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Review

# TPD52, a candidate gene from genomic studies, is overexpressed in testicular germ cell tumours

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ABSTRACT

Several genomic regions are recurrently over- or underrepresented in testicular germ cell tumours (TGCTs), but only a fraction of their genes change their expression accordingly. Two publications to date have studied DNA copy numbers and associated gene expression changes on a genome-wide level to identify key players in TGCT tumorigenesis. Here, we compare lists of significant genes in these studies, and show that 17 genes are common to both. These include concomitant gain and over-expression of *JUB*, *NRXN3*, and *TPD52*, and loss and under-expression of *C11orf70* and *CADM1*, in addition to 12 overexpressed genes located on the chromosome arm 12p. We performed immunohistochemical analysis of *TPD52* on a tissue microarray, which showed complete absence of TPD52 protein in normal germ cells and most intratubular germ cell neoplasias. TPD52 was expressed in two-thirds of seminomas and embryonal carcinomas, and at intermediate frequencies in the more differentiated non-seminomas.



**Contents**



## 1. Introduction

Although testicular germ cell tumours (TGCTs) have low overall incidence (1–2% of all neoplasms) in males, they represent the most common malignancy in young males. In the Norwegian population, for example, TGCT is the most common cancer type in men between 15 and 54 years of age (Bray, 2007). Of additional concern is the fact that TGCT incidence has increased markedly in the last 50 years, especially in the industrialized world (Huyghe et al., 2003). TGCTs are histologically complex due to the type and degree of differentiation, and can broadly be separated into seminomas and non-seminomas. The latter can be further subdivided into the undifferentiated and pluripotent embryonal carcinomas, and subtypes with extraembryonic (choriocarcinomas and yolk sac tumours) or embryonic (teratomas) differentiation patterns.

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Pronounced differences in TGCT incidence and trends between geographical populations and ethnicity point to a role for genetic factors (Huyghe et al., 2003). Previous studies have revealed the TGCT genome to be hypo- to hyper-triploid in nature, reviewed in Skotheim and Lothe (2003). The TGCT genome has very consistent patterns of gains and losses, and recurrent chromosomal changes that appear in more than 30% of the analysed TGCTs, include extra copies of whole or parts of chromosomes 1, 2, 7, 8, 12, 14, 15, 17, 21, and X, and a reduced copy number of whole or parts of chromosomes 4, 5, 11, 13, and 18 (Skotheim and Lothe, 2003). Regardless of histological subtype, parts of or the whole of chromosome arm 12p is overrepresented in close to all tumours. This is most commonly in the form of an isochromosome of 12p, although amplification of smaller regions within 12p have also been described (Kraggerud et al., 2002; Rodriguez et al., 1993; Suijkerbuijk et al., 1994; Zafarana et al., 2003). The non-random pattern of gains and losses is believed to be the result of the selective advantage conferred by the genes whose expression is correspondingly increased or decreased. Despite this, the identity of these driver genes in TGCT tumorigenesis is largely unknown. In an effort to identify these, we looked in detail at the two publications to date (Korkola et al., 2008; Skotheim et al., 2006) that report on high-resolution genomic aberrations in TGCTs with associated gene expression changes.

By comparing the lists of genes published in these papers that were either gained and overexpressed or lost and underexpressed, we established a shortlist of candidate genes involved in the development of TGCTs. A total of 17 genes were jointly identified by the two studies. Among these was the tumour protein D52 (TPD52) gene, not previously studied in the context of TGCTs. A third report of combined DNA copy numbers and gene expression changes in TGCT also indicated TPD52 as being potentially important for this disease (McIntyre et al., 2007). However, this study was carried out with lower resolution, and gene lists from across the genome were not presented. Although TPD52's exact function remains unknown, the gene has been frequently found to be overexpressed across a number of cancer types, also in concert with amplification (Boutros et al., 2004). Here, we employ a TGCT-specific tissue microarray to evaluate TPD52 expression *in situ* across the various histological subtypes.

## 2. Materials and methods

### 2.1. Bioinformatic methods

To our knowledge, there are two studies to date reporting simultaneously on DNA copy number changes in TGCTs and RNA expression levels on a genome-wide scale (Korkola et al., 2008; Skotheim et al., 2006). The Korkola et al. (2008) study utilized a BAC microarray from Spectral Genomics with 2621 clones to identify DNA copy number changes and Affymetrix U133A+B microarrays to study mRNA gene expression. The Skotheim et al. (2006) study utilized Agilent Human 1 cDNA microarrays with 12,557 clones to identify DNA copy number changes and Agilent Human 1A 22k oligo microarrays to study mRNA gene expression. The two studies correlated mRNA gene expression to changes in DNA copy number in a similar fashion: in Korkola et al. (2008), the correlation analyses were carried out region by region using Significance Analysis of Microarrays, or SAM (Tusher et al., 2001), whereas in Skotheim et al. (2006), the correlation analyses were carried out locus by locus using a previously described algorithm (Hyman et al., 2002).

Gene lists from both these papers were obtained either directly from the article or the supplementary material as appropriate. For Skotheim et al. (2006), this was Supplementary Table 1, while for Korkola et al. (2008) Tables 2–4 were used. Because none of the samples in either study had a normal 12p DNA copy number, there was no control group for the expression changes, and alternative statistical methods had to be used to analyse these genes. As such, lists of significantly overexpressed genes on this chromosome arm were obtained from Fig. 2 and Table 3 in Skotheim et al. (2006) and Supplemental Table 3 in Korkola et al. (2006). The annotation from Skotheim et al. (2006) was updated according to the original Agilent probe identifiers and information available at Biomart[1] (accessed June 2008). For Korkola et al. (2008), the published gene symbols and probeset identifiers were used to retrieve updated annotation from Biomart. Overlap between the resulting gene lists was established using the corresponding Ensembl gene identifiers (ENSG numbers), and visualized using the web application Gene Venn (Pirooznia et al., 2007).

### 2.2. In situ TPD52 protein expression on tissue microarrays

*In situ* protein expression analysis of TPD52 was performed using immunohistochemistry on a tissue microarray originally published in Skotheim et al. (2003), containing 510 testicular tissue cores punched from formalin-fixed and paraffin-embedded tissues from 279 patients who underwent orchiectomy due to testicular cancer. The use of the testicular tissue samples was approved by the Regional Committee for Medical Research Ethics in Norway.

The TPD52 antiserum used for immunohistochemical staining is described in Balleine et al. (2000). Paraffin-embedded tissue microarray sections (4 μm) were dewaxed twice in xylene, then washed twice in 100% ethanol and once in 75% ethanol, each for 5 min. Tissues were rehydrated in water and equilibrated in PBS for 5 min. After blocking with 10% normal goat serum (NGS) in PBS for 20 min, sections were rinsed with PBS and incubated with affinity-purified rabbit polyclonal anti-TPD52 antisera (1:100) in 2% NGS/PBS for 2 h. Sections were rinsed with PBS before incubation with biotinylated goat anti-rabbit secondary IgG (1:500) in 2% NGS/PBS for 1 h. Sections were then rinsed with PBS, incubated with 0.3% hydrogen peroxide to block endogenous peroxidases, rinsed again with PBS and stained with tertiary ABC reagent (Pierce) for 1 h. After rinsing with PBS, sections were incubated with 1 mg/ml DAB for 5 min, rinsed with PBS, counterstained with Nuclear Fast Red, and rinsed with distilled water. Slides were then dried at 60 °C, dehydrated in xylene for 5 min, and mounted.

Slides were scanned using the Virtual Microscope ScanScope Unit and ScanScope Console program (Aperio Technologies) at 200× magnification. Tissue microarrays were visualized using Image Scope (Aperio Technologies). Two alternative scoring schemes, automated and visual, were implemented. In the automated scoring scheme, the staining intensity was quantified using the Positive Pixel Count algorithm (Aperio Technologies). The number of strong pixels (NSP, defined as pixels of 175–220 intensity) was measured per tissue core. In the visual scoring scheme, each core was scored visually as positive or negative by two independent researchers. Discordantly scored cores were revisited for a final scoring agreed on by both researchers. Tissue cores present only partially, with only necrotic tissue, or with staining artefacts were excluded from analyses. The epithelial and mesenchymal components of the teratoma tissue cores were first scored separately. A teratoma core was considered positive for TPD52 staining if either the epithelial or mesenchymal components, or both, had scored positive. The immunohistochemistry results per histological subtype of the individual tissue cores were integrated with previously obtained molecular data, and the tissue microarray dataset was matched with clinical data for the corresponding patients. Fisher's exact tests (SPSS for Windows; version 15, SPSS Inc., Chicago, IL) were used to evaluate the statistical significance of differences in expression between groups of samples.

## 3. Results

### 3.1. Gene list comparison

134 gained and overexpressed or lost and underexpressed genes from Skotheim et al. (2006) were combined with 17 overexpressed

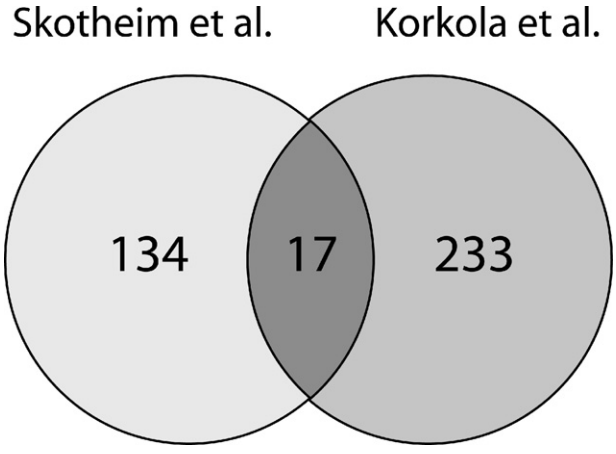


**Fig. 1.** Numbers of genes overlapping between three studies investigating TGCT for gene expression changes that are associated with DNA copy number changes.

[1] http://www.biomart.org/.

**Table 1**
The 17 overlapping genes between the two studies investigating gene expression changes in TGCT that are associated with DNA copy number changes (Skotheim et al., 2006; Korkola et al., 2008). *Genes located on chromosome 12p were analysed by different statistical methods than genes from other genomic regions.

| Gene symbol | Gene name | Chrom. band | Ensembl gene ID (ENSG number) |
|---|---|---|---|
| *TPD52* | Tumour protein D52 | 8q21.13 | ENSG00000076554 |
| *C11orf70* | Uncharacterized protein C11orf70 | 11q22.2 | ENSG00000137691 |
| *CADM1* | Cell adhesion molecule 1 precursor | 11q23.3 | ENSG00000182985 |
| *BCAT1** | Branched-chain-amino-acid aminotransferase | 12p12.1 | ENSG00000060982 |
| *CCND2** | G1/S-specific cyclin-D2 | 12p13.32 | ENSG00000118971 |
| *CD9** | CD9 antigen (p24) | 12p13.31 | ENSG00000010278 |
| *DERA** | Putative deoxyribose-phosphate aldolase | 12p12.3 | ENSG00000023697 |
| *GAPDH** | Glyceraldehyde-3-phosphate dehydrogenase | 12p13.31 | ENSG00000111640 |
| *GDF3** | Growth/differentiation factor 3 precursor | 12p13.31 | ENSG00000184344 |
| *GOLT1B** | Vesicle transport protein GOT1B | 12p12.1 | ENSG00000111711 |
| *LAML1_HUMAN** | LAMA-like protein 1 precursor | 12p13.1 | ENSG00000121316 |
| *LDHB** | L-Lactate dehydrogenase B chain | 12p12.1 | ENSG00000111716 |
| *NANOG** | Homeobox protein NANOG | 12p13.31 | ENSG00000111704 |
| *TEAD4** | Transcriptional enhancer factor TEF-3 | 12p13.33 | ENSG00000197905 |
| *YARS2** | Tyrosyl-tRNA synthetase, mitochondrial precursor | 12p11.21 | ENSG00000139131 |
| *JUB* | Ajuba isoform 1 | 14q11.2 | ENSG00000129474 |
| *NRXN3* | Neurexin-3-alpha precursor | 14q24.3 | ENSG00000021645 |

12p genes from the same dataset to give 151 candidate genes for the Skotheim et al. sample set. Similarly, 154 gained and overexpressed or lost and underexpressed genes from Korkola et al. (2008) were combined with 96 overexpressed 12p genes identified using the same samples giving 250 candidate genes for the Korkola et al. dataset.

The overlapping genes between the two studies constitute our candidate TGCT-specific genes and are summarized in Fig. 1 and listed in Table 1. A total of 17 genes were common to the Skotheim et al. and Korkola et al. datasets, and include *TPD52* on chromosome 8q. Of these 17, 12 (marked with an asterisk in Table 1) are overexpressed genes located on chromosome 12p.

### 3.2. *TPD52 expression in TGCT*

As *TPD52* was found to be overexpressed and present at increased copy number in both studies (Korkola et al., 2008; Skotheim et al., 2006), but had not previously been studied in TGCTs, *in situ* TPD52 protein expression was analysed in testicular samples in a tissue microarray format. These analyses yielded in total 425 scorable testicular tissue cores from 237 patients. Examples of testicular tissues positive and negative for TPD52 expression are shown in Fig. 2. The staining pattern was membranous and cytoplasmic, although we noted two seminoma and one yolk sac tumour tissue cores (out of the 425 scorable testicular tissue cores) with areas containing clear nuclear staining (data not shown).

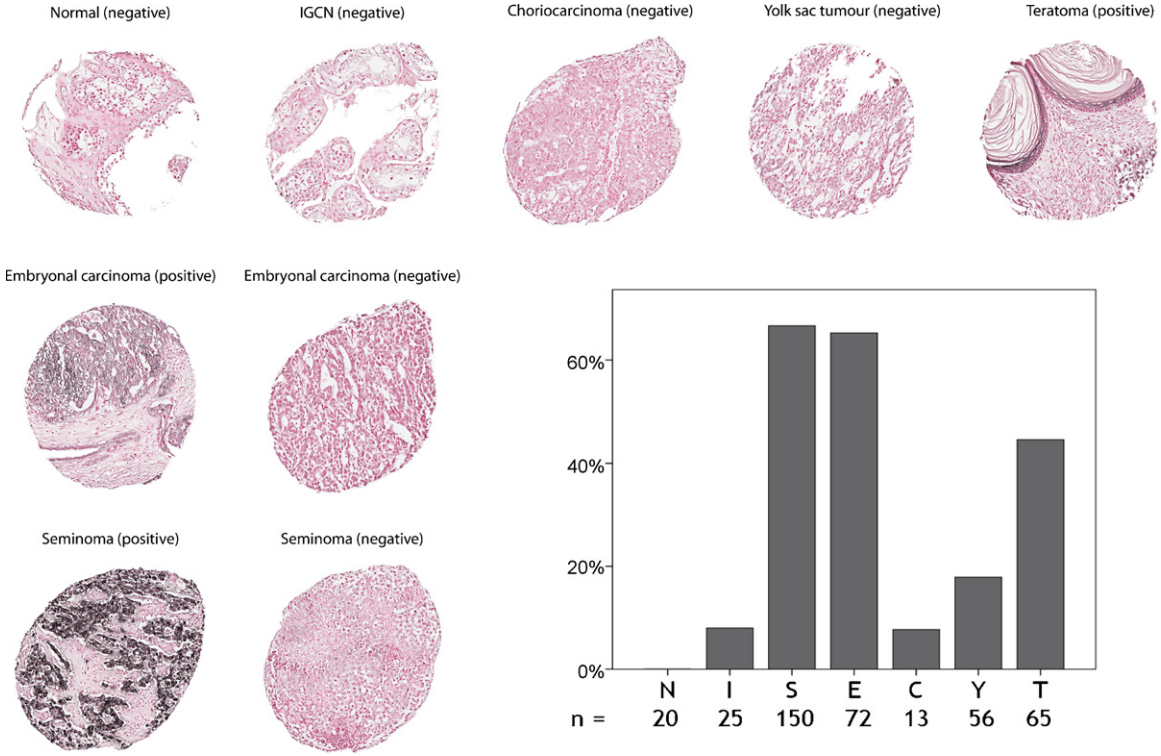


**Fig. 2.** *In situ* protein expression of TPD52 in testicular tissues. Tissue cores: histology and scoring as labelled per core. Histogram: frequencies of tissue cores positive for TPD52 protein expression for the following histological subtypes: N, normal testis; I, intratubular germ cell neoplasia; S, seminoma; E, embryonal carcinoma; C, choriocarcinoma; Y, yolk sac tumour; and T, teratoma.

**Table 2**
TPD52 expression in normal, premalignant and malignant testis subdivided by histological subtypes. Frequencies of positively scored samples by the visual and automated (NSP) scoring methods, *P*-values for association with metastasis and survival status, and *P*-values for pair-wise comparisons between histological subtypes by visual (upper right half) and automated (lower left half) scoring. Cells with *P*-values under 0.05 are shaded in grey.

| | | Normal | IGCN | Sem | EC | CC | YST | Ter |
|---|---|---|---|---|---|---|---|---|
| *n* = | | 20 | 25 | 150 | 72 | 13 | 56 | 65 |
| Freq visual score | | 0% | 8% | 67% | 65% | 8% | 18% | 45% |
| Freq NSP 20k | *p* value metastasis | NA | 0.513 | 0.514 | 0.449 | 0.308 | 1.000 | 0.282 |
| 0% | Normal | | 0.50 | $3.1\times10^{-9}$ | $3.8\times10^{-8}$ | 0.39 | 0.06 | $7.9\times10^{-5}$ |
| 0% | IGCN | NA | | $2.4\times10^{-8}$ | $4.1\times10^{-7}$ | 1.00 | 0.33 | $1.0\times10^{-3}$ |
| 48% | Sem | $1.1\times10^{-5}$ | $7.1\times10^{-7}$ | | 0.88 | $4.0\times10^{-5}$ | $2.7\times10^{-10}$ | $3.8\times10^{-3}$ |
| 14% | EC | 0.11 | 0.06 | $6.0\times10^{-7}$ | | $1.3\times10^{-4}$ | $6.1\times10^{-8}$ | 0.02 |
| 8% | CC | 0.39 | 0.34 | $6.7\times10^{-3}$ | 1.00 | | 0.68 | 0.01 |
| 7% | YST | 0.57 | 0.31 | $1.2\times10^{-8}$ | 0.27 | 1.00 | | $1.9\times10^{-3}$ |
| 9% | Ter | 0.33 | 0.18 | $1.3\times10^{-8}$ | 0.44 | 1.00 | 0.75 | |

Using the automated scoring system, the NSP values per core ranged from 0 to 370,000 with no clear division in the distribution between samples with many or few strong positive pixels. Thus, we chose to use an arbitrary cut-off at 20,000 strong positive pixels for a positive score. Frequencies of tissue cores positive for TPD52 protein expression at this cut-off level are shown in the left-most column of Table 2 for normal testis, IGCN, and TGCTs per histological subtype. The frequency of positive scores per histological subtype was compared to all others in a pair-wise fashion, for which *P*-values are shown in the lower left half of Table 2. By this scoring method, none of the normal testis ($n=20$) or the IGCN ($n=25$) cores were positive for TPD52 immunostaining. However, a considerable 72/150 (48%) of the seminoma cores ($P=1.1\times10^{-5}$ [seminoma vs. normal; two-sided Fisher's exact] and $P=7.1\times10^{-7}$ [seminoma vs. IGCN]) were positive. For the other histological subtypes, the frequencies of positive scores were: 10/72 (14%) for embryonal carcinoma, 1/13 (8%) of choriocarcinoma, 6/65 (9%) for the teratoma and 4/56 (7%) for the yolk sac tumour cores.

In addition, to enable scoring of tissue cores with weaker, though clear, expression of TPD52, we also examined all the images visually, scoring all cores with positive staining of smaller or larger regions as positive. Frequencies of tissue cores positive for TPD52 protein expression by this scoring scheme are shown both in Table 2 and Fig. 2 for normal testis, IGCN, and TGCTs per histological subtype. Of the cores that could be scored, none of the normal testis cores ($n=20$) and only 2/25 (8%) of the IGCN were positive for TPD52 immunostaining. However, a considerable 100/150 (67%) of the seminomas ($P=2\times10^{-8}$ [seminoma vs. IGCN, two-sided Fisher's exact]) and 47/72 (65%) of the embryonal carcinomas ($P=4\times10^{-7}$) expressed TPD52. The three differentiated non-seminoma histological subtypes expressed TPD52 at a range of frequencies, but all significantly lower than embryonal carcinomas. 1 of 13 (8%) of choriocarcinomas were positive for TPD52 staining ($P=1\times10^{-4}$ [choriocarcinoma vs. embryonal carcinoma], as were 10/56 (18%) of the yolk sac tumours ($P=6\times10^{-8}$) and 29/65 (45%) of the teratomas ($P=0.017$).

Finally, we evaluated whether the TPD52 expression according to the visual scoring scheme was associated to the patients' metastasis and survival status. From the patients with known metastasis and survival status and a valid TPD52 score, 89/237 (38%) had metastatic disease, while 10/220 (5%) patients were dead from the disease. Association tests were carried out for both the whole sample set and for the histological subtypes individually. No groups were found to have association *P*-values of <0.05 for either of the clinical parameters.

## 4. Discussion

Gene expression changes in TGCT have been investigated by numerous studies (Almstrup et al., 2004, 2005; Juric et al., 2005; Korkola et al., 2005; Okada et al., 2003; Skotheim et al., 2002, 2005; Sperger et al., 2003). Only a few of these have included samples from all major histological subtypes of TGCT, from which it has become evident that the genes up- or down-regulated in TGCT are mostly dependent on the histological subtype of the analysed specimen (Korkola et al., 2005; Skotheim et al., 2005).

Here, we have concentrated on the changes in the chromosome content of the samples, and investigated differences in gene expression associated with altered DNA copy number at the corresponding loci. The non-random nature of chromosome changes in the TGCT genome, with relatively similar patterns observed across the histological subtypes, suggests that these are key events in the TGCT tumorigenesis (Skotheim and Lothe, 2003). Despite this, the gene expression changes are often profoundly different between the histological subtypes. As a result, analysis of the genes whose expression changes are associated to the DNA copy number levels is required to detect more subtle changes, as DNA copy numbers may change by only one or few copies. This makes the inclusion of false positives unavoidable. By comparing results from two separate studies (Korkola et al., 2008; Skotheim et al., 2006), we significantly increase the likelihood of identifying the true-positive genes whose expression changes are important to the TGCT tumorigenesis. Unfortunately, the sample sizes are not

large enough for a similar analysis of the individual histological subtypes.

We found 17 genes in common between the two studies, including the tumour protein *TPD52*. We have validated the overexpression of this gene at the protein level with a testicular tissue microarray. The two different scoring regimes used enabled us to identify cores with strong expression (automated scoring) as well as to detect expression that was weaker or histology-specific within the tissue core (visual scoring).

Both scoring schemes showed that compared to normal testis, TPD52 is overexpressed in primary TGCTs. No TPD52 expression could be detected in normal testis, and only in a low proportion of premalignant IGCN samples detectably expressed TPD52 by visual scoring. By contrast, TPD52 expression was detected in a significant proportion of seminomas and embryonal carcinomas, thus molecularly distinguishing these invasive tumour subtypes from normal and the non-invasive IGCN. Automated scoring showed that seminomas stain strongly significantly more frequently than the other histological subtypes (Table 2). TPD52 expression additionally distinguishes these undifferentiated subtypes (seminomas and embryonal carcinomas) from the more differentiated subtypes of non-seminoma such as teratomas, yolk sac tumours and choriocarcinomas, which again have lower frequencies of TPD52 expression. As such, expression seems strongly correlated to the histological subtypes thought to be directly derived from the IGCN (seminomas and embryonal carcinomas), and not to the more differentiated non-seminomas, which in turn are thought to derive from embryonal carcinomas. One possibility is that TPD52 may contribute to the invasiveness or otherwise to a selective advantage outside of the seminiferous tubuli during the first invasive step of the TGCT tumorigenesis. With the current dataset, no association to metastasis or survival was found. However, given the good prognosis of this group of patients, the survival testing has low statistical strength.

The *TPD52* gene is located at the chromosome band 8q21.13, in a wider region (8q) that is frequently gained across a range of cancer types (Myllykangas et al., 2006), including TGCTs (Skotheim et al., 2006). Increased TPD52 expression and gene copy number has been previously reported in breast (Adélaïde et al., 2007; Balleine et al., 2000; Jonsson et al., 2007; Pollack et al., 2002; Rodriguez et al., 2007), prostate (Pollack et al., 2002; Rubin et al., 2004) and ovarian cancer (Byrne et al., 2005), and is predicted in additional tumours such as multiple myeloma (Largo et al., 2006). Specific targeting of *TPD52* by gene amplification is also supported by studies identifying narrow amplicons at chromosome band 8q21.13 which include the *TPD52* locus in breast (Hicks et al., 2006; Rodriguez et al., 2007), prostate (Kim et al., 2007) and lung cancer (Weir et al., 2007). Increased TPD52 expression has been found to increase cell proliferation *in vitro* (Lewis et al., 2007; Shehata et al., 2008; Wang et al., 2007; Zhang et al., 2007), and to confer or increase tumorigenicity in immunodeficient (Wang et al., 2007; Zhang et al., 2007), and immunocompetent mice (Lewis et al., 2007). However, little is known about the oncogenic mechanisms that overexpression of this gene may induce. The TPD52 coiled-coil motif indicates a role in protein–protein interactions, with a number of partners identified through yeast two-hybrid screening (Byrne et al., 1998; Wilson et al., 2001), although none are immediately recognizable as regulators of proliferation. Further insights into the mechanisms by which increased TPD52 expression initiates and/or promotes tumorigenesis will require further targeted biochemical studies. However the identification of TPD52 overexpression in TGCTs highlights that this is not restricted to tumours of epithelial origin, and predicts that TPD52 regulates oncogenic processes that are of generic rather than tissue-specific significance. Preliminary results from an ovarian germ cell tumour tissue microarray indicate that TPD52 was also expressed in a proportion of the tissue cores (own unpublished results).

Of the four remaining genes not on chromosome 12p identified in both the Skotheim et al. and Korkola et al. datasets as playing a role in testicular cancer tumorigenesis, two have roles in cancer described in the literature. The first, *JUB*, encodes Ajuba, an embryonic stage-specific cytosolic protein that is recruited to the cell–cell contact site of embryonal cells upon aggregate formation, before being shuttled to the nucleus (Kanungo et al., 2000). By this action, it is proposed that Ajuba can transduce signals that help regulate cell growth and differentiation decisions. In both the datasets examined (Korkola et al., 2008; Skotheim et al., 2006), JUB is gained and overexpressed, and in the Korkola dataset it is found to be specifically overexpressed in yolk sac tumours (Korkola et al., 2008). As nuclear accumulation of Ajuba was found to induce endodermal differentiation in embryonal cells (Kanungo et al., 2000), its expression in differentiated tumour types of TGCT is perhaps unsurprising.

The second gene, *CADM1* or Cell adhesion molecule 1, has been shown to function as a tumour suppressor gene in many solid tumours, such as non-small cell lung cancer (Kuramochi et al., 2001) and neuroblastoma (Nowacki et al., 2008). The gene is inactivated in between 30 and 60% of cancer types, including liver, pancreas and prostate (Murakami, 2005), and both the Skotheim et al. and Korkola et al. datasets show genomic loss and under-expression of *CADM1* in TGCTs. The membrane protein encoded for by *CADM1* not only promotes epithelial cell adhesion, but may also actively suppress tumour formation in concert with its binding partners, DAL-1/4.1B and MAGuK by inhibiting cell proliferation (Mao et al., 2003). In the context of TGCTs, CADM1 has also been shown to be essential for normal spermatogenesis in mice (van der Weyden et al., 2006).

The final two genes identified in our analyses are *NRXN3* and *C11orf70*. *NRXN3* is gained and overexpressed, while *C11orf70* is lost and underexpressed in both datasets (Korkola et al., 2008; Skotheim et al., 2006). Nothing is known to date about the function of *C11orf70*, but the protein encoded for by *NRXN3* is a cell adhesion molecule, in which single nucleotide polymorphisms are associated with substance addiction (Hishimoto et al., 2007). Further speculation on how these two genes may be involved in TGCT tumorigenesis will have to await the results of more functional studies.

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