Recurrent inactivation of STAG2 in bladder cancer is not associated with aneuploidy

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Urothelial bladder cancer (UBC) is heterogeneous at the clinical, pathological and genetic levels. Tumor invasiveness (T) and grade (G) are the main factors associated with outcome and determine patient management1. A discovery exome sequencing screen (n = 17), followed by a prevalence screen (n = 60), identified new genes mutated in this tumor coding for proteins involved in chromatin modification (MLL2, ASXL2 and BPTF), cell division (STAG2, SMC1A and SMC1B) and DNA repair (ATM, ERCC2 and FANCA). STAG2, a subunit of cohesin, was significantly and commonly mutated or lost in UBC, mainly in tumors of low stage or grade, and its loss was associated with improved outcome. Loss of expression was observed in chromosomally stable tumors, and STAG2 knockdown in bladder cancer cells did not increase aneuploidy. STAG2 reintroduction in non-expressing cells led to reduced colony formation. Our findings indicate that STAG2 is a new UBC tumor suppressor acting through mechanisms that are different from its role in preventing aneuploidy.

The most commonly mutated oncogene in UBC is FGFR3 (50–60% of cases): mutations are more frequent in non-muscle-invasive bladder cancers (NMIBCs) with a low risk of progression (stage Ta low-grade tumors), here designated ‘non-aggressive’ (Online Methods)2,3. PIK3CA mutations occur in 15–20% of tumors and tend to associate with FGFR3 mutations4. p53 and RB pathway inactivation has been associated with NMIBCs with a high risk of progression (stage Ta or T1 high-grade tumors) and with muscle-invasive bladder cancer (MIBC) (here designated ‘aggressive’)5,6. RAS mutations are less common and are mutually exclusive with FGFR3 mutations7. There is now extensive evidence indicating that NMIBCs of high grade are genomically similar to MIBCs8,9, non-aggressive UBCs are genomically stable, whereas aggressive UBCs are genomically unstable2,8–10. Recently, exome sequencing has identified chromatin remodeling as an important pathway involved in UBCs11; this study focused mainly on NMIBCs.

To discover new genes mutated in UBC, we sequenced the exomes of 17 tumors of variable stage and grade and corresponding normal leukocyte DNA; all neoplastic samples used had a tumor cellularity of >70% (Supplementary Table 1). Because there are major initiatives for the sequencing of MIBC (for example, The Cancer Genome Atlas (TCGA) project), we have focused mainly on NMIBC. Metrics for enrichment and depth of coverage are shown in Supplementary Table 2: the mean coverage for tumors and leukocytes was 79 ± 11 and 82 ± 18 x, respectively. We identified 2,927 somatic mutations, of which 1,263 and 798 were predicted to be relevant (nonsynonymous) and damaging (have a functional effect) (Supplementary Table 3), respectively (Online Methods). The average number of somatic mutations per tumor was 169 ± 114, with wide individual variation (range of 4–360 mutations) (Fig. 1a), a figure that falls in the midrange for exome studies in solid tumors in adults. C>T transitions were the most common nucleotide substitution (mean of 44%), followed by C>G transversions (Fig. 1b). The ratio of

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nonsynonymous to synonymous (NS:S) changes was <1 in 15 of 17 samples (Fig. 1c and Supplementary Fig. 1a). We compared the total number of single-nucleotide variants (SNVs), indels, transitions, transversions, synonymous mutations, non-damaging nonsynonymous mutations and damaging nonsynonymous mutations in aggressive versus non-aggressive tumors; all variables were highly similar in both tumor groups. We performed the same analysis according to smoking status: the number of damaging mutations was higher in tumors from smokers than in those from non-smokers, but differences did not reach statistical significance (P = 0.09). The number of mutations in tumors from individuals who were >60 years old was also slightly but not significantly higher than for younger individuals (Supplementary Fig. 2). The NS:S ratio was similar regardless of tumor aggressiveness and smoking status, but this ratio was slightly lower in individuals diagnosed at >60 years of age (Supplementary Fig. 1b). It will be necessary to sequence more tumors to further investigate these relationships.

We assessed the reliability of the exome analysis and strategies for somatic variant calling using Sanger sequencing: we assayed 226 variants and verified 219, of which 214 (94.7%) were confirmed to be somatic (Supplementary Table 3). The list of genes with nonsynonymous mutations in ≥3 tumors that were expressed in >30% of UBCs, on the basis of Affymetrix expression analyses of an independent tumor sample series (n = 43) covering the full spectrum of the disease, is shown in Table 1. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses identified chromatin modification, DNA repair and DNA damage response, apoptosis and the cell cycle among the most significant processes to which mutated genes were ascribed (false discovery rate (FDR) < 0.1) (Supplementary Table 4).

To extend our findings, we performed a mutation prevalence screen including mainly NMIBC (n = 60 tumors) (Supplementary Table 5) using the HaloPlex Target Enrichment System followed by sequencing. We included selected genes that were recurrently mutated in the discovery screen as well as additional genes from the pathways in which these genes participate (Supplementary Table 6). We identified 260 SNVs: 200 were predicted to be relevant, and 143 of these were predicted to be damaging. We analyzed 95 mutations identified by HaloPlex by Sanger sequencing; 73 were verified (76.13%), and 72 of these (98.6%) were confirmed to be somatic.

The joint distribution of mutations in the discovery and prevalence screens is shown in Figure 2 and Table 1. Among the genes recurrently mutated, we identify new genes involved in chromatin remodeling different from those reported by Gui et al. (11) (MLL2, ASXL2 and BPTF). The BPTF protein binds histone H3 that is trimethylated at lysine 4 (H3K4me3) and has been found to be mutated in hepatocarcinoma, but little is known about its function in cancer. We show that BPTF knockdown led to a marked reduction in colony formation in the three UBC lines tested (Supplementary Fig. 3), suggesting that it has a role in cancer cell proliferation. We confirmed recurrent mutations in ARID1A, KDM6A (UTX), CREBBP, EP300, MLL and MLL3 (ref. 11), in agreement with recent reports implicating mutations in a wide range of chromatin remodelers in human cancer (12–15). Notably, we identified recurrent, previously unreported somatic mutations in genes involved in DNA repair (ATM, ERCC2 and FANCA, among others) and in the cohesin subunits STAG2, STAG1, SMCIA and SMCIB, indicating that these pathways have an important role in UBC. FGF3, TP53, PIK3CA and RB1 are among the recurrently mutated genes, providing evidence of the representativeness of the tumors analyzed.

We have focused on STAG2 because it was significantly mutated in our exomes (Table 1); an additional mutation was found in 9 published UBC exomes (11), and we identified 2 mutations in 21 MIBC exomes from the TCGA project (overall damaging mutation rate of 13% (6/47)). Our prevalence screen identified nine additional somatic mutations predicted to be damaging. Altogether, we have identified damaging somatic STAG2 mutations in 12 of 77 tumors (15.6%; 5 nonsense, 4 exon junction, 2 missense and 1 indel) (Supplementary Fig. 4 and Supplementary Table 7), and 9 of 11 were verified by Sanger sequencing (Supplementary Fig. 5). Damaging mutations were found in both non-aggressive (6/29; 20.7%) and aggressive (5/47; 10.6%) tumors. STAG2-inactivating mutations leading to loss of protein expression have recently been reported in non-epithelial tumors (16). STAG2 expression was low or undetectable in 6 of 7 (85%) UBCs with damaging mutations and in 3 of 34 (9%) tumors with wild-type STAG2 (P = 0.0001) (Fig. 3, Supplementary Fig. 5 and Supplementary Table 7). Together with the exome significance analysis, these data indicate that STAG2 is a new gene commonly mutated in UBC.

We then analyzed STAG2 expression in tissue microarrays of incident tumors representative of the disease spectrum (Supplementary Tables 8 and 9). Loss of STAG2 expression in tumors, defined as a histoscore of ≤50 with detectable stromal expression, was observed in 197 of 671 tumors (29.3%) (Fig. 3 and Supplementary Fig. 5). Loss of STAG2 expression was significantly associated with multicentricity (P = 0.011), tumor size (P = 0.002), low stage (P = 5.7 × 10−17) and low grade (P = 1.96 × 10−15) (Supplementary Table 10). Abnormal
STAG2 expression patterns included focal losses within otherwise positive tumors and a predominant cytoplasmic distribution of the protein (Supplementary Fig. 6). Because non-aggressive tumors are more differentiated, lack of STAG2 might reflect urothelial cell maturation. Arguing against this possibility, STAG2 expression that only one hit is required for its inactivation, given its location on the X chromosome.

Recently, STAG2 mutations in glioblastoma, melanoma and Ewing sarcoma have been proposed to participate in tumor development by promoting aneuploidy16. This hypothesis is at odds with our finding was detected in all cell layers of the normal urothelium (Supplementary Fig. 7). We also analyzed whether mechanisms other than mutation might account for loss of STAG2 expression. Using SNP arrays, we found STAG2 losses in 1 of 18 TaG1 or TaG2 STAG2-negative tumors (5%) from male subjects. Similar findings have been reported in leukemia16,18–20 (Supplementary Fig. 8).

STAG2 encodes a subunit of cohesin, a complex that mediates sister chromatid cohesion to ensure accurate chromosome segregation and DNA repair. Cohesin also regulates gene expression through mechanisms involving DNA looping and interactions with transcriptional regulators such as Mediator and CTCF21,22. Somatic human cells contain two versions of this complex consisting of SMC1A, SMC3, RAD21 and either STAG1 or STAG2 (ref. 21). Although there is still an incomplete understanding of the functional redundancy of these complexes, differential roles in centromeric versus telomeric cohesion have been proposed, and STAG1 has been preferentially implicated in transcriptional control23,24. Using immunohistochemistry, we showed that STAG1 is expressed in the normal urothelium and in the majority of UBCs, including most tumors lacking STAG2 expression (Supplementary Figs. 7 and 9). Interestingly, all six tumors that lost both STAG1 and STAG2 expression were of high grade and were wild type for FGF3, suggesting partial functional compensation in the maintenance of integral chromosome segregation machinery. The more frequent loss of the expression of STAG2 in tumors (Supplementary Fig. 9a) may reflect the fact

**Table 1** Genes frequently mutated in UBC assessed through exome sequencing or targeted HaloPlex resequencing (*n* = 77)

| Gene      | Number of mutations (*n* = 17) | Number of mutations in all tumors (*n* = 77) | Number of non-aggressive mutant cases (*n* = 29) | Number of aggressive mutant cases (*n* = 47) | P value a | P value b | P value c |
|-----------|-------------------------------|---------------------------------------------|-----------------------------------------------|---------------------------------------------|-----------|-----------|-----------|
| ARID1A    | 7                             | 0.0001                                      | 10                                            | 3                                           | 0.732     |           |           |
| STAG2     | 3                             | 0.019                                       | 9                                             | 12                                          | 0.315     |           |           |
| KDM6A     | 4                             | 0.019                                       | 6                                             | 10                                          | 0.732     |           |           |
| POZD2     | 3                             | 0.019                                       | 0                                             | 3                                           | 0.521     |           |           |
| MYCBP2    | 3                             | 0.061                                       | 2                                             | 5                                           | 0.999     |           |           |
| LPHN3     | 3                             | 0.096                                       | 0                                             | 3                                           | 0.521     |           |           |
| CREBBP    | 2                             | 0.098                                       | 9                                             | 11                                          | 0.702     |           |           |
| EP300     | 2                             | 0.098                                       | 5                                             | 7                                           | 0.188     |           |           |
| ATM        | 3                             | 0.138                                       | 6                                             | 9                                           | 0.702     |           |           |
| TP53       | 3                             | 0.2117                                      | 8                                             | 11                                          | 0.315     |           |           |
| RREB1      | 3                             | 0.237                                       | 0                                             | 3                                           | 1         |           |           |
| PIK3CA     | 6                             | 0.239                                       | 4                                             | 10                                          | 0.289     |           |           |
| WHSC1L1    | 2                             | 0.241                                       | 1                                             | 3                                           | 2         |           |           |
| MYO5B      | 3                             | 0.430                                       | 3                                             | 0                                           | 0.521     |           |           |
| MLL2       | 2                             | 0.636                                       | 13                                            | 15                                          | 0.315     |           |           |
| FGF3       | 2                             | 0.659                                       | 12                                            | 14                                          | 0.011     |           |           |
| TEX15      | 3                             | 0.778                                       | 0                                             | 3                                           | 0.521     |           |           |
| BRAF       | 1                             | 1                                           | 6                                             | 7                                           | 0.701     |           |           |
| ERCC2      | 0                             | NA                                          | 8                                             | 8                                           | 0.040     |           |           |
| MAPK8IP3   | 1                             | NA                                          | 4                                             | 5                                           | 0.363     |           |           |
| MLL        | 1                             | NA                                          | 5                                             | 6                                           | 0.363     |           |           |
| NUP93      | 1                             | NA                                          | 4                                             | 5                                           | 1         |           |           |
| STAG1      | 0                             | NA                                          | 5                                             | 5                                           | 3         |           |           |
| RB1        | 1                             | NA                                          | 3                                             | 4                                           | 1         |           |           |
| FANCA      | 0                             | NA                                          | 4                                             | 4                                           | 0         |           |           |
| MLL3       | 1                             | NA                                          | 4                                             | 5                                           | 3         |           |           |
| NOTCH1     | 0                             | NA                                          | 4                                             | 4                                           | 3         |           |           |
| ASXL2      | 3                             | NA                                          | 1                                             | 4                                           | 1         |           |           |

Data are shown for the discovery screen and the prevalence screen. Discrepancies between number of mutations and numbers of mutant tumors result from the occurrence of ≥2 mutations in the same gene in a given tumor sample. NA, not available.

aP value calculations are based on the mutations identified in the discovery screen (Online Methods). bOne sample with a mutation in STAG2 is excluded from the non-aggressive versus aggressive tumor comparison owing to insufficient information for classification. cP value for the frequency of mutant tumors with non-aggressive versus aggressive features.

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**Figure 2** Distribution of mutations in genes recurrently mutated in UBC that are expressed in >30% of tumors, joint analysis of the discovery and prevalence screens. In 22 of 77 tumors (28.6%), none of the genes listed were found to be mutated. For aggressiveness, red indicates aggressive tumors, and blue indicates non-aggressive tumors. Aggressiveness was defined as described in the Online Methods. For smoking status, red indicates smokers, and blue indicates non-smokers. White squares indicate cases where information was not available.
that loss of STAG2 expression occurs mainly in non-aggressive UBCs that are genomically stable. To address this issue, we analyzed chromosome number changes in a panel of 23 TaG1 and TaG2 tumors using high-resolution SNP or BAC arrays. Of 11 tumors without STAG2 expression, 9 lacked aneuploidy, and 2 showed loss of 1 copy of chromosome 9; similarly, 9 of 12 tumors that expressed STAG2 had normal chromosomal content (Fig. 4a, Supplementary Fig. 10 and Supplementary Table 11). Consistent with these findings, mutations in STAG2 and other cohesin genes have recently been reported not to be associated with aneuploidy in acute myeloid leukemia16–20. We next knocked down STAG2 in three UBC lines displaying a broad range of phenotypes. Efficient knockdown was achieved in the three lines, but there were no consistent effects on chromosome number at metaphase (Fig. 4b,c, Supplementary Fig. 11 and Supplementary Table 12), unlike what was previously reported in HCT116 cells19. This discrepancy may reflect the fact that different cell types have variable tolerance to aneuploidy. We also introduced STAG2 cDNA in three cell lines lacking STAG2 protein expression: UM-UC-6 cells harbor a p.Arg305* stop-gain alteration (exon 11) and a p.Phe1228Leu alteration (exon 33), VM-CUB-3 cells harbor a 10-bp deletion in exon 6 and LGWO 1 G600 cells have wild-type sequence in exons 3–35. In the three cell lines, we observed a significant decrease in colony formation upon STAG2 lentiviral expression (Fig. 4d and Supplementary Fig. 12). Intriguingly, STAG2 knockdown was also associated with reduced colony formation in five different cell lines (Supplementary Fig. 13). Similar effects have been reported with knockdown of the tumor suppressor ARID1A in pancreatic and bladder cancer cells25,26.

To place these findings in the context of the known pathways of UBC progression, we assessed the association of STAG2 alterations with FGFR3 mutation or overexpression, p53 nuclear accumulation and Ki67 expression (Supplementary Tables 13–16). In NMIBC, loss of STAG2 was significantly more common in tumors with mutant FGFR3 (42.7% versus 27.2%; P = 0.001), tumors lacking p53 overexpression (P = 0.002) and tumors with a low Ki67 index (P = 0.002). These results indicate that loss of STAG2 is associated with less aggressive tumors. Within the low-risk NMIBC subgroup, loss of STAG2 expression was associated with FGFR3 mutant status (P = 0.059) and with low p53 expression (P = 0.011). In individuals with high-risk NMIBC, loss of STAG2 expression was associated with high FGFR3 expression (P = 0.037), FGFR3 mutation (P = 0.12) and a low Ki67 index (P = 0.049). In both high-risk NMIBC and MIBC, there was no association with p53 expression as detected by immunohistochemistry (Supplementary Tables 13–16).

We then analyzed the association of loss of STAG2 expression with recurrence and progression among individuals with NMIBC and with progression and mortality in individuals with MIBC. We applied both Kaplan-Meier curves and multivariable Cox regression analyses. The large sample size of our study allowed us to perform a more informative stratified analysis. Loss of STAG2 expression was associated with lower risk of tumor recurrence and progression in individuals with NMIBC (Fig. 5a,b). However, in multivariable analyses, STAG2 expression was not an independent predictor of recurrence or progression after adjusting for stage, grade and FGFR3 mutation status (Supplementary Tables 17 and 18), as these parameters were highly correlated. In individuals with MIBC, loss of STAG2 expression was associated with lower risk of progression.
(hazards ratio (HR) = 0.68; \( P = 0.244 \)), and it was an independent predictor of survival in the multivariable analysis (HR = 0.44; \( P = 0.018 \)) (Fig. 5c,d and Supplementary Tables 19 and 20). Therefore, we conclude that loss of STAG2 expression is associated with better prognosis in individuals with both NMIBC and in MIBC; additional studies are required to determine its clinical value.

In summary, we find that both previously reported and newly identified genes coding for proteins involved in chromatin modification are recurrently mutated in UBC. In addition, we identify mutations in genes involved in the cell cycle, DNA repair and the regulation of apoptosis. The frequent alteration of genes in these pathways may provide opportunities for novel therapies, including those based on synthetic lethality. STAG2 is significantly mutated in UBC; mutations and loss of expression are common, particularly in tumors of low stage and grade, and are associated with improved clinical outcome. In non-aggressive tumors, STAG2 alterations occur in the absence of chromosomal instability. Our findings strongly suggest that STAG2 is a new tumor suppressor in UBC through mechanisms that are distinct from its role in cohesion to prevent aneuploidy.

URLs. FAR, http://sourceforge.net/projects/flexbar/; R software, http://www.r-project.org/.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Sequencing data have been deposited in the Sequence Read Archive (SRA) database. Whole-exome sequencing data are accessible under SRP029936, and validation data are accessible under SRP029935. SNP array data have been deposited in the Gene Expression Omnibus (GEO).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.B.-M., E.L. and L.R. designed and performed in vitro functional studies. C.B.-M. performed immunohistochemical analysis of tumor samples. A.S. and C.B.-M. designed and performed mutation validation analyses. A.S. designed and prepared HaloPlex libraries for sequencing. E.C.-d.-S.-P., M.V., F.C.-G., S.B. and D.G.P. processed and analyzed exome sequencing and targeted resequencing data. J.E., E.L.-K., D.R. and S.C. performed gene copy number analyses of tumors. M.M. coordinated subject and sample data management. A.C., M.K., J.A.L. and A.T. contributed to subject recruitment and data collection. J.H. performed statistical analyses. X.L. provided technical support with subject samples. M.B. and M.G. coordinated library preparation and sequencing. O.D. contributed to library preparation and sequencing. I.C.C. and R.N.S. contributed to the in vitro analysis of the effects of STAG2 knockdown on aneuploidy. N.J. and J.L. performed pathological review of samples. I.G. coordinated exome sequencing and targeted resequencing. I.G., S.H. and A.V. supervised bioinformatics analyses. A.L. provided scientific insight and contributed with reagents. N.M. coordinated subject recruitment and the collection of clinical and pathological data and supervised clinical-pathological-molecular association and outcome analyses. F.X.R. and N.M. conceived the study. F.X.R. supervised the overall way in which the study was conducted. F.X.R. wrote the manuscript with N.M., C.B.-M., A.S., E.C.-d.-S.-P., A.V., A.L. and I.G. contributed to manuscript writing.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Sylvester, R.J. Natural history, recurrence, and progression in superficial bladder cancer. ScientificWorldJournal 6, 2617–2625 (2006).
2. Luis, N.M., López-Knowles, E. & Real, F.X. Molecular biology of bladder cancer. Clin. Transl. Oncol. 9, 5–12 (2007).
3. Hernández, S. et al. FGFR3 mutations as a prognostic factor in non-muscle invasive urothelial bladder carcinomas: results of a prospective study. J. Clin. Oncol. 24, 3664–3671 (2006).
4. López-Knowles, E. et al. PIK3CA mutations are an early genetic alteration associated with FGFR3 mutations in superficial papillary bladder tumors. Cancer Res. 66, 7401–7404 (2006).
5. Reali, F.X. p53: It has it all, but will it make to the clinic as a marker in bladder cancer? J. Clin. Oncol. 25, 5341–5344 (2007).
6. López-Knowles, E. et al. The p53 pathway and outcome among patients with T1G3 bladder tumors. Clin. Cancer Res. 12, 6029–6036 (2006).
7. Jebar, A.H. et al. FGFR3 and Ras gene mutations are mutually exclusive genetic events in urothelial cell carcinoma. Oncogene 24, 5218–5225 (2005).
8. Lindgren, D. et al. Combined gene expression and genomic profiling define two intrinsic molecular subtypes of urothelial carcinoma and gene signatures for molecular grading and outcome. Cancer Res. 70, 3463–3472 (2010).
9. Höglund, M. The bladder cancer genome; chromosomal changes as prognostic makers, opportunities, and obstacles. Urol. Oncol. 30, 533–540 (2012).
10. Blaveri, E. et al. Bladder cancer stage and outcome by array-based comparative genomic hybridization. Clin. Cancer Res. 11, 7012–7022 (2005).
11. Gui, Y. et al. Frequent mutations of chromatin remodeling genes in transitional cell carcinoma of the bladder. Nat. Genet. 43, 875–878 (2011).
12. Fujimoto, A. et al. Whole-genome sequencing of liver cancers identifies etiological influences on mutation patterns and recurrent mutations in chromatin regulators. Nat. Genet. 44, 760–764 (2012).
13. Guichard, C. et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat. Genet. 44, 634–638 (2012).
14. Varela, I. et al. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. Nature 469, 539–542 (2011).
15. Wilson, B.G. & Roberts, C.W. SWI/SNF nucleosome remodelers and cancer. Nat. Rev. Cancer 11, 481–492 (2011).
16. Solomon, D.A. et al. Mutational inactivation of STAG2 causes aneuploidy in human cancer. Science 333, 1039–1043 (2011).
17. Amaral, A.F.S. et al. Plasma 25-hydroxyvitamin D3 levels and bladder cancer risk according to tumor stage and FGFR3 status: a mechanism-based epidemiological study. J. Natl. Cancer Inst. 104, 1897–1904 (2012).
18. Welch, J.S. et al. The origin and evolution of mutations in acute myeloid leukemia. Cell 150, 264–278 (2012).
19. Walter, M.J. et al. Acquired copy number alterations in adult acute myeloid leukemia genomes. Proc. Natl. Acad. Sci. USA 106, 12950–12955 (2009).
20. Walter, M.J. et al. Clonal architecture of secondary acute myeloid leukemia. N. Engl. J. Med. 366, 1090–1098 (2012).
21. Nasmyth, K. & Haering, C.H. Cohesin: its roles and mechanisms. Annu. Rev. Genet. 43, 529–558 (2009).
22. Remeseiro, S. & Losada, A. Cohesin, a chromatin engagement ring. Curr. Opin. Cell Biol. 25, 63–71 (2013).
23. Remeseiro, S. et al. Cohesin-SAl deficiency drives aneuploidy and tumourigenesis in mice due to impaired replication of telomeres. EMBO J. 31, 2076–2089 (2012).
24. Remeseiro, S. et al. A unique role of cohesin-SA1 in gene regulation and development. EMBO J. 31, 2090–2102 (2012).
25. Shain, A.H. et al. Convergent structural alterations define SWItch/Sucrose NonFermentable (SWI/SNF) chromatin remodeler as a central tumor suppressive complex in pancreatic cancer. Proc. Natl. Acad. Sci. USA 109, E252–E259 (2012).
26. Balbás-Martínez, C. et al. ARID1A alterations are associated with FGFR3 wild type, poor-prognosis, urothelial bladder tumors. PLoS ONE 8, e62483 (2013).
**ONLINE METHODS**

**Subjects and samples.** Subjects and samples came from the Epicuro/Spanish Bladder Cancer Study (SBCS)3,27 and from the Integrated Study of Bladder Cancer (ISBLAC) (Supplementary Tables 1 and 5). STAG2 expression was analyzed using tissue microarrays containing tumors from the Epicuro/SBCS, including tumors from individuals with newly diagnosed UBC. Staging, grading and follow-up were performed as described1,27. Expert pathologists reviewed diagnostic slides from all tumor blocks. We categorized TaG1 and TaG2 tumors as low-risk NMIBC or non-aggressive; TaG3, T1G2 and T1G3 tumors were categorized as high-risk NMIBC; and ≥2T2 tumors were categorized as MIBC. The latter two groups were pooled as aggressive tumors. Subject characteristics are summarized in Supplementary Tables 8 and 9. In subjects with NMIBC, recurrence was defined as the reappearance of NMIBC following a negative follow-up medical evaluation. Progression was defined as a transition from NMIBC to MIBC or the development of new local or metastatic tumors after primary treatment for individuals with MIBC. Median follow-up time was 62.6 months (range of 1–98 months). All deaths were recorded, but only UBC-related deaths were considered for survival analysis. Cases dying from other causes were censored at the time of death. Survival was computed as the period comprised between diagnosis and death or last control. All subjects provided written informed consent1. The ethics committees of all participating institutions approved both studies.

**Exome sequencing, targeted resequencing, bioinformatic analyses and mutation verification.** The Agilent SureSelect Human All Exon plus v3 50Mb (samples 114, 116, 193, 251, 310, 331, 413, 418 and Esp66) or v4 51Mb (samples 062, 064, 179, 188, 274, 313, 343 and 451) was used for library preparation and enrichment. Libraries were applied to an Illumina flow cell; sequencing was performed on HiSeq 2000 instruments using paired-end 75-bp reads. Base calling and quality control were performed on the Illumina Real-Time Analysis pipeline. Sequence reads were trimmed to the first base with quality >10 that mapped to human genome build hg19 (GRCh37) using GEM, allowing ≤4 mismatches. Reads not mapped by Genome MultiTool Marker (GEM)27 (–4%) were submitted to a last round of mapping with BEAST. Results were merged; only uniquely mapping, non-duplicate read pairs were used. SAMtools suite version 0.1.18 with default settings was used to call SNVs and short indels. Variants on regions with low mappability, read depth <10, tail distance bias P < 0.05 or strand bias P < 0.001 were filtered out. Somatic mutations were called by comparing tumor and blood exomes; Fisher’s exact tests were performed using variant-supporting read counts. Only variants with Fisher’s exact test P value <0.0001 were considered.

All SNVs in exon junctions or that led to a nonsynonymous change were considered to be ‘relevant’. SNVs that led to an amino acid substitution were evaluated usingMutationAssessor28 and SIFT29 to predict their damaging effect; both scores were normalized into a range from 0 to 1. P values from SIFT were subtracted from 1. MutationAssessor predictions were scored as follows: high risk of damage was assigned 1, medium risk of damage was assigned 0.7 and low risk of damage was assigned 0.5. When both predictions were available, scores were averaged; if one prediction was missing, the other score was used. Variants with a final score >0.8 were considered to be damaging. Stop gains and frameshifts were considered to be damaging if they ablated >30% of the sequence or a protein domain annotated in InterPro. Variants close to an exon boundary were considered to be relevant and damaging if the distance from the exon junction was eight bases into the intron or two bases into the exon of donor junctions or if the distance from the exon junction was eight bases into the intron or three bases into the exon of acceptor junctions. Scores from both methods were used as input to calculate P value for the associated genes. We used the Oncodrive-fm approach28 that combines data provided written informed consent1. The ethics committees of all participating institutions approved both studies.

**Immunohistochemistry.** STAG2 was detected using clone J-12 (0.5 µg/ml) (Santa Cruz Biotechnology, sc-81852) and affinity-purified rabbit polyclonal antibodies (0.5 µg/ml) raised against a synthetic peptide (DPASIMDESVLGVSMF)21. Both antibodies yielded concordant results in 92% of tumors. To detect STAG1, we used affinity-purified rabbit polyclonal antibodies (2 µg/ml) raised against a synthetic peptide (EDDSFGGMPF)22. Antibodies D5/16B4 (1,200,000 dilution) and K20.8 (1:50 dilution) detecting KRT5/KRT16 and KRT20, respectively, were from Dako. Antigen retrieval and reactions were performed as described26,27. A histoscore was calculated as the product of the staining intensity (0–3) and the percentage of positive cells (0–100%). Unsupervised clustering analysis was performed using scores and the heatmap.2 function of the gplots package within the R 2.15.1 statistical environment.

**Gene copy number analyses.** Copy number changes were analyzed using manually microdissected fresh tissue samples containing ≥60% tumor cells (n = 55). DNA was hybridized to Illumina HumanHap 1M BeadChip SNP arrays; 20 tumors were TaG1 or TaG2. Copy number changes were called as described1. An additional 76 samples were analyzed using Human 2.0 BAC arrays (UCSF Cancer Center)10,34.

**STAG2 functional assays.** To knock down STAG2, control or STAG2-targeting lentiviral particles were produced in HEK293T cells using Sigma Mission plasmids. Viral supernatants were used to infect RT112, UM-UC-5, 639V, SW1710 and UM-UC-11 cells; after three rounds of infection with shRNA-encoding lentiviruses, cells were selected for 48 h in medium containing puromycin (2 µg/ml). To overexpress STAG2, human cDNA (b isoform; 1,231 residues) was amplified by PCR (Addgene pEGFP-STAG2 plasmid, ref. 31972) and subcloned into the pLVX-puro lentiviral vector. After three rounds of infection,
cells were selected for 48 h with puromycin (2 µg/ml). Protein blotting was performed as described17.

For colony formation assays, 8x10^3 puromycin-selected cells were seeded; 7 days later, cells were methanol-fixed and crystal violet-stained; after elution (10% acetic acid), 680 nm absorbance was measured.

For chromosome analyses, puromycin-selected cells with knockdown were arrested with colcemid (0.1 mg/ml) for 6 h, collected, swollen in 75 mM KCl for 15 min (RT112), 25 min (639V) or 30 min (UM-UC-11) at 37 °C and fixed. Images of metaphase were captured, and chromosomes per metaphase were counted (Axioplan II Imaging MetaSystem Microsoft and Ikaros software, Metasystems). Chromosome number was compared using the Wilcoxon rank-sum test.

Other statistical analyses. Categorical data were reported as numbers and percentages. Associations between STAG2 expression and subject characteristics were assessed using the χ² test. Associations between markers were evaluated using the χ² test and using the odds ratio (OR) and 95% confidence interval (95% CI) as a measure of association between categorical variables.

Outcomes considered were recurrence-free and progression-free survival (NMIBC) and progression-free and cancer-specific mortality (MIBC). Survival was represented using Kaplan-Meier curves; differences between curves were assessed with the log-rank test. Cox proportional hazards models were applied for multivariable analysis. The adjusting factors used are indicated in Supplementary Tables 17–20. Statistical significance was considered as 0.05. R software (version 2.14) was used for statistical analysis.

27. Marco-Sola, S. et al. The GEM mapper: fast, accurate and versatile alignment by filtration. Nat. Methods 9, 1185–1188 (2012).
28. Reva, B. et al. Predicting the functional impact of protein mutations: application to cancer genomics. Nucleic Acids Res. 39, e118 (2011).
29. Kumar, P. et al. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat. Protoc. 4, 1073–1081 (2009).
30. Gonzalez-Perez, A. et al. Functional impact bias reveals cancer drivers. Nucleic Acids Res. 40, e169 (2012).
31. Vazquez, M. et al. Chapter 14: cancer genome analysis. PLoS Comput. Biol. 8, e1002824 (2012).
32. Quesada, V. et al. Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. Nat. Genet. 44, 47–52 (2012).
33. Rodriguez-Santiago, B. et al. Mosaic uniparental disomies and aneuploidies as large structural variants of the human genome. Am. J. Hum. Genet. 87, 129–138 (2010).
34. Snijders, A.M. et al. Assembly of microarrays for genome-wide measurement of DNA copy number. Nat. Genet. 29, 263–264 (2001).