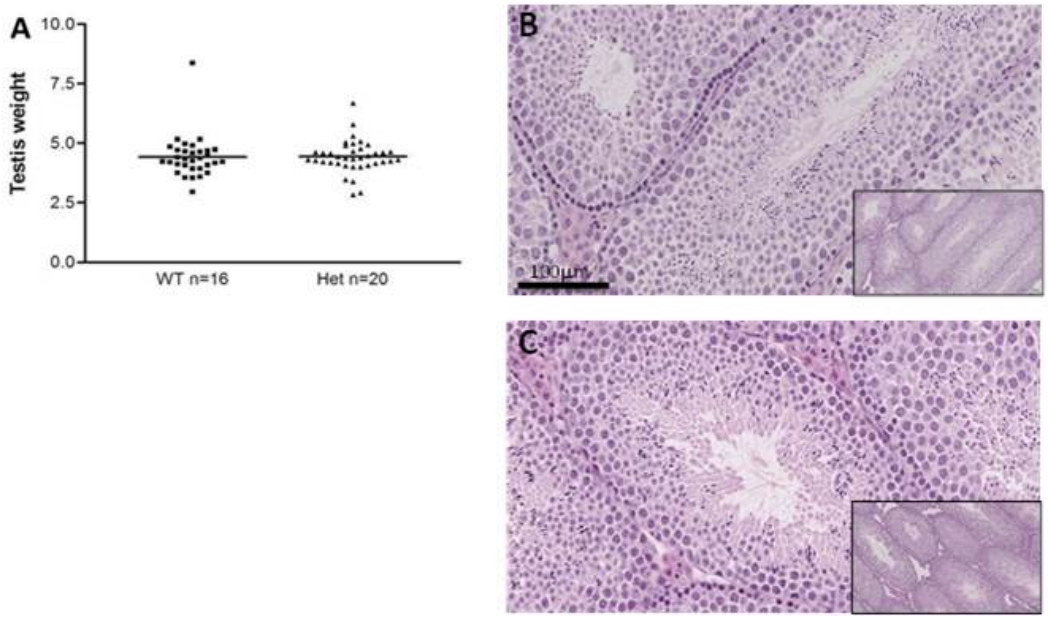


Figure 13. Testis weight and histology in *Dnd1*$^{+/+}$ and *Dnd1*$^{+/KO}$.

(A) Testes were removed and weighed from mice aged 6 to 10 weeks. Testes weights from adult heterozygous Dnd1-deficient males (Het, *Dnd1*$^{+/KO}$) and their wild-type control littermates (WT, *Dnd1*$^{+/+}$) were similar. Testes from wild-type males (WT) weighed an average of 4.43 ± 0.88g, compared to testes from heterozygous *Dnd1*$^{+/KO}$ males (4.44 ± 0.69g). Body weights did not differ significantly between WT (*Dnd1*$^{+/+}$) and Het (*Dnd1*$^{+/KO}$) (data not shown). Histology of these testes show no morphological differences between *Dnd1*$^{+/+}$ (B) and *Dnd1*$^{+/KO}$ (C). These results suggest that testes in *Dnd1*$^{+/KO}$ males do not show significant germ cell deficiency.

sense mediated decay (Maquat, 2004). The premature *Ter* stop codon is located 37 nucleotides upstream of the 3'-most exon-exon junction, within the 50-55 nucleotide range that fails to elicit nonsense-mediated decay (Figure 10, p.114). As a result, a translated DND1^{Ter} protein could be created consisting of *Dnd1* exons 1-2 and part of exon 3, with an intact RRM but a disrupted HRAAAMA.

The RRM within DND1^{Ter} may still recognize its target mRNAs, but any functional or regulatory capabilities normally derived from the deleted portion of DND1 would be absent. This supposition is supported by the work with the rat DND1^{Ter} mutation, where the GFP-DND1^{Ter} fusion protein is detectable in Huh7 cells (Northrup et al., 2012). The normal cellular processes that DND1^{Ter} might disrupt are numerous because RNA binding proteins have diverse functions, including alternative splicing, RNA stability, miRNA regulation, and translation control (Bandziulis et al., 1989; Fry et al., 1986; Query et al., 1989). To date, antibodies are not available for mouse DND1, thereby precluding many obvious studies.

Recent work revealed an essential role of a putative ATPase domain in DND1 (RAAAE) in PGC survival (Liu and Collodi, 2010). This previously un-annotated domain shares homology with RAAA (amino acids 178-181) in mouse DND1 where the arginine residue represents the amino acid substituted with a stop codon in *Dnd1^{Ter}* (Youngren et al., 2005). Thus any ATPase activity in mouse DND1 possesses would be lost in DND1^{Ter}. This region is also part of a larger motif in DND1, HRAAAMA, which is highly conserved and found in many paralogous RNA binding proteins, including Apobec complementation factor (A1CF) and suggests that this motif is functionally important in this gene family (unpubl.).

Although DND1^{Ter} appears to be a novel variant that increases TGCT risk; its tumourigenic effects are limited to the 129 strain of mice, which is not surprising, since this is the mouse strain that is susceptible to spontaneous TGCTs (Courage and Nadeau, 2012; Stevens and Hummel, 1957). This finding highlights the importance of genetic background in TGCT susceptibility, which has been observed in humans, particularly in the disparate TGCT rates among ethnic groups (Cancer Research UK, 2011; Chia et al., 2010b). The importance of genetic background is also evident in the interaction of DND1^{Ter} and mouse background; the 129 strain interacts positively with DND1^{Ter} to increase TGCT risk, whereas C56BL/6J and other strains only exhibit phenotypes related to some but not all aspects of mutations in genes such as *Kit*, *Kitl*, *Pten*, *Eif2s2I* and other TGCT modifier genes, but not TGCTs (Lam et al., 2004; Zhu et al., 2010; Krentz et al., 2013). However, the tumourigenic effects of *Dnd1^{Ter}* are not limited to PGC transformation in the 129 strain mice. C57BL/6J mice with the *Apc^{Min}* mutation are highly susceptible to intestinal polyps (Moser et al., 1990). However, when mice are compound heterozygotes for both *Apc^{Min}* and *Dnd1^{Ter}*, polyp numbers and polyp burden are significantly increased (Figure 12, p.126). These results suggest that DND1^{Ter} affects pathways that in both PGC transformation and intestinal polyposis.

Embryonic lethality is the usual interpretation for biased genotypic distributions in intercrosses, especially with complete loss of homozygous mutants. However

this interpretation is difficult to reconcile with normal litter sizes. In particular, loss of ~50% of intercross progeny (Table 8A) should lead to average litter sizes that are ~2 rather than ~4 pups per litter (Table 8C). We are aware of very few examples where substantial embryonic lethality does not affect litter size (Landrock et al., 2010). Instead, we propose that the unusual genotypic distributions result from biased allelic inheritance where specific combinations of oocyte and sperm are favoured (or disfavoured). Normal segregation in reciprocal backcrosses with the 129S1/SvImJ suggests that gametes are produced in sufficient numbers and are adequately functional (Table 8C). Instead results for intercrosses suggest that deficits in both females and males are needed to bias allelic inheritance. Together these results suggest that the bias arises at

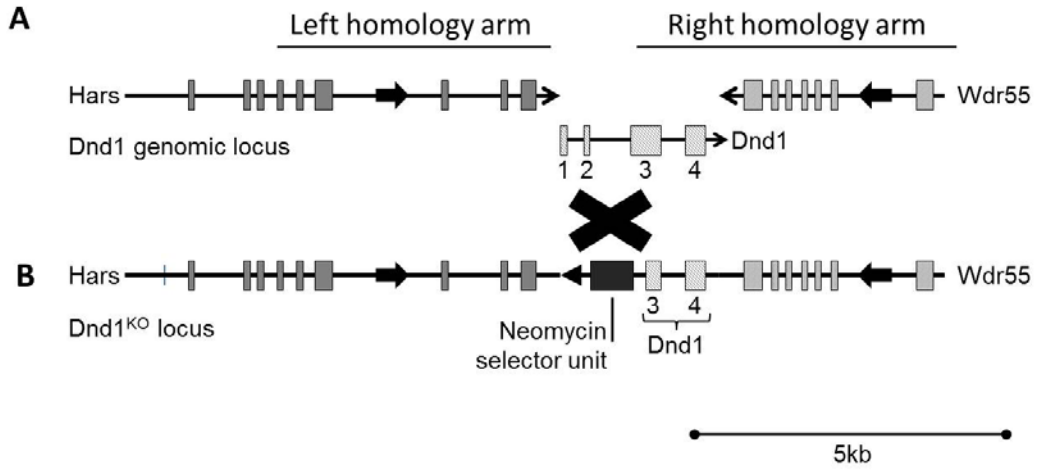


Figure 14. Generation of *Dnd1*^{KO} knockout mice.

(A) Genomic *Dnd1* locus showing flanking *Hars* and *Wdr55* genes. Exons are shown as boxes and arrows show the direction of transcription. The left and right homology arms of the targeting construct are indicated. (B) Following homologous recombination, exons 1, 2 and a portion of 3 of *Dnd1* are removed and a neomycin selection unit is introduced. The *Hars* and *Wdr55* genes remain intact.

fertilization. Interestingly, genetically and functionally related genes also show biased segregation, including *Apobec1* and *A1cf* (Blanc et al., 2005; Nelson et al., 2012).

The contrasting effects of different classes of *Dnd1* mutations may explain why sequencing studies failed to yield mutations within *DND1* in human TGCT cases (Linger et al., 2008; Sijmons et al., 2010). Complete loss of DND1 function is not sufficient to promote TGCT formation, even on the TGCT-susceptible 129S1/SvImJ inbred genetic background; reducing the chances that *DND1* mutations play a significant role in human TGCTs. Results from zebrafish support this interpretation. Loss of *Dnd1* inhibits PGC migration and results in PGC deficiency, but produced no TGCTs (Weidinger et al., 2003). TGCTs have been previously reported in zebrafish (Neumann et al., 2011b; Neumann et al., 2011a). *Dnd1*^{Ter} homozygotes also show reduced migration and loss of PGCs, with typically less than 20 PGCs arriving to the presumptive fetal gonads (Noguchi and Noguchi, 1985). Instead, our results show that it is the loss of the carboxy-terminus of DND1 protein that increases TGCT risk. The role of DND1^{Ter} in TGCT formation is further supported by recent results in a rat study where a spontaneous mutation producing a premature stop codon in exon 4, similar to *DND1*^{Ter}, resulted in TGCTs (Northrup et al., 2012). To date, human *DND1* mutations have not yet been reported that yield a similarly truncated DND1 protein.

Conclusions

Through intercrosses with mice carrying a complete loss of function *Dnd1*^{KO} allele, our study showed that DND1 is necessary for embryonic viability and results in abnormal allelic segregation. We have further shown that *Dnd1*^{Ter}, previously believed to be a loss-of-function allele, is likely translated into a protein that retains some normal DND1 function. Crosses with the *Dnd1* knock-out and *Ter* alleles revealed that the effects of the *Ter* allele on TGCT incidence depend on *Ter* dosage. Expression of the *Ter* allele in the *APC*^{Min} model of intestinal polyposis also significantly increased polyp burden. These results demonstrate that Dnd1Ter enhances tumourigenesis in two separate mouse models of cancer.

Acknowledgments

We thank Sabine Schaefer for her helpful comments on a draft of this paper. NCI grant CA75056 supported this work.

Chapter 4: Summary of Conclusions and Future Directions

Summary of conclusions and evidence

The incidence of testicular germ cell tumours is increasing throughout the world, more than doubling in the last 40 years (Bosl GJ et al., 1997). TGCTs are also one of the most heritable cancers (Nelson et al., 2012; DeRossi et al., 2006; Landrock et al., 2010; Edsgard et al., 2013; Heimdal et al., 1997), yet very few of the genetic contributors to the disease have been identified (Manolio et al., 2009). The missing heritability is due, in part, to the complexity of the disease (Stevens and Mackensen, 1961). Not only are there numerous genes, both known and unknown, that contribute to TGCT susceptibility; but there are additional epigenetic modulators that are capable of tempering an individual's risk of developing the disease. These epigenetic changes are just beginning to be deciphered, there remains a great deal of work to be done (Lind et al., 2007). Finally, because it is improbable that shifts in genetic or epigenetic profiles have occurred simultaneously throughout the world's male population, there is likely an environmental contribution to either the development of TGCTs directly or through modulation of the PGC epigenome.

The complex nature of TGCT susceptibility presents a significant challenge to the identification of the individual components that contribute to the disease. Because the majority of the susceptibility factors each contribute only a small portion of overall risk, it is possible that some contributors to disease predisposition are overlooked due to overall genetic diversity in human

populations. The questions addressed in my thesis minimize variation within study subjects by using genetically fixed mouse models of TGCTs.

Spontaneous metastasis of TGCTs in mouse models:

Chapter two dealt with spontaneous metastasis in a mouse model of TGCTs. Based on a fortuitous finding, this work set out to delineate the nature of these metastases. First we characterized the histological profile of these tumours to determine that they fulfilled the requirements to be called germ cell tumours. Next we demonstrated that these were true metastases that originated from within a primary TGCT and not extra-gonadal germ cell tumours that arose independently within the animal. Lastly, we surveyed a large number of animals to determine the rate of metastasis in animals with known TGCT rate modifiers (129-Chr19^{MOLF}, *Kitl*$^{Sl\text{-}J/+}$ and *Ter*) as well as in the 129S1/SvImJ background strain.

As a result of our large tumour surveys, we were able to determine the metastatic rates in mice with a number of different mutations that modify TGCT rates. Interestingly, we found that the rate of metastasis did not fluctuate according to TGCT rate; when the number of metastases was normalized to the number of primary tumours each strain tested exhibited a similar metastatic rate. This metastatic rate was also found in the background 129S1/SvImJ strain; suggesting that the susceptibility to spontaneous metastasis is a function of the 129 strain of mice. It is tempting to speculate that the genetic predisposition that is conducive

to TGCT formation in 129 strain mice is also responsible for these metastases; further work is needed to tease out the genetic contributions that the 129 strain makes to metastasis.

This study established a novel mouse model to study spontaneous TGCT metastasis. The ability to study metastasis on a genetically fixed background may provide the opportunity to identify mutations that contribute to metastatic potential separate from TGCT susceptibility. It also allows for controlled studies into the nature of TGCT metastasis and may one day be useful for treatment and drug studies.

The surveys in my study revealed that the tumours found outside of the gonads had initiated from within primary TGCTs and were not extra-gonadal in origin. In the literature, so-called extra-gonadal germ cell tumours (EGCTs) are very rare, making up 2-5% of all germ cell tumours (Pottern and Goedert, 1986; Schmoll, 2002). More recently the etiology of EGCTs is being questioned; advanced imaging and diagnostic techniques reveal that most EGCTs are actually the metastases from silent or "burned out" TGCTs (Fabre et al., 2004; Oosterhuis et al., 2005). My results are consistent with the hypothesis that EGCTs are in fact TGCT metastases; this distinction has important implications with regards to treatment modalities in human patients. The results of my study also contradict the assertion that EGCTs are the result of flaws in the PGC migration programme (Dixon and Moore, 1953; Fine et al., 1962; Hailemariam et al., 1997; Oosterhuis

et al., 2005). Mice with a known PGC migration defect (129S1/SvImJ-*Kitl*$^{Sl\text{-}J/+}$) have a similar number of metastases compared to mice with normal PGC migration.

The ability of mouse TGCTs to metastasize calls into question the long-held assertion that mouse model TGCTs are representative of pediatric TGCTs and do not accurately model the adult disease (Kersemaekers et al., 2002; Oosterhuis and Looijenga, 2005). Pediatric TGCTs, including infantile teratomas and yolk sac tumours rarely metastasize (see Table 1, p.26), whereas adult teratomas and other TGCTs have a high metastatic rate.

Questions and future directions:

The discovery of a spontaneous model of TGCT metastasis provides the opportunity to study the most relevant and deadly aspect of this disease in a normal physiological environment; prior studies used intravascular injection of cancer cells to simulate vascular spread of tumours (Kahan, 1987; Roomi et al., 2012) or chemical induction of metastasis (Tatsuta et al., 2001; Iishi et al., 1992). This mouse model may allow researchers to answer fundamental questions about TGCTs that will impact treatment modalities and have a positive effect on patients' outcomes. For example, due to the spontaneous nature of this mouse model it is possible to determine the critical time points of metastatic dissemination. Because the metastases occur on a fixed genetic background, it is

also feasible to establish if there is a particular molecular signature that can differentiate between metastatic and non-metastatic primary TGCTs. Together, the results of these experiments could allow clinicians to ascertain if a patient's primary TGCT is likely to metastasize and if those metastases are already disseminated. This information would affect treatment and follow up choices to minimize unnecessary procedures or treatment delays.

When do TGCTs acquire metastatic potential?

Cancer research has shown that there can be a long latency between the diagnosis of a primary tumour and the appearance of clinically recognizable metastases. In certain cancers, including breast cancer, this latency period may be several decades (Johansson et al., 2004; Margaret Lee, 1985; Triozzi et al., 2008); in other cancers the latency period may be virtually non-existent (Hess et al., 2006; Janne et al., 2002). In testicular cancer this latency period is typically very short, with the majority of relapses occurring within the first two years. Late recurrence is defined as recurrence more than two years after completion of the first line treatment in the absence of a second primary tumour (in the contralateral testicle). The average rate of late relapse is 2-4%; 3.1 per 1000 person-years of follow up for non-seminomas (Ronnen et al., 2005) and 1.4 per 1000 person-years of follow up for seminomas (Oldenburg et al., 2006). Although the median time to late relapse is 7-10 years the time interval can vary greatly, from 2-32 years (Ronnen et al., 2005; Oldenburg et al., 2006). Data also demonstrates that disseminated

metastases may lie dormant for many years prior to secondary growth (Croker et al., 2009).

Adult-onset TGCTs have a long quiescent phase, since they are known to originate in utero yet they do not actively proliferate and grow prior to the onset of puberty. This extremely long period of relative dormancy may also allow for the undetected dissemination of metastases that also enter a period of inactivity upon reaching a target tissue. These experiments are designed to determine if mouse TGCTs metastasize early in the disease course, or if they are a secondary event that is triggered by tumour growth. Identifying the timing of metastatic dissemination may help to alter treatment modalities and outcomes for patients.

These experiments will use the 129-M19 MOLF mouse model (see M19/M19, p.59). These mice have a very high rate of primary TGCTs, of which approximately 11% will have metastases. To determine the time of metastatic onset, live animal surgery will be performed to remove TGCT affected testicles at various time points in animal development, then animals will be sacrificed and surveyed for metastases. Complimentary experiments will be done using a line of 129–M19 MOLF mice that express an OCT4 promoter driven GFP reporter that has previously been characterized (Croker et al., 2009; Heaney et al., 2009). Cells that normally express OCT4 will be detectable via GFP fluorescence; and many metastases are OCT4 positive. Using this model we can perform serial whole

mount examinations of tissues and animals to look for GFP positive cells that may represent dormant TGCT metastases during development.

Do metastatic TGCTs have a genetic signature?

The treatment of TGCTs carries a wide range of physical and mental burdens; which are multiplied when additional treatments are required for metastases. The ability to identify the likelihood of metastasis from a genetic signature from within the primary TGCT would prevent unnecessary treatments in patients that do not have secondary tumours; and would eliminate delays in treatment for patients that have metastases. Although some progress has been made with regards to molecular and genetic signatures of metastases for other tumours (Ramaswamy et al., 2002; Carter et al., 2006; Wang et al., 2004) there is no such signature currently available for TGCTs.

These experiments will focus on comparisons between metastatic and non-metastatic primary TGCTs. Because it is evident from the discussion regarding risk factors for TGCTs that there are a number of factors that contribute to TGCT formation; it is likely that multiple elements also influence the acquisition of metastatic potential. Therefore our studies will seek to incorporate genomic and epigenetic alterations as well as gene and miRNA expression profiles into a single signature that identifies metastatic versus their non-metastatic counterparts.

To minimize the effects of genetic diversity on the analyses we will use two model systems: our mouse models of TGCTs and the WKY/Dnd1^{ter}/Ztm inbred

rat strain identified by Northrup (Northrup et al., 2012). Both of these models are useful because they occur on inbred strains and are known to be metastatic. These two models will be used to develop a test set that will then be used to query human TGCTs. Further refinement of the metastatic signature will then be necessary to accommodate for genetic differences between TGCT subtypes.

Some of this work has already begun using mouse model TGCTs from our lab. Results of copy number variations specific to metastatic primary TGCTs have been negative (see Appendix A, p84); but we have identified a copy number variant that includes two genes, *Adam6a* and *Adam6b*. Preliminary work done with *Adam6b* has shown that higher copy number of this gene may be an indicator of metastatic potential.

Chapters two and three are tied together by not only a common disease, but also by the nature of their results. These two chapters have demonstrated there remains a great deal to be learned about and from these mouse models, which is remarkable in light of the fact that they have been used in testicular cancer research since the early 1960s (Stevens and Hummel, 1957; Stevens, 1967a; Stevens, 1967c).

Dnd1^{Ter} *is not a loss of function allele*

Chapter three focused on the evidence that *Dnd1*^{Ter}, originally believed to be a loss of function allele (Youngren et al., 2005), likely produces a DND1^{Ter} protein that is responsible for the increased TGCT rate in 129 strain male mice. Through

crosses with mice from the intestinal polyp model, *APC*^{Min}, we also found that the effects of DND1^{Ter} are not limited to the testis. This dual impact may be the result of either a general pro-oncogenic function of DND1^{Ter}, or DND1^{Ter} interfering in a developmental pathway that is shared between TGCTs and intestinal polyps. In the course of this work, we were surprised to discover that *Dnd1* was essential for embryonic survival. More intriguing, we also found that the loss of a copy of *Dnd1* altered allelic segregation in intercrosses between animals with a single copy of *Dnd1*, but not in outcrosses between *Dnd1* heterozygous knockout animals and wild-type animals. These aberrations occurred before E3.5, suggesting that the loss of *Dnd1* may be conducive to a preferential fertilization environment. The findings from Chapter three raise many questions regarding the mechanisms responsible for the development of neoplasms in the intestine and testis and the observed irregular allelic segregation.

Questions and future directions

There are three fundamentally different aspects to the data that is presented in chapter three, and each of these represents the initial observation for new inquiries. Perhaps the most intriguing aspect of this work is that it is not limited only to testicular germ cell tumours, but also impacts embryonic development and intestinal polyposis.

Does loss of Dnd1 act through Geminin to influence embryonic survival?

These data reports first evidence DND1 may be necessary for embryonic survival as well as for PGC survival (Noguchi and Noguchi, 1985; Weidinger et al., 2003). Although DND1 has a number of described functions, the only known function it may play in embryonic survival is through the translational regulation of *Geminin* (*Gmnn*) mRNA (Chen et al., 2010). Loss of GMNN in mouse embryos results in embryonic death at the 8 cell stage and a failure to form an inner cell mass (Gonzalez et al., 2006; Hara et al., 2006). Importantly, *Gmnn* is expressed specifically in male germ cells (Eward et al., 2004) and helps to regulate the expression of the pluripotency genes SOX2, OCT4 and NANOG (Yang et al., 2011). Loss of DND1 may decrease or eliminate GMNN protein levels and play a role in the absence of $Dnd1^{KO/KO}$ embryos. These experiments would use embryonic stem (ES) cells that were wild-type for *Dnd1* and heterozygous for the *Dnd1* knockout to determine if reductions in DND1 negatively impact GMNN protein levels. Further work would include siRNA knock-down of DND1 to measure the effects of complete DND1 loss on GMNN protein levels.

Based on previous reports (Chen et al., 2010) and unpublished data (Figure 15, p.145), it is expected that loss of DND1 would decrease GMNN protein levels based on decreased translation and decreased mRNA levels. To determine if DND1 knockdown in ES cells can contribute to embryo development, stable

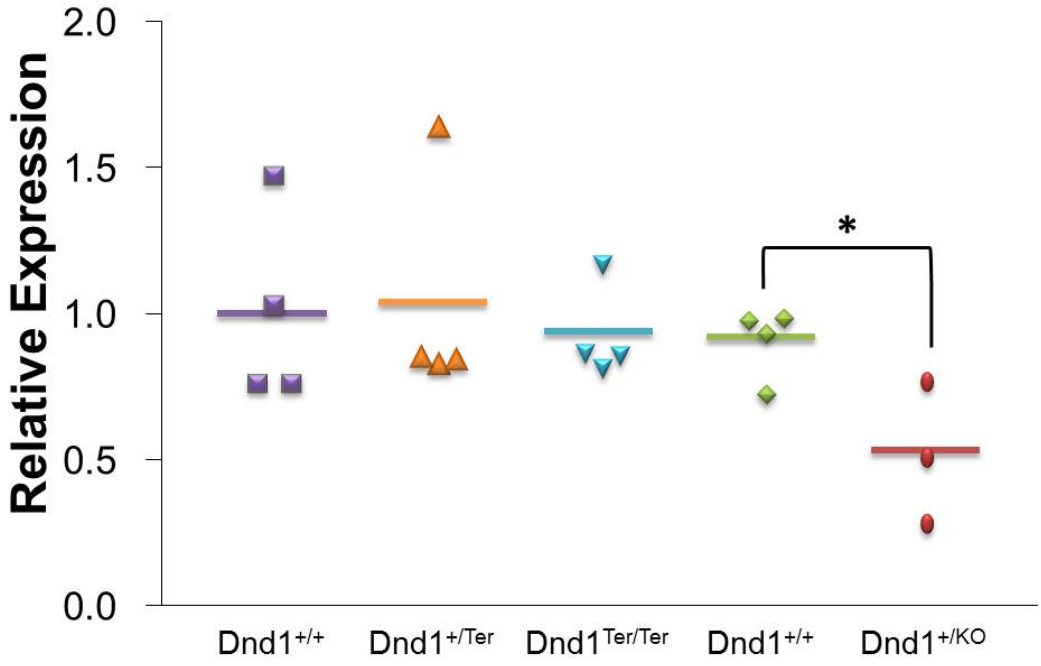


Figure 15. Geminin mRNA levels in ES cells.

Geminin mRNA levels were measured in ES cells derived from timed matings of *Dnd1*$^{+/Ter}$ × *Dnd1*$^{+/Ter}$ to generate *Dnd1*$^{+/+}$; *Dnd1*$^{+/Ter}$ and *Dnd1*$^{Ter/Ter}$ ES cells or from *Dnd1*$^{+/KO}$ × *Dnd1*$^{+/KO}$ crosses to generate *Dnd1*$^{+/+}$ and *Dnd1*$^{+/KO}$ ES cells. Relative mRNA levels are not different in ES cells from Dnd1+/Ter crosses, but mRNA levels are significantly decreased in *Dnd1*$^{+/KO}$ ES cells compared to their wild-type counterpart as measured by a t-test (*$p<0.05$; $t=2.8$).

knockdown of DND1 using shRNAs in GFP tagged ES cells would be used in blastocyst injections to develop chimeric mice.

Secondary experiments would focus on the role of $DND1^{Ter}$ in GMNN protein expression. Our own observations (Figure 15, p.145) show that ES cells homozygous for $Dnd1^{Ter}$ have Gmnn mRNA levels that are similar to wild-type animals (Figure 15), but because DND1 is known to act at the level of translation these results may not reflect the end result of $DND1^{Ter}$ expression on GMNN protein levels. These experiments would use the ES cells already described, as well as heart tissues from $Dnd1^{Ter}$ animals to probe GMNN levels by immunohistochemistry and western blot. If the *Ter* allele of *Dnd1* retains the ability to positively regulate GMNN protein levels, this may explain the ability of $DND1^{Ter}$ to rescue the loss of $Dnd1^{KO}$ embryos.

Does Dnd1KO affect allelic segregation through selective fertilization?

Loss of a copy of *Dnd1* alters *Dnd1* allele segregation when animals that are heterozygous for the $Dnd1^{KO}$ allele are intercrossed, but not when they are outcrossed with wild-type animals. Aberrations in allelic segregation are infrequently seen, but have been observed in other crosses (Nelson et al., 2012). The underlying mechanism is unknown, but may involve selective fertilization.

Selective fertilization has been described in many organisms with regards to sperm competition (Levine, 1967; Overstreet and Adams, 1971; Dunn, 1927) and the effect of maternal diet on sex ratios (Song, 2012; Wang et al., 2011; Anderson

et al., 2012). To test if selective fertilization is responsible for the abnormal segregation seen in *Dnd1*$^{+/KO}$ intercrosses, we will use a combination of molecular and physiological tests.

There is some suggestion that small RNAs, including miRNAs may influence spermatogenesis and sperm motility (Rajender et al., 2012; Lee et al., 2010; Curry, 2010) and may influence oocyte function (Suh et al., 2010; Yang et al., 2012). To determine if loss of DND1 affects either sperm production, motility or oocyte function we will analyze sperm and ovulated eggs from heterozygous *Dnd1*$^{+/KO}$ animals to determine if the overall allelic distribution is normal in these animals. Based on the results seen in outcrosses (Table 8C, p.116), we expect to see a normal 1:1 ratio of *Dnd1*$^{+}$ and *Dnd1*^{KO} alleles in these animals. Next we will test the overall motility of sperm from *Dnd1*$^{+/KO}$ males to determine if there differences between sperm with the *Dnd1*$^{+}$ and *Dnd1*^{KO} alleles.

If the ratios of *Dnd1*$^{+}$ and *Dnd1*^{KO} alleles are normal in sperm and oocytes, then we will need to determine the source of segregation bias. To examine the possibility of preferential fertilization, we will perform *in vitro* fertilization (IVF) using sperm and eggs obtained from *Dnd1*$^{+/KO}$ animals and genotype the resulting embryos to determine allelic distribution. If there no bias present in offspring from IVF, then the bias may be the result of the environment in the oviduct. Should this be the case, we will examine sperm and E1.0 embryos flushed from *Dnd1*$^{+/KO}$ intercrossed females. These experiments will help to delineate the

source of the segregation bias seen in *Dnd1*$^{+/KO}$ intercrosses and may uncover a novel role for DND1 in sperm or oocyte function.

Does DND1^{Ter} *share a common pathway between TGCT initiation and polyposis?* The data from chapter 3 shows that the *Ter* allele of *Dnd1* is not a loss of function allele, and that in tissues where DND1^{Ter} is likely expressed it acts in a pro-oncogenic manner to increase TGCT rates (in testes) or polyp numbers and burden (in *APC*^{Min}-driven colon polyposis). This provides the opportunity to test if DND1^{Ter} acts in a pathway shared between the two oncogenic processes or if its pro-oncogenic properties are multi-faceted.

To differentiate between these possibilities we will use a tagged DND1 and DND1^{Ter} proteins to bind proteins and mRNAs extracted from both PGCs sorted from fetal gonads and colon polyps. Identification of binding partners unique to DND1^{Ter} may provide clues to the pathways that are affected by DND1^{Ter}, and if the pathways are shared or different in TGCTs and intestinal polyposis. We will also examine the Wnt signalling pathway in these cells, with particular emphasis on CDKN1B (p27). Previous data has shown that DND1 regulates CDNK1B translation (Kedde et al., 2007) and CDNK1B interacts in the Wnt pathway with APC (Figure 16, p.149). CDNK1B may act as a modifier of polyposis (Kedde et al., 2007; Wilding et al., 2002) and play a role in TGCT development (Bourdon et al., 2002; Grygalewicz, 2001; Heaney et al., 2009).

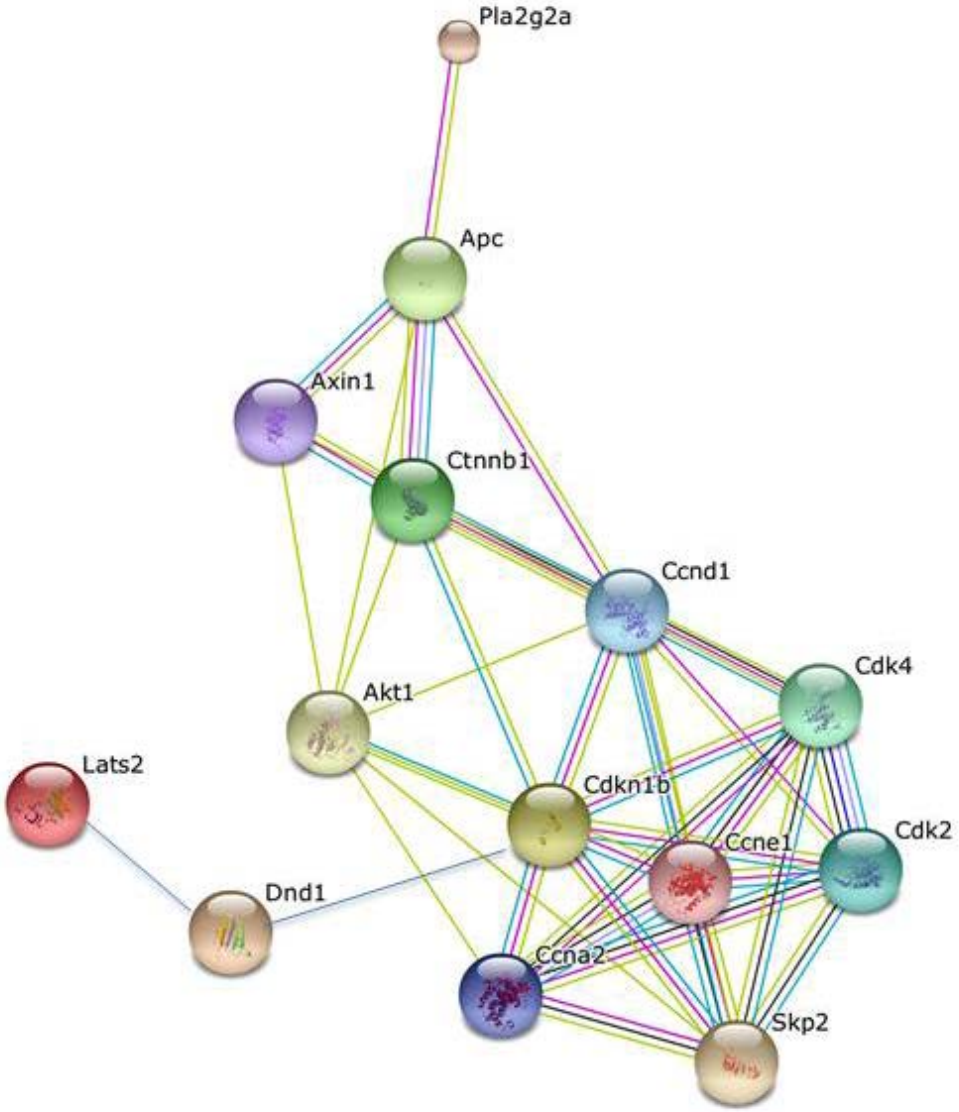


Figure 16. Interaction network that connects DND1 and APC.

DND1 is a known regulator of p27 (CDKN1B) mRNA levels (Kedde et al., 2007). CDNK1B interacts in the Wnt pathway above (from http://string-db.org) with APC and may act as a modifier of polyposis (Kedde et al., 2007; Wilding et al., 2002). CDNK1B may also play a role in TGCT development (Bourdon et al., 2002; Grygalewicz, 2001; Heaney et al., 2009).

Does Dnd1^{Ter} act differently to regulate CDKN1B (p27)?

It is known that Dnd1 binds to the 3'UTR of CDKN1B (p27), and regulates its translation by inhibiting the access of miRNAs (Kedde et al., 2007). We obtained the p27 reporter construct used in those assays, and measured the effects of DND1 and DND1^{Ter} on the p27 reporter in HEK293 cells. Preliminary data shows that transfection with *Dnd1* may increase reporter activity, since it prevents miRNA binding and inhibition of translation. DND1^{Ter} reduces reporter activity (Figure 17, p.151). Co-transfection of *Dnd1* and *Dnd1^{Ter}* reduces activity compared to control levels. The mechanism by which this occurs is unclear. We will used tagged *Dnd1* and *Dnd1^{Ter}* constructs to determine if DND1^{Ter} is able to bind to *Cdkn1b* mRNA, preventing access of wild-type DND1 but allowing miRNA access; or that (2) DND1^{Ter} acts to hetero-dimerize with DND1 and prevent its binding to *Cdkn1b* mRNA. These experiments may provide some mechanistic evidence regarding the function of DND1^{Ter} in translational control of mRNA targets.

~ *Fin*

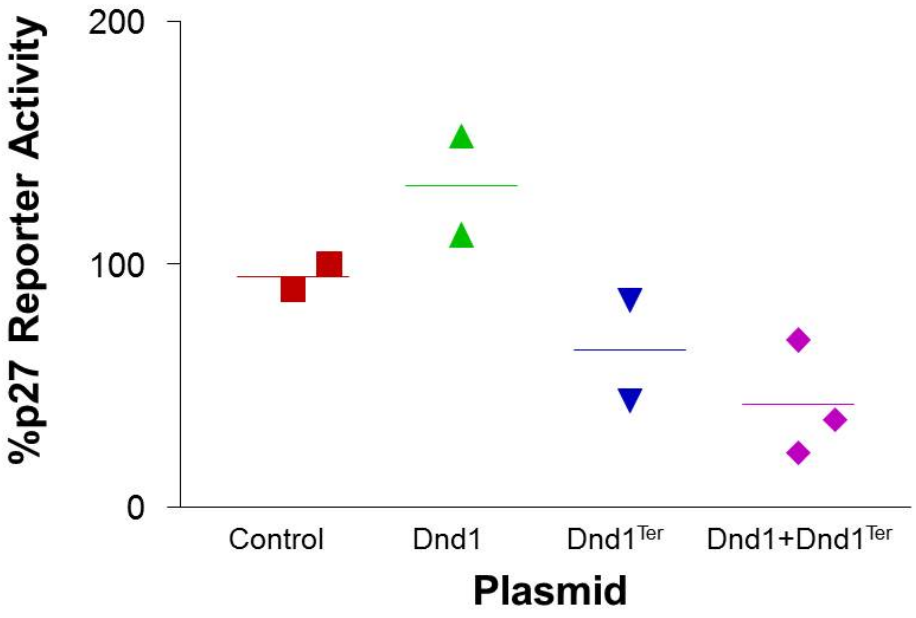


Figure 17. p27 reporter activity in *Dnd1* and *Dnd1*^{Ter} transfections.

HEK293 cells were transfected with a p27 reporter construct obtained from R. Agami and either empty plasmid (control) or *Dnd1*, *Dnd1*^{Ter} or both *Dnd1* and *Dnd1*^{Ter} together. As expected, transfection with *Dnd1* increases the reporter activity, since it prevents miRNA binding and inhibition of translation. *Dnd1*^{Ter} reduces reporter activity when transfected alone or in conjunction with *Dnd1*.

Appendices

Appendix A – Common genomic changes in mouse TGCTs

Introduction

Morbidity and mortality that are associated with preventative treatment of TGCTs may be avoided if genomic DNA alterations that predict metastasis are identified. There is currently very little data from human TGCT patients that has examined DNA alterations as function of metastasis. To determine if there were any genomic DNA copy number changes that were present in primary TGCTs that subsequently metastasized we used comparative genomic hybridization (CGH) from the DNA of 4 primary TGCTs that were known to have generated metastases and 5 primary TGCTs with no detectable metastases.

Results:

In our samples there were no DNA changes unique to primary TGCTs that generated metastases compared to non-metastatic primaries. However, we did identify a region of DNA copy changes that were found in all 9 primary TGCTs.

The first region identified is from chr12, with a common region from 113 879 566-114 602 383 (mm8). There are very few genes found in this region: *Ighg*, *Adam6*, *Adam6a* and *Adam6b*. The Adams (**a d**isintegrin **a**nd **m**etalloproteinases) are a relatively new family of proteins that share a metalloproteinase domain with matrix metalloproteinases (MMPs), and have a disintegrin domain (for review, see (Mochizuki and Okada, 2007). Very little is known about ADAM6/6a/6b, except that it is restricted to the testis and has a role in fertilization (Han et al.,

2009). ADAMs are known to be involved in cancer cell proliferation and progression, and so we initially chose to verify these changes using real-time PCR quantifying *Adam6b*.

Initial results showed that each of the primary TGCTs had amplifications in this region (Figure 18, p.156). Further analysis determined that primary TGCTs that were known to have generated a metastasis (metastatic) had a higher copy number (mean = 3.7 ± 0.2) compared to TGCTs that did not generate metastases (benign; mean = 2.6 ± 0.1). Examination of 3 metastases themselves demonstrated that they exhibited small copy number losses (mean = 1.6 ± 0.02). The difference between the means in each of these groups is significant as analyzed by one-way ANOVA.

Although these results are preliminary, they do suggest that Adam6b may be involved in TGCTs, and that level of copy number change of this gene may be a marker for metastatic potential.

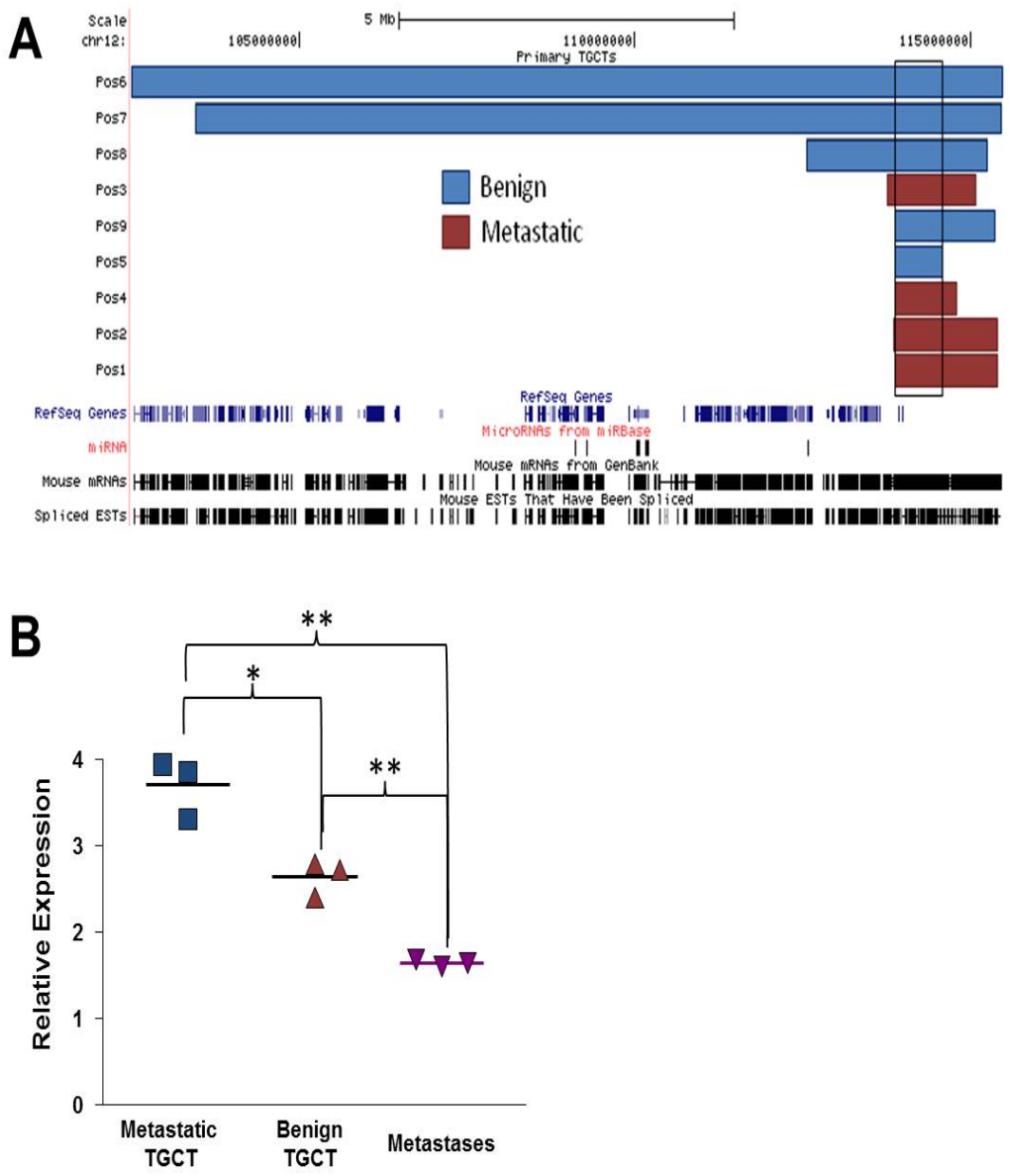


Figure18. Adam6b as a gene with copy number variation in TGCTs and metastases.

(A) Schematic display of the overlapping regions on Chr.12 from 9 primary TGCTs that were amplified. TGCTs are classified as benign (non-metastatic; blue) or metastatic (red). The common region is outlined in black. Copy number variation of Adam 6b within metastatic TGCTs, benign TGCTs (no metastases detected), and metastases. Metastatic TGCTs had significantly higher relative copy numbers of Adam6b (3.7 ± 0.2) compared to benign TGCTs (2.6 ± 0.1) and metastases (1.6 ± 0.02). Benign TGCTs also had a significantly higher Adam6b copy number compared to metastases. * $p<0.01$, ** $p<0.001$.

Appendix B. Does DND1 affect RNA editing?

Introduction

RNA editing

RNA editing is the changing of one or more positions in an RNA molecule, by insertion, deletion or base substitution. One of the first examples of RNA editing is also the best characterized. Through C-to-U substitution in the apolipoprotein B transcripts in mouse intestine, a stop codon is generated and results in a truncated ApoB48 protein (Anant and Davidson, 2001). This editing event requires single-stranded DNA and protein cofactors, including at a minimum APOBEC-1, which functions as the catalytic deaminase; and the adaptor protein apobec complementation factor (ACF) (Figure 19, p.161). Other APOBEC proteins include AID, which functions in activated B cells (Muramatsu et al., 1999); APOBEC2, which may function in inflammatory response (Matsumoto et al., 2006); and APOBEC3, which appears to have an antiviral role (Sheehy A, 2007).

A second type of substitutional RNA editing involves the substitution of an A-to-I through the involvement of adenosine deaminases (ADARs) (Gott and Emeson, 2000). A-to-I editing has a strict requirement for double-stranded RNA template and no protein co-factor requirement, which distinguishes it from C-to-U editing. In 2004, Levanon *et al.* identified 1637 potential targets of A-to-I editing alone (Levanon et al., 2004), and more recently Barak et al. described RNA editing of Alu repeats in human transcripts (Barak et al., 2009).

Mammalian development is dependent on RNA editing to generate alternative transcripts (Bass, 2002). Deletion of the ADAR1 gene is embryonic lethal (Wang et al., 2000) as is the deletion of ACF (Blanc et al., 2005), and ADAR2 knockout mice die shortly after birth (Higuchi et al., 2000), although Apobec1 knockout animals are normal and fertile (Nakamuta et al., 1996). Given the importance of RNA editing, it is to be expected that aberrant function of this process would be involved in disease. Several neurological diseases are associated with alterations in RNA editing, including schizophrenia (Sodhi et al., 2001), ALS (Takuma et al., 1999), and potentially Alzheimer's disease and Huntington's disease (Akbarian et al., 1995). Abnormal RNA editing has also been associated with cancer (for review, see (Skarda et al., 2009). Hyper-editing through over-expression of Apobec1 leads to carcinomas in transgenic animals (Yamanaka et al., 1997), and under-editing of the GluR-B receptor mRNA has been found in human malignant gliomas (Maas et al., 2001).

Editing of Wilm's Tumour mRNA

Wilm's Tumour gene (WT1) has also been identified as a target of RNA editing (Sharma et al., 1994). WT1 mRNA and protein is expressed in the testis and has been shown to be critical for germ cell proliferation or survival (Natoli et al., 2004) as well as for Sertoli cell differentiation (Klattig et al., 2007b). In the kidney, WT1 protein affects transcriptional repression efficiency and is

developmentally regulated; however the function of edited WT1 has not been described in the testis.

Editing of the mouse WT1 mRNA occurs at nucleotide 637. The edited mRNA has a non-synonymous change, converting a CTC to a CCC. In the kidney, the amino acid change, from a leucine to a proline affects the function of the WT1 protein as a transcriptional repressor (Sharma et al., 1994). The edited mRNA also loses a *MnlI* restriction enzyme recognition site in exon 6, which is conserved in mouse, rat and human WT1 mRNAs, making it a useful method to distinguish editing events.

Relative to the significant role of RNA editing in the generation of protein diversity and its potential role in disease, little is known about the targets of RNA editing. In fact, the list of genes known to undergo C-to-U editing is remarkably short (Klattig et al., 2007), leaving many potential targets of editing in germ cells unknown, any of which may play a role in TGCT development.

DND1 is most similar to APOBEC complementation factor (ACF) (Figure 20, p.162), which is involved in Apobec mRNA editing of apolipoprotein B (ApoB) and other mRNAs (Chester et al., 2004). These sequence similarities suggest a potential role for DND1 as a component of the editosome. Supporting this hypothesis, DND1 has been shown to interact with APOBEC3 *in vitro* (Bhattacharya et al., 2008), although the function of DND1 in this complex is unknown. Due to these similarities, we tested ApoB and WT1 mRNA for editing

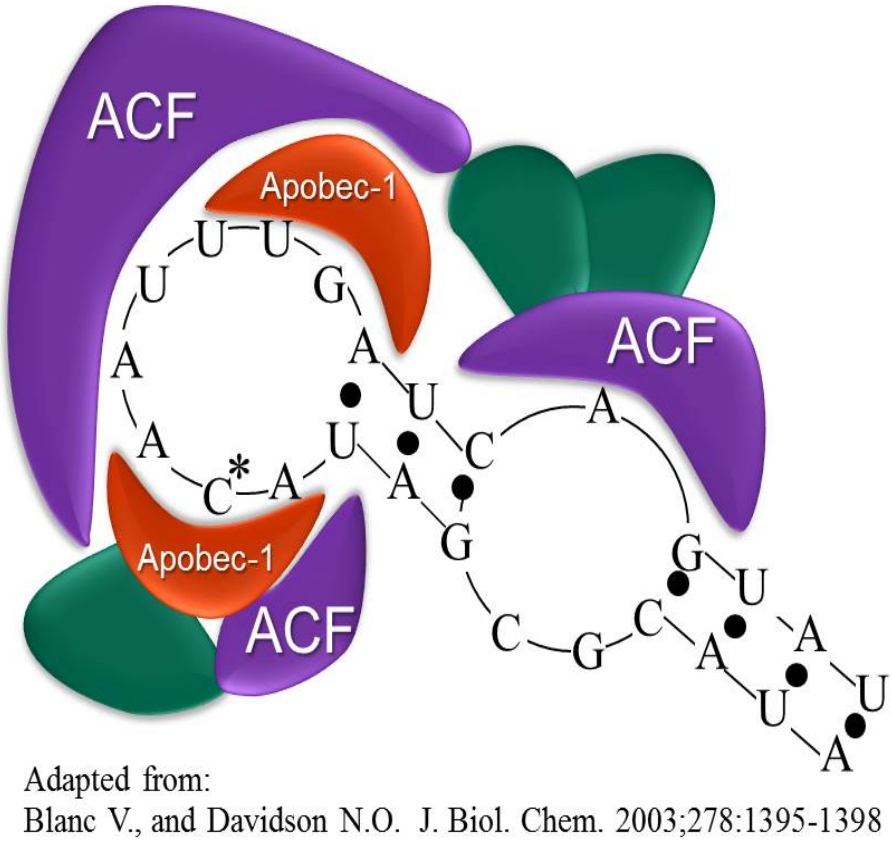


Figure 19. The model of ApoB RNA editing.

The edited base C (asterisk) is shown in association with Apobec-1. The schematic illustrates the core components of ApoB editing: apobec-1 (red) and ACF (purple) binding to RNA both 5′ both up-and downstream of the edited base. Additional proteins (green) may contribute to the editing complex. Adapted from (Blanc and Davidson, 2003).

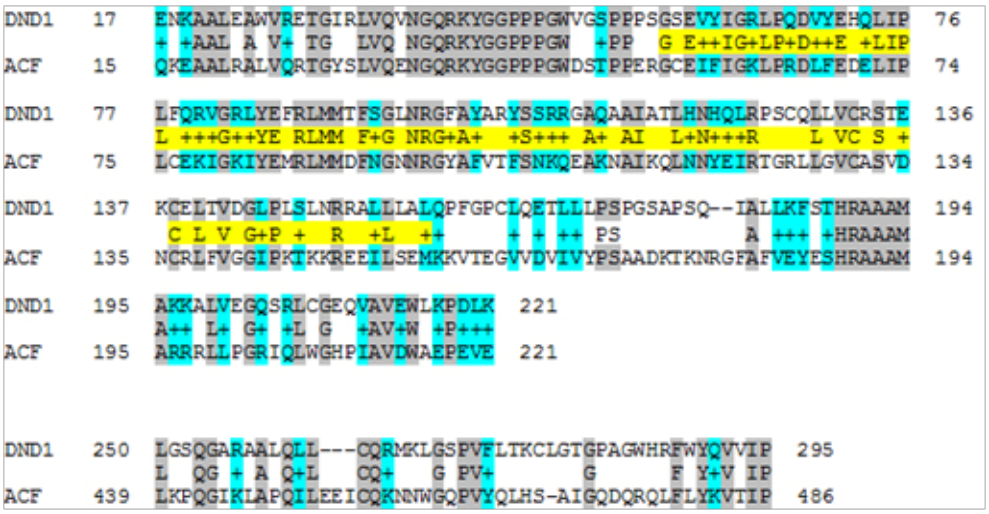


Figure 20. Homology of DND1 and ACF proteins.

Alignment of DND1 (gi:207446695) and ACF protein (gi:124487289) sequences. Sequences were aligned using Blastp alignment, showing 41% identical residues, and 67% homology. Gray highlights identical amino acids, and blue highlights indicate conserved residues. The RRM of both proteins is highlighted with yellow.

in wild-type 129S1/SvImJ and 129S1/SvImJ-*Dnd1*^{1er} to determine if the presence of DND1^{Ter} alters normal editing.

Results

Dnd1^{Ter} *affects ApoB mRNA editing*

To test whether sequence similarity between *Dnd1* and *Acf* reflects conserved functions in RNA editing, we measured apolipoprotein B (ApoB) editing in the

hearts of wild-type and *Dnd1*$^{Ter/Ter}$ mice because heart is one of the few tissues that expresses both DND1 and ApoB. Results suggest that ApoB editing is adversely affected in *Dnd1*$^{Ter/Ter}$ mutants.

129/Sv males with wild-type (WT) *Dnd1* or with one (+/*Ter*) or two copies (*Ter/Ter*) of the *Dnd1*^{Ter} mutation were used for the ApoB mRNA editing assays (Fig. 21). Samples from mice homozygous for the targeted deficiency of *Apobec1* were used as a negative control (9.6±1.4%). The assay was done using a SNaPshot assay, which is based on the single nucleotide extension of a primer specific to ApoB mRNA, interrogating the edited base.

Dnd1$^{Ter/+}$ heterozygotes showed editing levels that were similar to WT littermates (77.1±4.2% and 75.7±6.4%, respectively), whereas *Dnd1*$^{Ter/Ter}$ homozygotes had lower levels of editing (48±8.9%) and were considerably more heterogeneous (Figure 21). The wide variation of editing levels in the *Dnd1*$^{Ter/Ter}$ homozygotes approximates a bimodal distribution, although the mechanism by which this occurs remains unclear. Although each of the *Dnd1*$^{Ter/Ter}$ males used for this

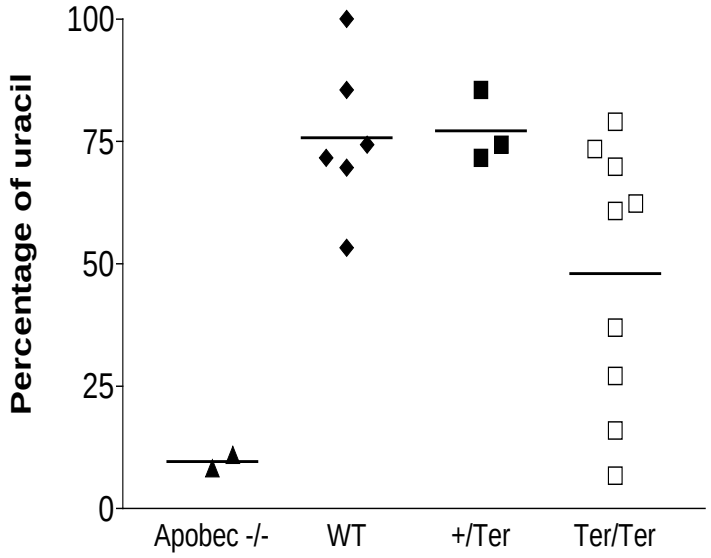


Figure 21. Uracil incorporation into ApoB cDNA SNaPshot assays.

Editing of the ApoB mRNA at codon 2153 converts cytidine to uracil. Heart mRNA from wild-type 129/Sv males (WT), and littermates with one (+/*Ter*) or two (*Ter/Ter*) copies of the *Ter* mutation was used in the assay. 129/Sv males deficient for Apobec1 (Apobec$^{-/-}$) were used as a negative control for ApoB editing. The percentage of uracil incorporation was calculated as the area under the dye peak corresponding to the edited uracil base divided by the total area of the uracil and cytidine (unedited) dye peaks.

study had bilateral TGCTs, it is possible that variation in physiological responses to testicular cancer affect RNA editing.

Dnd1^{Ter} affects WT1 mRNA editing

To test for editing changes in WT1, mRNA was isolated from adult male *Dnd1*$^{+/+}$ and *Dnd1*$^{Ter/Ter}$ heart tissue. After reverse-transcription, primers specific for WT1 were used to amplify a portion of the WT1 cDNA containing the edited nucleotide (Sharma et al., 1994). Sequence information for WT1 mRNA predicts a product of 277bp, with 3 *MnlI* restriction sites, giving fragments of 151bp, 53bp, 47bp and 27bp. The resulting product was digested with MnlI restriction enzyme, and then visualized on a 4% agarose gel with ethidium bromide. The 53bp and 47bp products are indistinguishable on an agarose gel, and the 27bp product is difficult to visualize as well. There were no apparent differences in WT1 mRNA editing in heart tissues between *Dnd1*$^{+/+}$ and *Dnd1*$^{Ter/Ter}$ (Figure 22A, p.167).

To determine if editing might be present in the gonads, male and female 129/S1$^{Dnd1+/Ter}$ were time-mated. The genital ridges from E13.5 male embryos were dissected and used to isolate mRNA. WT1 amplification and digest were performed as described above. There were no differences in WT1 mRNA editing between the E13.5 gonads from *Dnd1*$^{+/+}$, *Dnd1*$^{+/Ter}$ and *Dnd1*$^{Ter/Ter}$ (Figure 22B, p.167).

WT1 mRNA is known to be edited in adult kidney (Sharma et al., 1994). We used a SNaPshot reaction to determine if the shift in WT1 editing levels is more subtle than can be detected using DNA cleavage and electrophoresis. We chose to use kidneys from adult male 129S1*Dnd1*$^{Ter/Ter}$, 129S1*Dnd*$^{+/+}$ and 129S1*Apobec*$^{-/-}$ mice to measure the levels of WT1 editing. Preliminary results (Figure 23, p.168) from these assays suggests that there may be a slight increase in the percent of unedited WT1 mRNA in *Dnd1*$^{Ter/Ter}$ kidneys compared to *Dnd1*$^{+/+}$ kidneys (30.57 ± 0.42% and 28.13 ± 0.98%, respectively). Apobec1 deficient males have unedited WT1 mRNA levels that are similar to *Dnd1*$^{+/+}$ animals (27.92 ± 1.47%). Although ANOVA analysis of these results is not significant (p=0.0544) due to the limited number of samples per group; a two tailed t-test between the *Dnd1*$^{Ter/Ter}$ and *Dnd1*$^{+/+}$ groups does show a significant change (p=0.027).

The possibility that WT1 mRNA editing may be altered in *Dnd1*$^{Ter/Ter}$ animals suggests that this alteration may contribute to TGCT formation. Among the known targets of WT1 are Splicing Factor 1 (*Sf1*), the absence of which has been shown to suppress TGCT rates (Zhu et al., 2010) and *Sprouty1*, which is confined to the testis and brain (Sabatel et al., 2010).

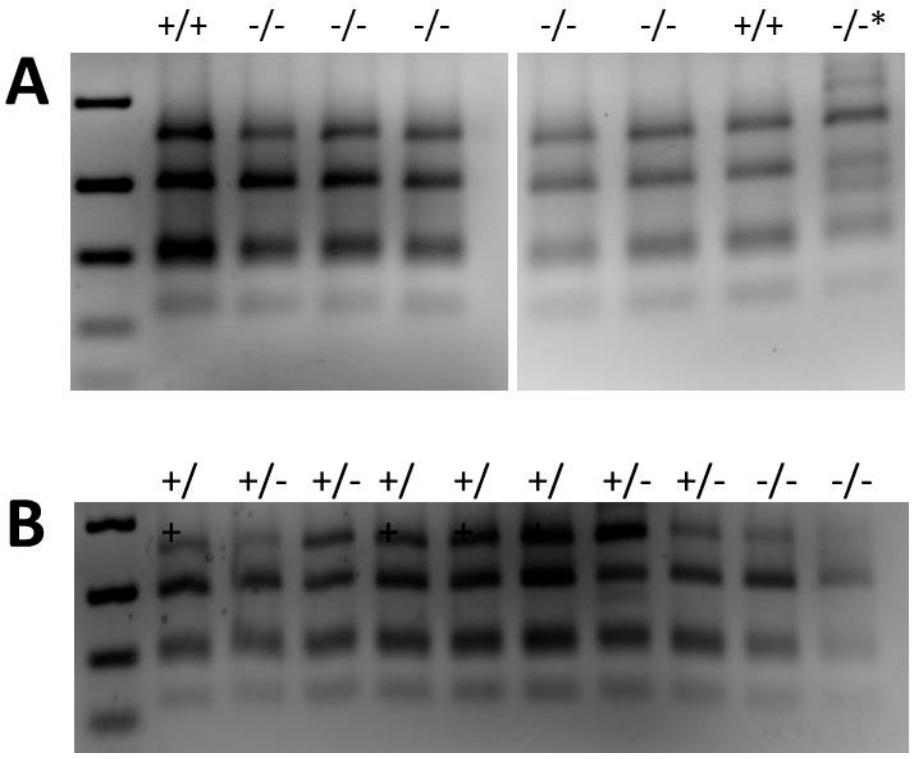


Figure 22. WT1 mRNA is not differentially edited in *Dnd1*^{Ter} animals as assessed by *Mnl1* restriction digest.

(A) WT1 mRNA is not differentially edited in heart tissue from adult 129/S1 *Dnd1* wild type (+/+) and *Dnd1*$^{Ter/Ter}$ (-/-) males. *Heart tissue from a B6 *Dnd1*$^{Ter/Ter}$ male. (B) WT1 mRNA is not differentially edited in whole129/S1 E13.5 male genital ridges from *Dnd1* wild type (+/+), *Dnd1*^{Ter} heterozygotes (+/-) and *Dnd1*^{Ter} homozygotes (-/-).

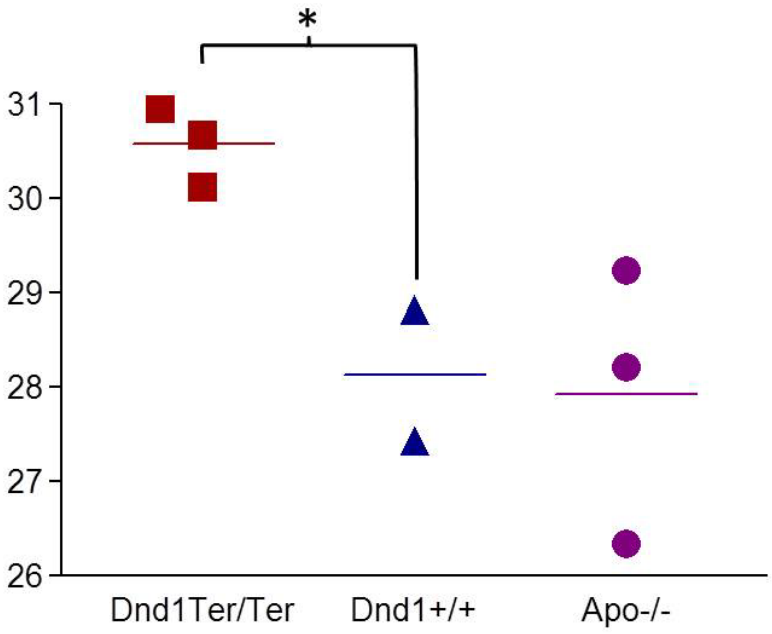


Figure 23. The percent of unedited WT1 mRNA in adult kidneys.

WT1 mRNA in *Dnd1*$^{Ter/Ter}$ kidneys is significantly lower (more unedited WT1) compared to *Dnd1*$^{+/+}$ kidneys (30.57 ± 0.42% and 28.13 ± 0.98%, respectively). Apobec1 deficient males (*Apo*$^{-/-}$) have unedited WT1 mRNA levels that are similar to than Dnd1$^{+/+}$ animals (27.92 ± 1.47%). *$p<0.05$

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