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Title:

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Date:

2020-06-01

Citation:

McEvoy, C. R., Fox, S. B. & Prall, O. W. J. (2020). Emerging entities in NUTM1-rearranged neoplasms. *Genes Chromosomes and Cancer*, 59 (6), pp.375-385. <https://doi.org/10.1002/gcc.22838>.

Persistent Link:

<https://hdl.handle.net/11343/275458>

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Emerging entities in *NUTM1*-rearranged neoplasms

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as doi: [10.1002/gcc.22838](https://doi.org/10.1002/gcc.22838)

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Abbreviated title: NUTM1-rearranged neoplasms

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Abstract

Structural alterations of *NUTM1* were originally thought to be restricted to poorly differentiated carcinomas with variable squamous differentiation originating in the midline organs of children and adolescents. Termed NUT carcinomas (NCs), they were defined by a t(15;19) chromosomal rearrangement that was found to result in a *BRD4-NUTM1* gene fusion. However, the use of DNA and RNA-based next generation sequencing has recently revealed a multitude of new *NUTM1* fusion partners in a diverse array of neoplasms including sarcoma-like tumors, poromas, and acute lymphoblastic leukemias (ALLs) that we propose to call NUTM1-re-arranged neoplasms (NRNs). Intriguingly, the nosology of NRNs often correlates with the functional classification of the fusion partner, suggesting different oncogenic mechanisms within each NRN division. Indeed, whereas NCs are characterised by their aggressiveness and intransigence to standard therapeutic measures, the more positive clinical outcomes seen in some sarcoma and ALL NRNs may reflect these mechanistic differences. Here we provide a broad overview of the molecular, nosological, and clinical features in these newly discovered neoplastic entities. We describe how aberrant expression of NUTM1 due to fusion with an N-terminal DNA/chromatin binding protein can generate a potentially powerful chromatin modifier that can give rise to oncogenic transformation in

numerous cellular contexts. We also conclude that classification, clinical behaviour and therapeutic options may be best defined by the NUTM1 fusion partner rather than by tumor morphology or immunohistochemical profile.

1. Introduction

In the early 1990's a series of case reports emerged that described poorly differentiated carcinomas with a t(15;19)(q13, p13.1) translocation that occurred in the mid-line epithelial structures of young people. These tumors were highly aggressive, refractory to chemotherapy and radiation, and rapidly fatal.¹⁻³ Molecular studies confirmed that the translocation fused the 5' region of the bromodomain-containing protein 4 (*BRD4*) gene on chromosome 19 to almost the entire nuclear protein in testis (*NUTM1*) gene on chromosome 15.^{4,5} Subsequent studies determined that approximately 80% of NUT carcinomas (NCs) harbored a *BRD4-NUTM1* fusion while the remaining NCs were termed "NUT variant carcinomas".⁶ Later reports revealed the *BRD3-NUTM1* fusion to represent around 10% of NCs,⁷ while nuclear receptor binding SET domain protein 3 (*NSD3*)-*NUTM1* fusions were also found to be present in a small number of cases.⁸⁻¹⁰

Until very recently NC was considered a self-contained tumor subgroup. Apart from the defining molecular feature of a *BRD-NUTM1* rearrangement, it also typically had a midline location, distinct morphological features and immunohistochemistry (IHC) staining patterns, and a dismal clinical outcome. However, the entity of NC has now expanded to encompass carcinomas outside of the midline,^{6,11-13} and with novel *NUTM1* fusion partners.^{14,15} Most

strikingly, NC can now be considered as just one class of many *NUTM1*-rearranged neoplasms (NRNs), with *NUTM1* structural alterations having now been reported in undifferentiated sarcoma-like tumors, poromas, porocarcinomas, and acute lymphoblastic leukemias (ALLs). The advent of next generation sequencing technologies has helped to greatly expand the repertoire of *NUTM1* fusion partners in NRNs to include members of the MAX dimerisation (*MAD*) gene family in *NUTM1*-associated sarcoma-like tumors, transcriptional enhancer domain (TEAD) activators in poromas, and numerous other DNA binding proteins in *NUTM1*-associated ALLs (Table 1). Interestingly, the clinical outcomes for non-NC NRNs often appear to be more favourable, while new treatment options for NC have undergone recent development and testing with promising results.

Although several recent overviews of NC exist,¹⁶⁻¹⁸ we here summarise the entire spectrum of NRNs, placing an emphasis on their molecular and clinical aspects. In particular, we stress that many NRNs are less aggressive than NC and that the *NUTM1* fusion partner largely dictates biological behaviour. Determining its identity can therefore be important in clinical and therapeutic decision making.

2. *NUTM1*

NUTM1 is located on chromosome 15q14 and comprises seven exons spanning approximately 12kb. The encoded protein is 1132 amino acids in length and contains nuclear localisation and nuclear export signals that allow it to shuttle between the nucleus and cytoplasm.⁷ It also possesses a highly conserved acidic domain (AD1) that is capable of binding and activating the histone acetyltransferase (HAT) p300.¹⁹ Although the fusion point of *NUTM1* in NRNs may vary, all *NUTM1* fusions analysed retain an intact AD1 domain along with all downstream sequence. Until recently, very little was known regarding the normal function of *NUTM1*. It is now known to be critical for male fertility and is expressed in post-meiotic male germ cells where it recruits the HATs p300 and/or CBP and enables histone H4K5 and H4K8 acetylation.²⁰ This stimulates the transcription of a subset of genes involved in transcriptional shutdown in preparation for the striking genome-wide chromatin remodelling process within condensing spermatids, whereby histones are evicted and replaced by transition proteins and small basic proteins called protamines. Notably, post-meiotic

spermatids coexpress NUTM1 and the BRD family member bromodomain testis-specific protein (BRDT),²¹ thus the megadomain-spanning chromatin hyperacetylation and transcriptional repression of genes involved in differentiation observed in NCs (see below) may be seen as a somatic aberration of this crucial male germline phenomenon.

NUTM1 IHC has been exploited for diagnostic purposes with the development of a NUTM1-specific monoclonal antibody. Aside from weak focal expression in some germ cell tumors IHC with this antibody so far appears specific to NRNs. There is near 100% sensitivity in NCs but sensitivity in other NRNs is unknown. A recent paper reported negative NUTM1 IHC in one *NSD3-NUTM1* NC and in two other NRNs harbouring *MXD1-NUTM1* and *BCORL1-NUTM1* fusions.²² *NUTM1* mRNA levels were high in all of these cases. It is currently not clear whether the negative IHC was due to a post-translational modification or a technical reason. Apart from germ cells in the testis and ovary,²³ adult tissues do not express NUTM1. Therefore, lack of an internal positive control for NUTM1 IHC may unknowingly lead to false negative IHC due to user/laboratory-dependent variability and interfere with its usefulness as a screening tool. Until this query over IHC sensitivity is resolved, cases that are suspicious for a NRN but with a negative NUTM1 IHC should undergo *NUT* FISH and/or molecular sequencing where possible.

3. NUT Carcinoma

NC is a rare subtype of undifferentiated squamous cell carcinoma that was originally termed “NUT midline carcinoma” since it arose in the midline of the mediastinum or head and neck, often in children or young adults.²⁴ It is undoubtedly frequently misdiagnosed as a poorly differentiated squamous cell carcinoma or carcinoma of unknown primary due to its rarity and lack of access to NUTM1 IHC. For example, a recent comprehensive report on the impact of gene fusions in cancer cell fitness included two cell lines derived from patients originally diagnosed with small-cell lung carcinoma and lung squamous cell carcinoma. These were found to harbor *BRD4-NUTM1* and *NSD3-NUTM1* fusions respectively, indicating that they were misclassified NCs.²⁵ Furthermore, there are now many exceptions to the originally reported age range and anatomical location, and unfamiliarity with this may also contribute to

misdiagnosis. NCs are now known to arise outside of the midline and in diverse sites including lung,¹¹ bladder,⁶ brain,²² kidney¹² and bone.¹³

NCs are typically composed of sheets of predominantly relatively monotonous cuboidal cells with high N/C ratios that show small foci of “abrupt” keratinisation, considered an important morphological “clue” to the diagnosis.¹⁶ However, some NCs appear to completely lack keratinising foci,²⁶ which is likely absent due to the inherent sampling error of small biopsies. Other morphological variants of NC include gland-like structures (presumably pseudoglands),^{26,27} small round cells,^{22,28} rhabdoid cells,^{22,28} and chondroid differentiation.²⁷ The tumor cells usually diffusely express high molecular weight cytokeratins and p63/p40 (ΔNp63) by IHC, consistent with squamous differentiation. SOX10 and MYC are also often expressed. Nevertheless, some NCs lack expression of cytokeratins and/or p63/p40,^{22,26,28} or show neuroendocrine differentiation by IHC.^{22,26}

NC is both extremely aggressive and remarkably resistant to standard chemotherapeutic and radiation therapies. Most patients rapidly succumb to the disease, with a median overall survival of between six and 10 months.^{29,30} Aggressive surgical resection with negative margins may be associated with improved survival rates,³⁰ although these findings are not consistent.³¹ Therapeutic success has also been reported in rare cases by employing combined modality therapy according to the Scandinavian Sarcoma Group (SSG) IX protocol for inoperable Ewing sarcomas.^{13,32}

Unlike most other carcinomas, NCs are defined by the presence of a *NUTM1* fusion rather than by their anatomical site of origin. The *BRD4-NUTM1* fusion is present in approximately 80% of NCs¹⁶ while the remaining 20% have been found to harbor *NUTM1* fusions with *BRD3*,⁷ *NSD3*,⁸ *ZNF532*,^{14,28} and *ZNF592*.¹⁵ BRD4 and BRD3 are members of the bromodomain and extra terminal domain (BET) protein family. Like other BET family members they contain two bromodomains that target acetylated chromatin. Many of the details regarding the oncogenic mechanism of BRD4-NUTM1 have been described in a series of compelling papers by French and colleagues^{14,19,33-36} and You and colleagues.³⁷⁻³⁹ Briefly, the BRD4 region of the fusion oncoprotein is thought to bind to acetylated lysine residues on histones. This is followed by the recruitment of the histone acetyltransferase p300 by the NUTM1 AD1 region which, in turn, acetylates neighbouring histones. This leads to a positive feedback loop of further tethering of

the BRD4 region to the newly acetylated histones and subsequent NUTM1-associated p300 histone acetylation. This process is able to progress over large (>1.5Mb) genomic regions or “megadomains”, often encompassing entire topologically associated domains.³⁴ In *BRD-NUTM1* expressing cell lines these megadomains are visible microscopically as several hundred foci per nucleus that are associated with p300 and histone acetylation.^{19,39} Altered gene regulation within the localised hyperacetylated transcriptionally-activated regions is responsible for oncogenic transformation. In particular, *MYC*, *SOX2* and *TP63* are key targets of this process and their dysregulation plays a major role in the pathogenesis of NC.^{14,33-35,38} In contrast to the p300-associated transcriptional activation observed in *BRD4-NUTM1*-associated megadomains, p300 is sequestered away from pro-differentiation genes leading to their silencing and genome-wide hypoacetylation.^{34,36} *BRD3-NUTM1* is thought to act in an analogous manner to *BRD4-NUTM1*. Notably, all of the alternative *NUTM1* fusion partners in NC so far identified (*NSD3*, *ZNF532* and *ZNF592*) are predicted to form a chromatin binding complex with *BRD4/3*.^{8,14,15} Thus, these *NUTM1* fusions likely enable a linkage between *NUTM1* and *BRD4/3* and result in a similar mechanistic process.

While NC may sometimes exhibit complex chromosomal rearrangements⁴⁰ it is also notable for the absence of additional known oncogenic drivers,^{40,41} (though see reference⁴² for an exception) and low overall mutation frequency,^{43,44} a fact that coincides with its frequent occurrence in young people. The scenario of a single gene fusion being sufficient to drive oncogenesis is reminiscent of *EWSR1-FLI1* in Ewing's sarcoma or *BCR-ABL* in chronic myeloid leukemia. This lack of additional driver mutations is also seen in most other NRNs as described below.

4. MAX Dimerization Protein (*MAD*)-*NUTM1* tumors

Over the last several years evidence has accumulated that *NUTM1* rearrangements can be found outside the standard clinicopathologic setting of NC. Of particular interest, several members of the MAX Dimerization (*MAD*) gene family, including *MXD1*,²² *MXD4*,^{26,45} and *MGA*^{26,46-48} have now been identified as *NUTM1* fusion partners. Tumors with *MAD-NUTM1* fusions have overlapping histopathological features with NCs, but overall are less differentiated as determined by both morphology and IHC. They are typically composed of

sheets of round or mixed round/spindled cells,^{22,26,45-48} sometimes with rhabdoid or giant cells.²² Importantly, morphological evidence of epithelial differentiation, including the abrupt keratinising foci seen in NCs, is absent in *MAD-NUTM1* tumors. A few are associated with distinctive matrix including collagenous “amianthoid” fibres^{26,46-48} or chondroid differentiation.²⁶ The undifferentiated morphology is borne out by IHC, which typically shows variable expression of the non-specific markers CD99, CD34 and BCL2, and no expression of cytokeratins, p63/p40, EMA, S100, GFAP, neuroendocrine markers or lymphoid markers.^{22,26,45-48} Since only very few *MAD-NUTM1* tumors have been identified, variations on this expression profile may be encountered in the future. Indeed, at least one *MAD* fusion tumor (*MXD1-NUTM1*) has already been described with weak expression of cytokeratins,²² and occasional tumors express desmin, but not myogenin.⁴⁶ In general, *MAD-NUTM1* tumors appear best classified as sarcomas, but in practice their differential diagnosis includes NC, as well as *CIC-NUTM1* sarcoma (see below), Ewing family sarcoma, myoepithelial carcinoma, synovial sarcoma, and myxoid chondrosarcoma amongst others.

MAD proteins compete with the proto-oncogene *MYC* for binding to *MYC*-associated factor X (*MAX*). *MYC/MAX* heterodimers transactivate promoters containing E-box sequences, typically activating genes involved in cellular proliferation. *MAD* proteins also form heterodimers with *MAX* that bind promoter E-boxes. However, *MAD/MAX* represses *MYC*-dependent activation by tethering the mSin3 transcriptional repressor and associated histone deacetylases (HDACs).^{49,50} The competition between *MYC* and *MAD* for binding to *MAX* therefore has a large impact on determining the proliferative state of the cell. Notably, apart from binding to E-boxes when in association with *MAX*, *MAD* proteins have no known involvement in chromatin binding, either directly or indirectly. Furthermore, they are functionally distinct from BRD proteins and are not members of the BRD-NUTM1 (‘Z4’) protein complex seen in NC.^{14,15} Thus the oncogenic mechanism of *MAD-NUTM1* tumors appears incompatible with the accepted model of NC formation. This fact also has important potential therapeutic implications as discussed below.

The fact that *MAD* proteins are negative regulators of *MYC*, and that *MYC* is a crucial target of BRD-NUTM1 in NC is intriguing. In order to account for these findings we propose an oncogenic mechanism whereby the N-terminal region of *MAD* in the *MAD-NUTM1* fusion protein forms heterodimers with *MAX* and bind to promoter E-boxes at *MYC* target genes.

Following E-box binding, the p300 HAT activity mediated by the C-terminal NUTM1 AD1 domain overrides the Sin3 HDAC activity mediated by the N-terminal MAD domain, leading to localised hyperacetylated chromatin and activated gene transcription (Figure 1). We note that this model predicts that MYC target genes will be activated in the absence of MYC expression. Furthermore, unlike the model for *BRD4-NUTM1* this model does not include a positive feedback system leading to megadomains of hyperacetylated chromatin. This model remains speculative and gene expression studies along with mechanistic studies involving recombinant *MAD-NUTM1* constructs will be required in order to ascertain its validity.

Although information is currently limited it appears that, like NCs, additional somatic cancer driver mutations are rare in these tumors. One case of whole genome sequencing⁴⁶ and one of whole exome sequencing⁴⁵ have both failed to find additional significant cancer driver mutations or evidence of chromoplexy. Although the total cases are again limited, the clinical course of *MAD-NUTM1* tumors seems more positive than NCs. Both *MGA-NUTM1* tumors reported by Diolaita and colleagues were successfully treated with surgical resection and radiation and the patients remained disease free 11 years and 15 months following diagnosis.⁴⁶ Furthermore, in a cohort of six patients with *NUTM1*-associated soft tissue and visceral tumors, the only patient remaining alive after 108 months harbored a *MXD1-NUTM1* fusion despite peritoneal dissemination and lymph node metastases.²²

5. Other *NUTM1*-associated solid tumors

The Ewing family of tumors includes sarcomas with recurrent fusions of *EWSR1*, capicua transcriptional repressor (*CIC*), or BCL6 corepressor (*BCOR*). Tumors with *CIC* and *BCOR* fusions have overlapping but distinct clinical and morphological features to those with *EWSR1* fusions, and tend to pursue a more aggressive course. *CIC* is typically fused to *DUX4* or *FOXO4*. However, *NUTM1* has also recently emerged as a recurrent *CIC* fusion partner.⁵¹⁻⁵⁴ *CIC* is a transcriptional repressor that interacts with DNA via a high motility group (HMG) box in conjunction with a C-terminal C1 domain.⁵⁵ It has also been shown to be a tumor suppressor in some lymphoid malignancies.⁵⁶

CIC-NUTM1 tumors represent an interesting nosological dilemma, as fusions involving each gene are already defining features of distinct tumor types; *NUTM1* fusions in NCs and *CIC*

fusions in Ewing family sarcomas. *CIC-NUTM1* tumors show variable predominance of round, rhabdoid, spindled and epithelioid cell morphologies,^{48,52-54} often in a myxoid matrix,⁵²⁻⁵⁴ but sometimes with chondroid differentiation or hyalinised stroma.⁵³ Their IHC staining patterns appear highly variable, but as for *MAD-NUTM1* tumors, with limited case numbers this may become more refined in the future. They often express CD99,^{48,52,53} and sometimes stain for WT1 and ETV4, which are typically expressed in *CIC*-fusion Ewing family tumors,^{48,52,54} but also cytokeratins as in NC.⁵² Based on the partial similarity to NCs, Schaefer and colleagues concluded that a case of *CIC-NUTM1* NRN was best considered as a NC rather than a Ewing family tumor.⁵³ However, others have shown that *CIC-NUTM1* tumors have transcriptome profiles similar to *CIC-DUX4* and *CIC-FOXO4* Ewing family tumors and are distinct from *BRD4/3-NUTM1* NCs.^{51,52} Moreover, their methylome profiles and overall morphology has also been reported to be extremely similar to *CIC-DUX4* and *CIC-FOXO4* tumors.^{52,57} Therefore, on current evidence they appear to represent sarcomas from the Ewing family of tumors. A similar approach to help classify *MAD-NUTM1* tumors is yet to be reported. While mechanistic studies have not yet been reported it has been proposed that genes targeted by *CIC* become transcriptionally activated via *NUTM1*-induced HAT recruitment (Figure 1d).⁵¹ *CIC-NUTM1* sarcomas appear to be associated with a more aggressive course and a poorer outcome than *CIC-DUX4* sarcomas⁵² although larger case series are required for confirmation. As in other NRNs, two studies of *CIC-NUTM1* tumors suggest that additional cancer drivers are rare.^{53,54}

An interesting likely addition to this tumor family has recently been reported in a frontal brain tumor with a *ATXN1-NUTM1* fusion in a 21 year old woman. The tumor was composed of spindle cells in a chondromyxoid matrix.⁵⁸ IHC showed expression of GFAP and CD56, and *ETV4* RNA was overexpressed. DNA methylation analysis classified the tumor closest to Ewing family sarcomas with *CIC* alterations. DNA sequencing using a 571-gene targeted sequencing panel failed to detect any additional pathogenic variants. *CIC* and *ATXN1* combine to form a transcriptional repressor that is part of the potent *CIC-ATXN1-ATXN1L* cell cycle regulator⁵⁹ suggesting that this tumor is most closely related to *CIC*-associated NRNs. In this single case the tumor was surgically removed and the patient showed no sign of recurrence 16 months later.

Dickson and colleagues have recently described a primary undifferentiated intramuscular tumor containing a *BCL6* corepressor-like 1 (*BCORL1*)-*NUTM1* fusion.²² *BCORL1* is a

transcriptional corepressor that associates with several different class II HDACs and the CtBP corepressor.⁶⁰ It is also a putative tumor suppressor gene and inactivating mutations have been detected in a range of myeloid malignancies.⁶¹ Like *CIC*, *BCORL1* has also been associated with other oncogenic fusion partners⁶²⁻⁶⁵ although only one of these cases has found *BCORL1* as the N-terminal partner, with *BCORL1* exons 1-11 fused to *ELF4* exon 8 in a hepatocellular carcinoma.⁶² Interestingly, in the single reported *BCORL1-NUTM1* fusion case²² only the first exon of *BCORL1* is retained in the fusion protein. This contains just 29 amino acids and is not known to contain any domain involved in its transcriptional modification activities. It has been recently demonstrated that the majority of tumor-associated gene fusions are non-functional,^{25,66} including those involving known cancer driver genes.²⁵ *BCORL1-NUTM1* may therefore represent a simple stochastic passenger event.

The repertoire of solid tumors harboring *NUTM1* fusions was recently expanded with a report demonstrating their common occurrence in poromas and porocarcinomas.⁶⁷ Poromas are benign sweat gland-derived skin tumors. Porocarcinomas are rare invasive tumors that can produce metastatic disease and may form via the malignant transformation of poromas or may arise de novo.⁶⁸ Gene fusion analysis of 104 poromas revealed 71 *YAP1-MAML2*, 21 *YAP1-NUTM1*, and one *WWTR1-NUTM1* fusions, while analysis of 11 porocarcinomas revealed one *YAP1-MAML2* and six *YAP1-NUTM1* fusions. Unpublished results from our laboratory have also confirmed *NUTM1* expression in poromas. *YAP1* and *WWTR1* are paralogous transcriptional regulators of TEAD proteins. They are negatively regulated by the Hippo signalling pathway and their activation, often caused by Hippo pathway inactivation, is commonly observed in tumorigenesis.⁶⁹ The alternate *YAP1* fusion partner, *MAML2*, is a commonly observed C-terminal fusion partner to *CRTC1* in mucoepidermoid carcinoma of the salivary gland. Like *NUTM1*, *MAML2* interacts with p300 and this is crucial for *CRTC1-MAML2* transforming ability.⁷⁰ Mechanistically, it is notable that all the *YAP1* and *WWTR1* fusions detected in poromas and porocarcinomas harbored the N-terminal TEAD-binding domain of *YAP1* or *WWTR1* fused to a C-terminal *MAML2*- or *NUTM1*-derived region that interacts with CBP and p300 transcriptional coactivators.⁶⁷ The *YAP1* and *WWTR1* fusions were able to transactivate a TEAD reporter and demonstrated their transformational capacity by promoting anchorage-independent growth in NIH3TC and dermal cells.⁶⁷ Furthermore, *YAP1-NUTM1* fusions were enriched in porocarcinomas and poromas with primarily dermal localization, implying a link between specific fusions and clinicopathologic features.

A targeted sequencing panel against 114 cancer-associated genes was used to analyse 23 poromas and nine porocarcinomas.⁶⁷ Unusually for NRNs a significant number of protein-altering variants were detected, with poromas and porocarcinomas averaging one and three mutations per lesion respectively. The altered genes were heterogeneous with only *KRAS*, *SETD2*, and *TP53* recurrently mutated in porocarcinomas. Porocarcinomas are known to be related to UV exposure⁷¹ and this result fits with the older median patient age (67 for poromas and 75 for carcinomas) observed in this study and suggests a more typical oncogenic mechanism involving the stochastic accumulation of mutations over time.

6. *NUTM1*-associated ALLs

Comparable to the recent rapid expansion of novel *NUTM1* fusions identified in solid tumors has been their identification in several subtypes of ALL. They have been detected in ALLs of both B-cell and T-cell lineages and disproportionately affect pediatric and infant cases. Although they are rare, many of the fusion partners have now been detected multiple times and they appear to represent novel oncogenic drivers.

Evidence for ALL-associated *NUTM1* fusions first emerged from a comprehensive genetic and transcriptomic study of *MLL*- and non *MLL*-rearranged ALL in infants and children.⁷² Two new fusions, *BRD9-NUTM1* and apoptotic chromatin condensation inducer 1 (*ACIN1*)-*NUTM1*, were detected in non *MLL*-rearranged infants. The same year, Nordlund also reported a *BRD9-NUTM1* fusion in an infant ALL patient⁷³ and previous reports of the (5;15)(p15;q14) rearrangement produced by *BRD9-NUTM1* in infant ALL suggests that this is a recurrent phenomenon.^{74,75} Interestingly, *BRD9*, while not categorised as a BET protein, contains a single bromodomain and binds the lysine residues of acetylated histones. *ACIN1-NUTM1* fusions have now also been reported on several more occasions in infant and pediatric ALLs.^{72,76-79} Other *NUTM1* fusion partners reported in ALL are *IKZF1*,^{76,77,80,81} *ZNF618*,^{76,81-83} *AFF1*,^{81,82} *SLC12A6*,^{76,77,81,84} *CUX1*,^{76,77,84} and *BPTF*.⁸⁴

With a single exception all of these *NUTM1* fusion partners are predicted to associate directly with DNA and/or participate in chromatin remodelling. The exception, solute carrier family 12 member 6 (*SLC12A6*), also known as K-Cl cotransporter C (*KCC3*), is a member of the K-Cl cotransporter family.⁸⁵ Our analysis of the *SLC12A6-NUTM1* fusion described by Hormann et

al⁷⁷ reveals that it is in-frame and that the fusion protein is expressed. The predicted protein comprises only a short N-terminal region of *SLC12A6* (32 – 91 amino acids depending on the transcript) that contains no known functional domain. Interestingly, we note that *SLC12A6* is located on chromosome 15, less than 8 kb upstream of *NUTM1* in reverse orientation. This raises the possibility that *SLC12A6-NUTM1* fusions are non-oncogenic stochastic passenger events caused by localised inversions.

Almost no details are known regarding the oncogenic function of the other ALL-associated *NUTM1* fusions. Gene expression studies comparing *NUTM1*-expressing vs non-expressing pediatric B-other ALL cases have found upregulation of the genes in cytoband 10p12.31, including the oncogene *BMI1*.⁷⁷ Since p300 preferentially binds a risk allele of *BMI1* associated with an increased likelihood for BCP-ALL the authors speculate that *NUTM1* fusion proteins contribute to leukemogenesis by stimulating p300 which leads to the upregulation of *BMI1* and other 10p12.31 genes. Also upregulated in *ACIN1*- and *CUX1-NUTM1* ALLs was the *HOXA4* gene cluster on chromosome 7p.⁷⁷ The detection of upregulated *HOXA4* genes in *ACIN1*- and *CUX1*-, but not *SLC12A6*- or *IKZF1-NUTM1* ALLs has been documented twice,^{77,81} providing further evidence that specific *NUTM1* fusion partners drive oncogenesis via distinct, though likely related, mechanisms.

Though numbers are again limited, sequencing evidence^{72,77} and the high pediatric incidence suggests that *NUTM1*-associated ALLs lack the additional mutational drivers seen in most other cancer types. Regarding prognosis, all seven B cell precursor ALL patients demonstrating high *NUTM1* expression (including confirmed *SLC12A6*-, *ACIN1*-, *CUX1*-(x 2) and *IKZF1-NUTM1* fusions) in the study of Hormann and colleagues, achieved continuous complete remission with a median follow-up time of 8.3 years (range 4.8 - 13.8 years).⁷⁷

7. Diagnostic and therapeutic implications

Solid NRNs are predominantly poorly differentiated and have overlapping morphological features. Some features are characteristic, e.g. keratinising foci, cytokeratins and p63/p40 expression in NCs, and WT1/ETV4 in *CIC-NUTM1* tumors. However, occasionally characteristic features are absent, or even “aberrantly” present in other NRNs as described above, e.g. cytokeratin expression in *MAD-NUTM1* and *CIC-NUTM1* tumors. Therefore,

identification of the *NUTM1* fusion partner appears to be the most diagnostic feature for NRNs. For some cases it may be challenging for a pathologist to achieve the correct diagnosis from what is typically a small biopsy specimen, due to sample error, tissue exhaustion and lack of access to appropriate molecular technologies. The diagnostic challenges presented by NRNs have important potential clinical implications. If *NUTM1* expression in isolation is interpreted as diagnostic for NC there is the potential for inappropriate treatment as treatment for a *MAD*- or *CIC*-fused sarcoma may be more appropriate. Indeed, at least one lung *CIC-NUTM1* sarcoma has been previously misdiagnosed as a NC.⁵²

NC is notoriously resistant to standard therapeutic interventions and though occasional cures have been reported^{13,32} the large majority of patients rapidly succumb to the disease. However, the involvement of BET proteins makes it a candidate for targeted therapy using BET inhibitors (BETis). These are targeted direct inhibitors of BET proteins and function by inhibiting their binding to the acetylated lysines of histones.⁸⁶ They can be effective against *BRD4-NUTM1* NCs in vivo, with a response rate of 20 – 30%,^{87,88} and the finding that they are able to halt proliferation and induce differentiation in a *NSD3-NUTM1* cell line⁸ suggests that they may be effective against NCs harbouring any BRD4/3 complex member fused to *NUTM1*. Interestingly, differences in the clinical effectiveness of BETis have been reported to exist between different fusion variants of *BRD4-NUTM1*⁴³ and so differences between different *NUTM1* fusion partners are also to be expected. HDAC inhibitors have also shown promise in vitro, in animal models, and in a single case administration to a pediatric patient.³⁶ It is hoped that the next generation of BETis and their use in combination with CDK4/6,⁸⁹ CDK9⁹⁰ or HDAC^{36,91,92} inhibitors will improve the response rate along with the severe resistance and toxicity issues currently observed.^{16,17} However, BETi and HDAC inhibitors may be of limited benefit in *MAD-NUTM1* and *CIC-NUTM1* tumors for which there is no demonstrated connection to the BRD complex and HAT-sequestration model of NC carcinogenesis, although this should be explored in vitro.

Other NRNs appear to have a generally more favourable response to the standard therapies for their specific tumor type. Their rarity and recent discovery currently precludes any definitive statement regarding their susceptibility to specific therapeutic regimens. A recent report has demonstrated the susceptibility of synovial sarcoma cells harbouring a *SS18-SSX* fusion to a small molecule degrader of the *NUTM1* fusion partner *BRD9*.⁹³ *BRD9* is a crucial component

of the oncogenic SWI/SNF (BAF) chromatin remodelling complex and its degradation induces downregulation of oncogenic transcriptional programs and inhibits tumor progression in vivo. Several additional studies have described the development of potent small-molecule inhibitors of the BRD9 bromodomain⁹⁴⁻⁹⁶ though how these or the BRD9 small molecule degrader would act in the context of *BRD9-NUTM1* ALL is unclear. A recent report has also characterised the mechanism and cellular activity of a BPTF bromodomain inhibitor⁹⁷ but studies are still in their infancy.

The finding that most NRNs are likely driven by a single structural mutation, with low somatic mutation rates and limited input from additional drivers, may be positive in terms of therapeutic potential. Such tumors are less likely to possess the clonal heterogeneity common to most tumors and pre-existing treatment-resistant clones that can undergo positive selection and expansion upon treatment administration are therefore less likely to exist. We also note one other potential positive aspect for future NRN therapeutics. Because of its highly specific and limited tissue distribution, drugs that are able to specifically target NUTM1 would be expected to possess limited toxicity apart from transient male infertility. We are unaware of any work currently being conducted on NUTM1-targeting drugs but this could represent an attractive future direction.

8. Concluding remarks

In this review we have sought to summarise the recent rapid increase in knowledge regarding NRNs. Much of this has been due to the increased use of whole genome and transcriptome sequencing, which has enabled the discovery of *NUTM1* rearrangements in unexpected cancer types. Furthermore, the use of RNA-based Anchored Multiplex PCR (AMP) analysis⁹⁸ has also allowed for “fusion discovery”, where no prior knowledge of the *NUTM1* fusion partner is required. Despite the extreme rarity of NRNs these methods have resulted in the discovery of over 15 novel *NUTM1* fusion partners within the past five years (Table 1). Some NRNs appear to be able to be classified as carcinomas or sarcomas. However, other NRNs are essentially undifferentiated and “primitive”, with variable, weak or mixed expression of genes that are typically used by pathologists to broadly classify tumors into carcinoma or sarcoma subtypes. This cautions that classification for some NRNs may remain problematic, and

indeed inappropriate, and classification by the *NUTM1* fusion partner may be of greater relevance to the clinical behaviour and therapeutic options.

The cataloguing of NRN entities and their associated *NUTM1* variants is, however, the low hanging fruit and uncovering the oncogenic mechanisms involved and relating them to potential therapeutic options will be more considerably more difficult. Admirable progress has been made in unravelling the mechanistic details of NC and in determining its susceptibility to BETis. Indeed, these studies have helped to reveal the importance of BET proteins in a wide range of cancers and are a good example of how the detailed study of a seemingly rare mechanism and cancer type can lead to the identification of a therapeutic target in multiple other tumors. Currently however, the corresponding studies for other NRNs are either in their extreme infancy or are yet to commence. While high quality functional studies using *NUTM1* fusion constructs are still required for an understanding of their basic oncogenic mechanisms, it appears that most, if not all, NRNs arise from the expression of *NUTM1* in a somatic rather than germline context and that a DNA/chromatin-binding fusion partner allows for NUTM1-associated HATs to aberrantly acetylate histones (Figure 1d). The outcome of this process is dysregulated gene expression and subsequent oncogenic transformation. Depending on the number and location of DNA/chromatin binding sites and for the potential for hyperacetylated megadomains to form, this single mutational event may result in a massive disruption of normal gene regulation. This is presumably the reason for the lack of additional driver mutations and often young age of incidence seen across most of the NRN spectrum. Furthermore, we predict that differences in the chromatin binding locations of the fusion oncogenes lead to the molecular, phenotypic, and clinical differences observed between the different NRN entities. This process must also be considered in the context of the cell type and developmental stage in which the fusion occurs. NRNs often appear to stimulate oncogenesis in the context of stem and progenitor cells. Interestingly, this observation applies to many fusion-driven malignancies that target chromatin complexes.⁹⁹ French and colleagues have speculated on causes for the rarity of NCs, noting that the genomic location of BRD4-NUTM1-associated hyperacetylated megadomains varies with cell type and that the prolonged overexpression of BRD4-NUTM1 is lethal in all cell types tested.³⁴ They have proposed that in the case of NC the pre-existing chromatin landscape of the squamous precursor cell may have the necessary accessible chromatin seed sites for transformation by *BRD4-NUTM1*, whereas in other cell types, which have alternate chromatin landscapes, a *BRD4-NUTM1*

fusion is lethal. To extend this analogy, it is possible that the chromatin landscape of certain sarcoma progenitor cells is, for example, susceptible to transformation by *MAD-NUTM1* fusions and lymphoblasts to transformation by *CUX1-NUTM1* fusions, whereas outside of these cellular contexts they are lethal.

The coming years will likely see the recognition of new categories of NRN and the identification of additional *NUTM1* fusion partners. The translation of initial discovery to subsequent mechanistic understanding and a final endpoint of effective therapeutics will undoubtedly be challenging. However, encouraging results have already emerged regarding NCs and we anticipate that a repeat of this process will be stimulated by the newly recognised NRN classes described here.

Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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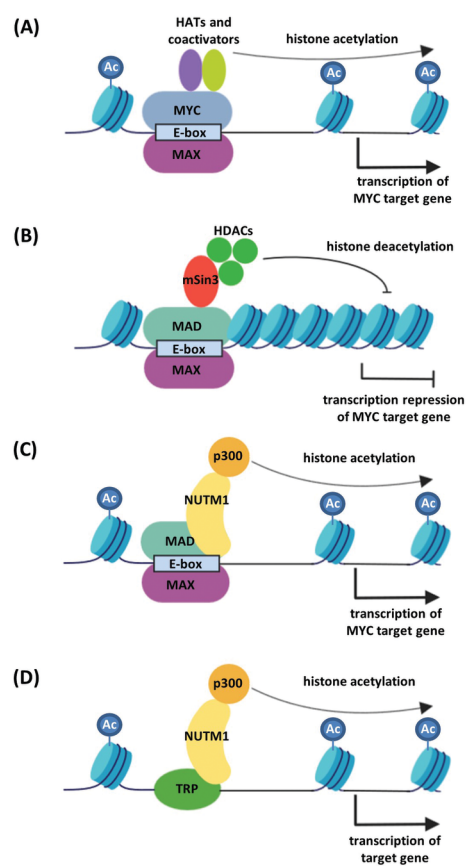
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Figure 1. A speculative model for MAD-NUTM1-mediated oncogenic transformation. A, Simplified model of typical transactivation of a MYC target gene. The MYC/MAX heterodimer binds an E-box upstream of a MYC target gene. MYC-associated HATs and coactivators induce histone acetylation and subsequent transcription of MYC target. B, MAD-associated repression of a MYC target gene. MAD competes with MYC for binding to MAX while the MAD/MAX heterodimer competes with MYC/MAX for binding to E-boxes. MAD/MAX binds the mSin3 transcriptional repressor and associated HDACs, leading to transcriptional repression of MYC target genes. C, MAD-NUTM1-associated activation of MYC target genes. MAD-NUTM1 forms a heterodimer with MAX via the retained MAD HLH-Zip sequence. Following E-box binding, NUTM1 recruits the p300 HAT leading to histone acetylation and activation of MYC targets. D, In a generic sense, transcriptional repression by any TRP (transcriptional repressor protein) could potentially be overridden by fusion to NUTM1 and recruitment of p300.



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Table 1. Categories of NRNs including all currently described *NUTM1* fusion partners.

Classification	<i>NUTM1</i> fusion partner	Normal function	Notes	References
NUT Carcinoma	<i>BRD4</i>	BET family. Recognizes and binds acetylated histones. Transcriptional regulator.	Found in approx. 80% of NCs. Sensitive to BETis.	4
	<i>BRD3</i>	BET family. Recognizes and binds acetylated histones. Transcriptional regulator.	Paralog of <i>BRD4</i> .	7
	<i>NSD3</i>	Transcription factor. Forms complex with <i>BRD4/3</i> .	<i>NUTM1</i> may be negative by IHC. <i>NSD3-NUTM1</i> sensitive to BETi in vitro.	8
	<i>ZNF532</i>	Transcription factor. Forms complex with <i>BRD4/3</i> .	Two reports, one in an atypical case exhibiting monotonous epithelioid and rhabdoid cytomorphology.	14,28
	<i>ZNF592</i>	Transcription factor. Forms complex with <i>BRD4/3</i> .	Single case. Undifferentiated malignant round cell tumor.	15
Sarcoma	<i>MXD1</i>	MAD family. Suppresses MYC-mediated transcription.	Single case. Stomach wall rhabdoid/polygonal cells. <i>NUTM1</i> negative by IHC.	23
	<i>MXD4</i>	MAD family. Suppresses MYC-mediated transcription.	Reported in undifferentiated small round cell ovarian and colon (cecum) sarcomas.	26,45
	<i>MGA</i>	MAD family. Suppresses MYC-mediated transcription.	Lung myxoid spindle cell sarcoma. Chest wall/pleural undifferentiated sarcoma. High-grade spindle cell sarcoma.	26,46,48

Round cell sarcoma	<i>CIC</i>	DNA binding transcriptional represso involved in cell cycle regulation.	Possible subset of <i>CIC</i> -fused Ewing sarcomas.	51-54
Favor sarcoma	<i>ATXN1</i>	Binds to <i>CIC</i> to form transcriptional repressor involved in cell cycle regulation.	Most closely related to Ewing family sarcomas with <i>CIC</i> alterations	58
Favor sarcoma	<i>BCORL1</i>	Interacts with histone deacetylases. Transcriptional modulator.	NUTM1 negative by IHC. Single case involves only <i>BCORL1</i> exon 1 (29 aa).	23
Poromas and porocarcinoma.	<i>YAP1</i>	Transcriptional TEAD regulator. Parologue of <i>WWTR1</i> .	Transformational ability confirmed in vitro.	67
	<i>WWTR1</i>	Transcriptional TEAD regulator. Parologue of <i>YAP1</i> .	Transformational ability confirmed in vitro. Single example in poroma.	67
Pediatric/infant ALL	<i>BRD9</i>	Involved in chromatin remodelling and regulation of transcription. Recognizes and binds acetylated histones.	Bromodomain-containing.	72,73
	<i>ACIN1</i>	Induces apoptotic chromatin condensation after activation by caspase-3.		72,76-79
	<i>IKZF1</i>	Zinc finger, DNA binding chromatin remodelling functions. Interacts with HDACs.		76,77,80,81
	<i>ZNF618</i>	DNA binding zinc finger protein. Likely role in transcriptional regulation.		76,81-83

	<i>AFF1</i>	DNA-binding/chromatin remodelling functions. Transcriptional regulation.		81,82
	<i>SLC12A6</i>	K-Cl cotransporter family.	No known DNA binding activity. Close genomic proximity to <i>NUTM1</i> . Possible non-oncogenic passenger event.	76,77, 81,84
	<i>CUX1</i>	Homeodomain family of DNA binding proteins. Involved in transcriptional regulation.		76,77,84
	<i>BPTF</i>	Contains a C-terminal bromodomain. DNA-binding domain and a zinc finger motif. Likely transcriptional regulator.	Bromodomain-containing.	84

Abbreviations: BET, bromodomain and extra terminal domain; BETis, bromodomain and extra terminal domain inhibitors; IHC, immunohistochemistry; TEAD, transcriptional enhancer domain; HDAC, histone deacetylase.

Title Page

Emerging entities in *NUTM1*-rearranged neoplasms

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Abbreviated title: NUTM1-rearranged neoplasms

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