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SUPPLEMENTARY RESULTS

Initial studies to identify chromatin/nuclear matrix-bound proteins in G0 and G1

In initial studies, extracts of chromatin and nuclear matrix-bound (C/NM) as well as unbound (free) proteins were isolated from quiescent (G0) T cells and T cells stimulated with PMA/ionomycin for 40h (see main text). The extracts were trypsinized and proteins were identified by LC-MS/MS (Washburn et al., 2001) by standard 2D MudPIT approach (Surveyor/DecaXP+, ThermoFinnigan). These studies identified 611 proteins across four biological replicates of T cells in G0 as well as 40h +PMA/ionomycin. 250 proteins increased in C/NM binding as cells entered the cell cycle for the first time (Table S1), and these were functionally annotated by reading the primary literature (Figure S2A). A dynamic protein interaction map of proteins that increase in binding to C/NM in G1 was generated by mapping the proteins to the human protein interactome (Figure S2B) and MCODE analysis of the most highly interconnected sub-networks reveals clusters of proteins involved in (1) RNA splicing, structure and transport, (2) ribosome proteins and (3) nucleolar proteins including proteins involved in ribosome biogenesis (Table S1, Figure S2B). The network is also available as a file which can be viewed in Cytoscape (.cys File 1) and as a high resolution PDF (Figure S2C).

To determine whether the proteomic data reflect changes in protein abundance, we analyzed a subset of these proteins by western blotting (See main text; Figure 2). Analysis of LSP-1 and MeCP2 showed that they were predominantly bound in G0 not G1, as indicated by the mass spectrometry analyses (Figure 2A and B). In initial analyses of a further 31 proteins predicted to be C/NM-bound in G1 but not G0, all but one (nuclear scaffold protein, SAFB1, Figure 2D) were correctly assigned.

Effect of detergent removal on detection of proteins using the LTQ-Orbitrap mass spectrometer

We observed some changes in protein detectability highlighted by comparing the two mass spectrometry analyses. For instance, the percentage of proteins involved in ribosome biogenesis was higher in the LTQ-Orbitrap dataset than in the Surveyor/DecaXP+ dataset, while the opposite was true for ribosome proteins. The MCM proteins bound to C/NM were observed to bind to C/NM in the LCQ-Deca dataset, but not in the LTQ-Orbitrap dataset. We found that this effect derived from the removal of detergent from the LTQ-Orbitrap samples following lysis and fractionation. To determine if a detergent removal step could account for the loss of MCM proteins, cell lysates were treated with Extracti-Gel D (Pierce, see Materials and Methods). Western blot samples were taken of the fractions prior to detergent removal (Pre) and of the proteins remaining afterwards (Extract). The gel used in the extraction was also heated in SDS lysis buffer in order to analyse the proteins that were removed by the
detergent extraction process (gel). The western blot data show that if a reaction volume of 50µl was used then the amount of MCM7 detected in the extract was reduced to almost undetectable levels (Figure S3). The same was also true of MCM2 and cdk6. To determine if this would explain the loss of some C/NM proteins, but not all, the blots were also probed for ALY (THOC4) which was detected C/NM extracts using the LTQ-Orbitrap instrument. The western blot showed that detergent removal did not significantly reduce the amount of ALY detected (Figure S3A), suggesting that some proteins, such as the MCM proteins, are more likely to be lost during the detergent removal process. A possible reason for this is that proteins are being sequestered in detergent micelles. To investigate this, the extraction volume was increased to 250µl by the addition of PBS to reduce the Triton X-100 concentration and reduce the formation of micelles. This resulted in a marked increase in the amount of MCM7 recovered after detergent removal, with the amounts detected prior and subsequent to detergent extraction being comparable (Figure S3B). The amount of ALY (THOC4) detected was not affected by dilution of the detergent. These observations suggest that certain proteins e.g. the MCMs are sequestered in detergent micelles during a detergent removal step and this could explain why these proteins are absent from the LTQ-Orbitrap dataset.

Analyses of functionally annotated sub-networks
We wished to determine whether sub-networks of functionally-related proteins could be extracted from the protein interaction network described in the main text (Figure 3B; Tables S2-S4) and, in addition, whether predicting interacting partners would identify proteins that had been identified by the mass spectrometry screen but were annotated as being involved in other functions. We chose to analyse “seed-sets” of proteins in C/NM-bound and free datasets annotated in six categories, for example RNA helicases, nuclear pore complex proteins, solute carriers and others described below. Proteins from these functional groups were analysed independently using HumanNet (http://www.functionalnet.org/humannet/; (Lee, 2011)) to initially produce a seed network for each. Predicted interactions between the seed proteins and other proteins in the HumanNet protein interaction database were then mapped to produce an extended network, which was visualised using Cytoscape. The proteins predicted by HumanNet to interact with the seed set were compared with those proteins identified by the mass spectrometry experiments described in the main text (Figure 3). The nodes corresponding to proteins that were identified in our mass spectrometry experiments (in both the C/NM-bound and -free datasets) are depicted as triangles, while those which were not found are depicted as circles. Proteins that are significantly up-regulated in the C/NM-bound fraction as cells progress from G0 to G1 have previously been allocated to functional groups (see main text; Figure 3) and the nodes corresponding to
these proteins are shown with the colours that correspond to these functional groups (annotated in Figure 3B). HumanNet uses evidence from many different sources to predict the interaction between protein pairs. These include data on orthologues in other species, as well as the results of experiments with human cells (http://www.functionalnet.org/humannet/HumanNet.v1.evidence_code.txt). In order to ascertain which of these evidence codes was used to determine the presence of proteins in the extended networks, we combined the seed protein dataset from each of the functional groups being analysed with the extended interaction set produced by HumanNet. This combined dataset was then re-analysed using HumanNet to produce a table showing the interactions between all the proteins in a functional group, along with their predicted interaction partners. All seed-set and predicted proteins for each functional category analysed are shown in Tables S6A-E and the sub-networks produced are depicted in Figures S4A-E.

These analyses show that there are a significant number of proteins predicted by HumanNet that were identified by our mass spectrometry analyses. The percentages are:

| Functional annotation of sub-network | % Identified by MS | % C/NM-bound & up-regulated in G₁ |
|-------------------------------------|--------------------|----------------------------------|
| A. DNA damage & repair              | 139 of 209 (66.5%) | 57 of 209 (27.2%)                |
| B. Nuclear pore complex             | 115 of 208 (55.3%) | 12 of 208 (5.8%)                 |
| C. Proteasome                       | 106 of 204 (52%)   | 4 of 204 (2%)                    |
| D. Solute carriers                  | 116 of 211 (55%)   | 2 of 211 (1%)                    |
| E. Ubiquitin pathways               | 114 of 209 (54.6%) | 6 of 209 (2.9%)                  |

% Identified by MS: Percentage of the predicted proteins identified by mass spectrometry.

% C/NM-bound and up-regulated in G₁: Percentage of the predicted proteins that are C/NM-bound and identified by mass spectrometry to be up-regulated during the G₀→G₁ transition and so have an assigned functional annotation.

Because there are proteins predicted by HumanNet to interact with the seed sets that were also identified in our mass spectrometry screen, and in particular are up-regulated in the C/NM-bound fraction as cells progress from G₀→G₁, these data support the use of predicted sub-networks to infer interacting proteins with probable functional relevance.

For each predicted sub-network, we will illustrate in the Discussion below why certain components are known to be important in T cell functions as well as some of the predicted network components that are of interest due to their presence in networks with different annotated functions.
Induction and phosphorylation of proteins involved in regulating translation

Normal human primary T cells isolated from single donor buffy coats by negative selection were stimulated with PMA/Ionomycin for up to 72 hours. Proteins that are involved in the control of 5’ cap dependent protein synthesis were analysed by western blotting as a measure of entry into the growth cycle. mTOR became phosphorylated on S^{2448} after approximately 24 hours (Figure S4). This is consistent with the increase in cellular protein detectable between 8 and 24 hours following mitogenic stimulus (Lea, 2003) and is consistent with these cells having entered the growth cycle (Nave et al., 1999). Although the MNK1 and MNK2 mediated phosphorylation of eIF4E has been shown to be non-essential for growth and development in mouse fibroblasts (Ueda et al., 2004), western blotting of extracts from normal primary human T cells shows phosphorylation of eIF4E occurring between 8 and 24 hours after PMA/Ionomycin stimulation. There is also an increase in the amount of total eIF4E after 24 hours. The apparent increase in the phosphorylated form could be due to an overall increase in the amount of eIF4E protein. An increase in both the phosphorylated and non-phosphorylated forms of eIF4E is an indication of an up-regulation of 5’ cap dependent protein synthesis. The non-phosphorylated form of 4EBP1 was not detectable in non-stimulated (0 hours) T cells and was induced by 2 hours, when the cells would be in early G_{1}, before the G_{0} → G_{1} commitment point (Lea, 2003). Thereafter, levels of this protein were constant for the remainder of the experiment (up to 72 hours) as the cells progressed from G_{0} → G_{1} → S-phase. A phosphorylated form of 4EBP1 was detected between 24 and 48 hours after PMA/Ionomycin stimulation, consistent with a release from inhibition of eIF4G and subsequent activation of 5’ cap dependent protein synthesis. These data show the kinetics of induction and phosphorylation of proteins involved in regulating protein synthesis in T cells post stimulation with PMA/Ionomycin.
SUPPLEMENTARY DISCUSSION
Supplementary review of T cell proteomics

As described in the main text, the control of the transition from a quiescent (G₀) state into the cell cycle requires switching from a quiescent, maintenance set of programs to programs involved in the cell cycle (proliferation), growth cycle (blastogenesis) and cellular (functional) activation. Such analyses are shown here for the first time. Previous proteomics analyses of primary T cells have reported, for example on cord blood CD4+ Th1 & Th2 cells (Moulder et al., 2010; Rautajoki et al., 2004), the components of lipid rafts (Lin et al., 2010) and surface proteins (Loyet et al., 2005), cytoskeletal proteomics of HIV-infected CD4+ cells (Chan et al., 2009), in response to IL-12 (Rosengren et al., 2005), in disease states (Borro et al., 2007) and phospho-proteome analyses (Brill et al., 2004; Carrascal et al., 2008). For the non-specialist, we provide here a review of the major processes represented in our dataset and their roles in T cell biology. All proteins discussed were identified in our mass spectrometry analysis.

Processes in the complete network of proteins identified by mass spectrometry

Chromatin Structure and remodelling

In quiescent cells, genomic DNA is packaged into the nucleus by being compacted into highly condensed heterochromatin. This is a barrier to gene transcription as it prevents access by transcription factors and other components of the transcriptional machinery. Remodelling of chromatin to allow gene transcription to occur is one of the first steps required by a cell when entering the cell cycle. De-condensation of chromatin has been shown to occur rapidly in T cells after signalling via the TCR (Zhao et al., 1998), with the Brg- or hBrm-associated factor (BAF) complex becoming located in the nuclei of T cells within 10 minutes of antigenic signalling. ACTL6A/BAF53 and BAF170/SMARCC2 are both components of the SWI/SNF chromatin remodelling complex and these become bound to C/NM as T cells enter G₁. BAF53 is required for maximal ATPase activity of BRG1/SMARCA4 (Zhao et al., 1998), suggesting that an active process of chromatin remodelling is occurring in these cells during the G₀→G₁ transition. Recently it was shown that the Jmjd3 protein recruits the BRG1/BAF155/BAF170 chromatin remodelling complex as well as Tbet to the IFNγ promoter in T cells. This is independent of the H3K27 demethylase activity of Jmjd3 (Miller et al., 2010) and illustrates how important chromatin remodelling is in regulating gene expression. Decondensation of chromosomes after mitosis in HeLa cells has been shown to be dependent on the Histone acetyl transferase NAT10 (hALP) (Chi et al., 2007). A similar process will be necessary in cells exiting quiescence and this protein increases in binding to C/NM during entry into G₁. These data indicate that chromatin re-modeling is an important cellular function as cells transit from G₀ to G₁. Data from the study by Miller et al. (Miller et
al., 2010) showed that the chromatin remodelling activity of Jmd3 is sufficient to regulate some but not all of the 5 genes analysed. We showed that some genes up-regulated in G1 are epigenetically “primed” in G0 ready for induction (Smith et al., 2009), but this is not true in all cases. For example MCM3 is primed (ibid), while the MCM7 promoter is remodelled prior to induction (Orr et al., 2010). It will be interesting to determine how many genes that are activated during the G0→G1 transition are dependent on chromatin remodeling for induction.

**Transcription**

The action of the chromatin re-modeling complexes, such as BAF, is to cause a topological change in the chromatin from a repressive, heterochromatin state to the euchromatic state that allows transcription to occur. The activation of core transcriptional machinery is shown by the increase in C/NM binding of α and β subunits of RNA polymerase II (POLR2A and POLR2B). These proteins were only found in the LCQ-Deca dataset and it is probable that they were lost from the LTQ-Orbitrap dataset during the detergent removal step, which was necessary for sample preparation for this instrument (see section 3.2.10). This increase in the binding of core transcriptional machinery shows the importance of de novo gene transcription in T cell cycle entry and activation. There is also increased binding of a number of transcription factors e.g. NFκB1, NFκB2, IRF4, ILF2 and ILF3 which have T cell-specific functions. NFκB1 and NFκB2 are members of the ubiquitous NFκB family of transcription factors, which induce the expression of a wide variety of genes, including ones that are important for T cell function. These include genes encoding chemokines and cytokines such as IL2 (Lai et al., 1995) and IFNγ (Wingren et al., 1993) and also the immunoreceptor B7.1 (CD80) (Fong et al., 1996). IRF4 (Interferon Regulatory Factor 4) is also required for T cell activation, and interacts with NFATc to specifically enhance the transcriptional activation of the IL4 promoter (Rengarajan et al., 2002). IRF4 also competes with IRF5 in binding to MyD88 in response to Toll-like receptor signalling and acts to negatively regulate the induction of proinflammatory cytokines (Negishi et al., 2005). ILF2/NF45 and ILF3/NF90 (both found in the LCQ-Deca dataset, see above) are the subunits of the NFAT (Nuclear Factor of Activated T cell) transcription factor. NFAT regulates IL2 expression by binding to the antigen receptor response element (Kao et al., 1994) and ILF3 also binds and stabilises IL2 mRNA. Along with induction of NFAT, TCR signalling also induces the expression of EGR2 and this protein was found to increase in binding to C/NM. EGR2 induction acts as part of a negative feedback loop responsible for dampening the immune response (Safford et al., 2005), thus preventing over stimulation and possible activation induced cell death. Members of the AP-1 transcription factor family were also found to increase in binding to C/NM, e.g. JUNB and JUND. JUNB has an important role in the regulation of T cell
proliferation and differentiation by controlling the expression of IL4 (Flavell et al., 1998; Hartenstein et al., 2002; Li et al., 1999) and IL2 (Bunting et al., 2006; Jain et al., 1995; Jain et al., 1992a; Jain et al., 1992b). JUND is also involved in T cell differentiation, having a key role in the induction of IL5 expression (Schwenger et al., 2002). Other transcription factors were also increased in binding to C/NM. CBFB (Core Binding Factor β) is the obligate partner of the RUNX transcription factor proteins and is involved in a number of important cellular programmes. It is necessary for normal haematopoiesis (Okuda et al., 2000) and development of T cells, which is dependent on the correct amount of CBFB being present (Talebian et al., 2007). Loss of function caused by the CBFB-MYH11 fusion, which occurs in acute myeloid leukemia M4Eo, results in a large reduction in survival of CD4+CD8+ thymocytes in mice (Zhao et al., 2007). CBFB, in conjunction with RUNX1 and RUNX3, is also critical for the development of regulatory T cells (Tregs). A defining feature of Tregs is the expression of FoxP3, which establishes stable transcriptional and functional programmes in this cell type. Loss of this protein leads to reduced suppressive function. A complex of CBFB and RUNX is required for FoxP3 mRNA stability and protein expression (Rudra et al., 2009). RUNX3 (found to increase in binding to C/NM in the LCQ-Deca dataset) is necessary for the correct maturation of double positive CD4+CD8+ thymocytes into single positive CD8+ T cells, through epigenetic silencing of the CD4 promoter (Woolf et al., 2003). RUNX3 also co-operates with another transcription factor, T-bet, in T-helper cells to induce production of IFNγ and to repress IL4 production (Djuretic et al., 2007).

These findings are in keeping with the significant up-regulation of gene transcription programmes that take place as T cells enter the cell cycle. The product of this de novo gene transcription is hnRNA (heteronuclear RNA) which contains both introns and exons and requires further processing before mature mRNA can be exported from the nucleus.

**RNA Processing**

The increase in gene transcription that occurs as T cells enter the cell cycle can be viewed as a first step that leads to the synthesis of proteins required for cell proliferation and functional activation. The transcribed hnRNA has to be processed and transported out of the nucleus to be translated into proteins. Large numbers of proteins involved in RNA processing (RNA splicing, transport, decay, RNA binding and RNA helicase proteins) showed an increase in binding to C/NM.

The up-regulation of mRNA splicing activity is demonstrated by an increase in C/NM binding of proteins that are core components of the spliceosome, e.g. SNRP70 (also known as U1RNP) which is the first component of the spliceosome to bind to the pre-mRNA, at the 5’ splice site. SNRPA was also found and a large scale siRNA screen showed that this protein,
along with the RNA helicase DDX5, is essential for cell division in HeLa cells (Kittler et al., 2004). PPAN, also found in this study, is the human orthologue of the *Saccharomyces cerevisiae* gene SSF1, which is required for the second step of mRNA splicing (Ansari and Schwer, 1995). This protein may be important for controlling differentiation as PPAN mRNA is rapidly up-regulated in HL60 cells following treatment with granulocyte-colony stimulating factor (Suarez-Huerta et al., 2000). There was also an increase in C/NM binding of components of the SF3b complex, which forms part of the U2 snRNP. The U2 snRNP binds to the 3’ splice site of the pre-mRNA, to form the E complex which is then stabilised by an ATP dependent process to form the A complex (Reed, 1996). The binding of each of the SF3B1, SF3B2, SF3B3 and SF3B5 proteins to C/NM increased during the G0 → G1 transition. The requirement for SF3B2 in cell cycle entry is analysed further in the main text. SR splicing proteins were also found to increase in binding to C/NM in G1, including SFRS1 (SF2), SFRS3, SFRS6 and SFRS14. SFRS1 is required for constitutive splicing and for cell viability *in vivo* (Graveley, 2000). There was also a concomitant increase in the C/NM binding of the SR protein specific kinase SRPK1. SR protein phosphorylation is essential for spliceosome assembly (Xiao and Manley, 1997), so this finding indicates that there is also an increase in SR-dependent splicing activity during cell cycle entry. 

There was also an up-regulation of 16 hnRNPs (heterogeneous nuclear ribonuclear proteins). These are involved in mRNA splicing, export, telomere biogenesis and control of translation (Dreyfuss et al., 2002). For instance, hnRNP K (found by LCQ-Deca) has been implicated in the control of a wide range of cellular processes (Karol Bomsztyk, 2004; Micha et al., 2006). In mice, it interacts with the Polycomb group protein Eed (Denisenko and Bomsztyk, 1997), and is found at higher density at transcribed gene loci as compared with silent loci in rat hepatoma cells (Ostrowski et al., 2003), suggesting a role in chromatin remodelling. hRNP K has also been shown to have a role in gene transcription. It can act to both induce the expression of certain genes and to repress others. Co-expression of hnRNP K and the TFIID TATA box-binding protein, TBP, increases the activity of the c-myc promoter (Michelotti et al., 1996). hnRNP K can interact with TBP *in vitro* (Shnyreva et al., 2000) and exists as a complex with it *in vivo*, so the induction of *c-myc* could depend on this interaction. The expression of c-src is also increased by the interaction of hnRNP K with the Sp1 transcription factor (Ritchie et al., 2003). hnRNP K can also act to repress gene transcription. For instance it represses the thymidine kinase gene promoter through the CT motif (Hsieh et al., 1998). hnRNP K also interacts with the splicing factors 9G8 and SRp20 (Shnyreva et al., 2000) and may also be involved in the regulation of mRNA stability by interaction with YB-1 and hnRNP E1 (Ichimiya et al., 2000). In addition to the above, hnRNP K is also involved in regulating translation. Binding of hnRNP K, along with hnRNP E1/2 to a 3’ UTR DICE
element blocks the recruitment of the 60S ribosomal subunit and hence prevents the initiation of translation.

Proteins involved in transport of mRNA out of the nucleus were also found to increase in binding to C/NM. In metazoans there is a physical and functional link between the transcription and RNA transport machinery (Reed, 2003). This is partly through the formation of Exon Junction Complexes (EJCs) (Le Hir et al., 2001). These complexes include THOC4/ALY and UAP56/DDX39 and both of these were found to increase in binding to C/NM as cells enter G1. The recruitment of THOC4/ALY to mRNA enhances the export of spliced mRNA over that of unspliced mRNA (Zhou et al., 2000). eIF4AIII/DDX48, found in the LCQ-Deca dataset, also forms part of the EJC and plays a role in nonsense mediated decay in mammalian cells (Ferraiuolo et al., 2004; Shibuya et al., 2004). Transcription and RNA transport are also functionally linked. THOC4 and UAP56 form part of a transcription/export complex (TREX), which is specifically recruited to transcribed genes and facilitates RNA export (Strasser et al., 2002). NXF1, also called TAP, is the human orthologue of the yeast MEX67p and this was also found in this study. This protein, along with its cofactor Nxt1/p15 mediates the export of most cellular mRNAs in Saccharomyces cerevisiae and Caenorhabditis elegans (Tan et al., 2000). In yeast, there is another mRNA export factor, Yra1, and this mediates the export of a different sub-class of mRNAs than MEX67p. These sub-classes show enrichment for mRNAs encoding proteins involved in different cellular functions (Hieronymus and Silver, 2003). This may be significant given recent studies, which show that nuclear pore complex proteins bind to various genomic sites (reviewed in (van Steensel and Dekker, 2010)), although the cell cycle-dependence of these interactions has yet to be studied. NXF1 and Nxt1/p15 are conserved in higher eukaryotes and it is thought that this complex also functions in a similar manner in human cells. NXF1 directly interacts with the nuclear pore complex via nucleoporins (Matzat et al., 2008), leading to the export of the mature mRNA from the nucleus. An increase in the C/NM binding of several nuclear pore proteins (NUP88, NUP93, NUP107, NUP153, NUP155, NUP160 and NUP205) was also found as cells entered G1. NXF1 then dissociates from the RNA and is returned to the nucleus. The observation of nuclear matrix bound NXF1 is evidence that significant levels of active mRNA export is taking place as T cells transit from G0 to G1. Co-ordinate export of groups of mRNAs involved in different processes (Keene and Lager, 2005) may co-ordinate transcriptional programmes with the translation of groups of mRNAs, ensuring the temporal synthesis of proteins during the G0→G1 transition (see discussion of Nuclear Pore complex sub-network below).
There was also an increase in binding to C/NM of the multi-functional protein, NONO/p54<sup>intb</sup>. This has been shown to be involved in nuclear RNA processing and regulation of transcription (Shav-Tal and Zipori, 2002). p54<sup>intb</sup> also associates with the paraspeckle protein, PSP1/PSPC1 (Fox et al., 2005a), which also becomes bound to C/NM in G<sub>1</sub>. This provides a link between RNA processing and the formation of particular nuclear structures. These structures, seen as “speckles” by immunostaining, are enriched in splicing factors and are located in the interchromatin regions of the nucleus (Lamond and Spector, 2003). Blocking transcription with Actinomycin-D disrupted the formation of paraspeckles (Fox et al., 2002), suggesting that these structures are associated with active transcription and RNA processing. It has been proposed that these are nuclear “factories” that process RNA, so not only do the programmes that a cell engages change during the transition from a quiescent state, there is also a change in the spatial organisation within the nucleus. This will serve to bring together proteins, DNA and RNA that require co-processing.

Proteins involved in RNA editing also increased in binding to C/NM during the G<sub>0</sub> to G<sub>1</sub> transition. APOBEC3C (Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3) is induced during T cell activation (Refsland et al., 2010). Members of the APOBEC family may play a role in cell cycle control via C to U RNA editing (Jarmuz et al., 2002). The major role of APOBEC3C appears to be the inhibition of replication of exogenous retroviruses e.g, HIV-1 (Goila-Gaur and Strebel, 2008) and also DNA viruses such as HPV (Bonvin et al., 2006). Anti CD3/CD28 stimulation of T cells has been shown to induce the expression of ADAR1 (Laxminarayana et al., 2007) and this protein was found to have increased binding to C/NM in G<sub>1</sub>. This protein is involved in site selective A to I editing of RNA and may alter the expression of several genes. A to I editing of gene transcripts, which is regulated by ADAR1, may also play a role in regulation of the immune response as it has been shown to occur in T cells stimulated with TNFα and IFNγ (Yang et al., 2003). ADAR1 also has an RNA editing-independent role in the regulation of ILF3 (interleukin enhancer binding factor 3) mediated gene expression (Nie et al., 2005). The discovery of ADAR1 and ILF3 in this study illustrates how different cellular functions, in this case Transcription and RNA editing, may be inter-related.

**Ribosome biogenesis**

The processes discussed above lead to the production of mature mRNA. The next stage required for successful cell cycle entry is the translation of these mRNAs into proteins. In order for this to occur, it is necessary for the cell to synthesise many more ribosomes. Quiescent cells have a low, basal translation rate and there is a significant increase in the number and activity of ribosomes during cell cycle entry into G<sub>1</sub> (Ahern and Kay, 1971). This is borne out in our study with the discovery of over 30 ribosomal proteins that increased in
binding to C/NM. Components of both the 40S and 60S subunits were found. Although mature ribosomes are located in the cytoplasm, ribosome assembly occurs in the nucleolus (Todorov et al., 1983), so it is probably components of ribosomes undergoing assembly or transport that are being detected. Proteins involved in ribosomal RNA (rRNA) processing increased in binding to C/NM.

The assembly of snoRNPs (small nucleolar ribonuclear proteins) is a critical step in ribosome biogenesis. The core box C/D proteins NOP56 and NOP58 were found in our study. These are associated with the U3 snoRNA, which is necessary for rRNA processing (Terns and Terns, 2002). RRP9 (U3-55K) which also associates with the U3 snoRNA was also found to increase in binding to C/NM, along with other members of the RRP family (RRP7, RRP8, RRP9, RRP12 and RRP15). IMP3, IMP4 and MPHOSPH10 also increased in binding to C/NM. These proteins form a complex which associates with the U3 snoRNA and is required for the early stages of pre-rRNA processing (Granneman et al., 2003).

Factors essential for the maturation of the large (60S) ribosomal subunit were also found, including PES1, BOP1 and WDR12, the three components of the PeBoW complex (Rohrmoser et al., 2007). The PeBoW complex is also essential for rRNA processing. Expression of N-terminal or C-terminal truncations of either PES1 (Grimm et al., 2006; Holzel et al., 2007; Lapik et al., 2004), WDR12 (Holzel et al., 2005) or BOP1 (Strezoska et al., 2002) block the conversion of 32S pre-rRNA into mature 28S rRNA and triggers p53-dependent cell cycle arrest.

There was also an increase in binding to C/NM of proteins involved in biosynthesis of the small (40S) ribosomal subunit. NOP14 and EMG1 have been shown to interact in *Saccharomyces cerevisiae* and depletion of either of these proteins leads to a reduction in levels of mature 18S rRNA and the 40S ribosomal subunit (Liu and Thiele, 2001). BYSL (Bystin) and NOC4L increased in binding to C/NM in G1. The nucleolar protein NOC4L is required for the synthesis and nuclear export of the 40S subunit (Milkereit et al., 2003) and siRNA knockdown of BYSL leads to a delay in processing of 18S rRNA and a defect in 40S ribosomal subunit production (Miyoshi et al., 2007).

There was also a significant increase in binding to C/NM of eIF6. This protein is of particular interest as it has a dual role in regulating translation and ribosome biogenesis. The requirement for the induction of this protein for increasing cell size during the G0→G1 transition is investigated in greater detail in the main text.

Ribosome biogenesis was amongst the most up-regulated cellular functions in both the LCQ-Deca and LTQ-Orbitrap datasets. Taken across both datasets, proteins involved in
ribosome biogenesis account for 10.3% of the proteins identified that increase in binding to C/NM in G₁ and form a large part of the most significant sub-network. This shows the importance of this cellular process during cell cycle entry.

**DNA Replication**

There was an increase in binding to C/NM during the G₀→G₁ transition of proteins involved in DNA replication. Included in these were members of the MCM protein family (Mcm2, 3, 5, 6 and 7), which are required for the formation of the pre-replicative complex that “licenses” DNA for replication (Prokhorova and Blow, 2000). The requirement for the precise induction of MCM proteins during the G₀→G₁ transition in maintaining genome stability during the subsequent cell cycle was investigated in a previous study by our laboratory (Orr et al., 2010). The MCM proteins are also members of the AAA+ ATPase family (Iyer et al., 2004) and two other members of this family (ATAD2 and ATAD3A) also become bound to C/NM during G₁. Although the function of ATAD2 in T cells is unclear, it is known to interact with the estrogen receptor alpha (ESR1) and is required for the estrogen induced expression of c-myc and E2F1 (Zou et al., 2007). Both c-MYC and E2F1 transcription factors are known to be involved in cell cycle control and ATAD2 could play a similar role in T cells. There was also an increase in binding to C/NM of PCNA (proliferating cell nuclear antigen). This protein is required for assembly of active replication origins and enhances the activity of DNA Polδ and Polε (Moldovan et al., 2007). A number of proteins involved in nucleotide excision DNA base repair are also recruited at the replication fork. Components of this DNA repair sub-network are discussed in more detail below. Our findings demonstrate an up-regulation and binding of the molecular machinery of DNA replication to chromatin and nuclear matrix which is known to occur in G₁ so that cells become “licensed” to enter S-phase (Chong and Blow, 1996)

**Nuclear Matrix and Actin Binding Proteins**

In addition to the processes outlined above, it is likely that an extensive re-organisation of the nuclear matrix is required to facilitate cell cycle entry. hnRNP proteins have been implicated as components of the nuclear matrix and several examples were found to increase in the C/NM fraction during progression from G₀ into G₁. Proteins classified as Actin binding proteins also increased in binding to C/NM. Nuclear actins are involved in regulating transcription (Grummt, 2006; Percipalle et al., 2003; Percipalle and Visa, 2006), chromatin remodelling (Chen and Shen, 2007; Olave et al., 2002) and are implicated in RNA processing through an association with pre-60S ribosomes and mature mRNPs (Percipalle et al., 2001).
The protein that increased most in binding to C/NM as cells entered the cell cycle was XIRP1 (xin actin-binding repeat containing protein 1). This is of particular interest because this protein has not previously been described in T cell nuclei. It was originally found to be involved in the development of chick and mouse hearts, where it associates with N-cadherin and connexin-43 (Wang et al., 1999). Further studies revealed that it has 16 actin binding motifs that were shown to bind to and stabilize cytoskeletal actin in cardiac and skeletal muscle (Pacholsky et al., 2004). XIRP1 has also been shown to bind to filamin C in cardiac myocytes, where it plays an important role in the organisation of the actin cytoskeleton during cardiac morphogenesis (van der Ven et al., 2006). The study presented here is the first to show that XIRP1 is expressed in human T cells, is localised to the nucleus and increases in binding to C/NM during cell cycle entry. Filamin C was also found in the C/NM fraction of human T cells although the levels of binding do not increase significantly. Filamin C is involved in the organisation of Actin filaments and the anchoring of membrane proteins to the Actin cytoskeleton. This suggests a role for XIRP1 in the polymerisation and organisation of the Actin cytoskeleton. The presence of both XIRP1 and Filamin C in the C/NM fraction of human T cells shows that this role is not restricted to the cytoplasm and that this protein may play a central role in the re-organisation of the nuclear cytoskeleton, which would need to occur to allow the processes necessary for cell entry.

**Processes in predicted sub-networks**

Proteins identified by mass spectrometry in five annotated categories were used as seed-sets to predict protein interaction sub-networks, using HumanNet (Lee, 2011) (Figure S4A-E and Table S6). We will describe some of the functional groups of proteins highlighted by these analyses and the cross-talk with other cellular functions. These review some of what is known about the mechanisms controlled by each sub-network. Much of this will be known by those in the respective fields, but may help those outside those fields to understand details of the mechanisms that are up-regulated in T cells during the $G_0 \rightarrow G_1$ transition as well as aspects of those mechanisms that may not be well known.

**A. DNA damage and repair**

The genome is at constant risk of damage from both exogenous and endogenous events including DNA depurination as a result of hydrolysis, base oxidation by reactive oxygen species (ROS), mismatches during replication and strand breaks due to replication fork collapse (Sancar et al., 2004). This DNA damage must be repaired to prevent mutation, cancer or cell death. DNA damage is sensed by DNA damage sensor proteins, such as ATM, ATR, the Rad17-RFC complex, and the 9-1-1 complex which then initiate signal
transduction cascades. The repair mechanism used by the cell depends on the type of DNA lesion and the cell-cycle phase of the cell. The integrated network of DNA-repair processes with transcription and apoptosis is known as the DNA-damage response (DDR) and is controlled by checkpoint proteins (Branzei and Foiani, 2008) which are in turn regulated by post-translational modifications including CDK-dependent phosphorylation, ubiquitylation and sumoylation.

We have identified proteins involved in a number of different processes in the DDR (Figure S4A and Table S6A). These are summarised below.

**Base excision repair (BER)**

BER occurs throughout the cell cycle and removes small lesions from the genome which may otherwise cause mutations or breaks during replication. DNA glycolases remove the base(s) and the single strand break is then processed by short or long-patch BER (Fortini and Dogliotti, 2007).

*The short patch BER pathway.* DNA glycolases recognise and remove the base leaving an AP site which is cleaved by an APE1 endonuclease and results in a single strand break. DNA Polβ is then recruited to fill a nucleotide gap that is ligated by Lig3/XRCC1 complex.

*The long patch BER pathway.* APE1 cleaves the 5’ phosphodiester bond, the RFC/PCNA-Polδ/ε complex carries out repair synthesis and nick translation displacing several nucleotides. FEN1 endonuclease cleaves the 5’ overhang and the long-repair patch is ligated by Ligase 1.

We have identified several proteins important in BER, including FEN1 (flap structure specific endonuclease 1) which removes 5’ overhang in DNA repair. Three out of the five subunits which make up the Replication factor C (RCF) complex RFC 2, RFC 3, RFC 4 and PCNA (proliferating cell nuclear antigen). Both PCNA and the RCF complex are required for the elongation of primed DNA templates by DNA polymerase delta and DNA polymerase epsilon. The RCF complex may also play a role in transcription regulation as well as DNA replication and repair.

**Nucleotide excision repair (NER)**

During G1, nicks or gaps such as those resulting from UV induced pyrimidine dimers (Sancar et al., 2004) are processed by nucleotide-excision repair enzymes and lead to ATR activation. Defects in this pathway cause the photosensitivity syndrome xeroderma pigmentosum (XP) and result in high incidences of light-induced skin cancer.
NER removes bulky lesions which would otherwise block DNA polymerases. Damaged bases are removed by a multi-subunit enzyme called the excision nuclease. It is thought that the multi-subunit enzyme recognises the phosphodiester backbone conformation created by the lesion rather than specific chemical group lesions. This is sometimes known as an indirect readout (Sancar et al., 2004). The enzyme then makes dual incisions ‘bracketing’ the lesion on the damaged strand, forming a 24-32nt oligomer which is then released. Repair synthesis fills the gap which is then ligated. The three damage recognition factors are RPA, XPA and XPC (Mu et al., 1997; Wakasugi and Sancar, 1998, 1999). These proteins bind DNA with some preference for damaged DNA. Specificity is achieved through co-operative binding of the repair factors and kinetic proof reading of TFIIH. TFIIH is a multi-subunit transcription/repair factor (Egly, 2001) whose subunits XPB and XPD provide 3'-5' and 5'-3' helicase activity. TFIIH is recruited by the three damage recognition factors and forms a pre-incision complex and about 20 bp of DNA is unwound. At a damage site, the pre-incision complex is stable and the XPG nuclease replaces XPC in the complex. The XPF-ERCC1 nuclease may then join the complex and create irreversible dual incisions in the damaged strand. The gap is filled by Polδ/ε along with accessory proteins PCNA and RFC.

We have identified several proteins important in NER including, RPA1 (70kDa), RPA 2 (32kDa) and RPA 3 (14kDa) which form a heterotrimer that is one of the repair and damage recognition factors which assembles at the damage site. XAB2 (XPA binding protein2) involved in transcription-coupled repair and transcription (Nakatsu et al., 2000), the damage recognition factor XPC, RAD23 homologue A and RAD23 homologue B (which stabilises XPC) ERCC3 also known as XPB, an ATP dependent 3'-5' DNA helicase and a subunit of basal transcription factor 2 (TFIIH). The accessory proteins RCF and PCNA were also identified.

**Non homologous end joining (NHEJ)**

During G1, ionising radiation (IR) can cause double stranded breaks (DSBs) which lead to activation of DNA-PK and ATM. DSB or single strand breaks during G1 are mostly repaired through NHEJ due to the highly compacted nature of chromatin and the lack of sister chromatids available for homologous recombination (HR).

In NHEJ, the Ku heterotrimer (Ku70- Ku80) functions as a single-stranded DNA-dependent ATP-dependent helicase, it binds to two ends of a DSB and recruits protein kinase, DNA-activated, catalytic polypeptide (DNA-PKcs), a molecular sensor of DNA damage and the ligase4-XRCC4 heterodimer which ligates the two duplex termini. The Mre11/Rad50/NBS1 (MRN) complex may also be involved in NHEJ in the processing the termini of the double stranded break.
We have identified a number of proteins important in NHEJ including Ku70 (XRCC6), Ku80 (XRCC5), DNA-PKcs which form a complex involved in end recognition, and MRE11A and Rad50 which are involved in end processing.

**B. Nuclear pore complex**

Transfer of macromolecules between the nucleus and cytoplasm occurs via nuclear pores (Feldherr, 1962; Watson, 1959). Detailed freeze-etched electron microscopy studies with human T cells showed that the number of nuclear pores per nucleus approximately doubles as they enter the cell cycle from quiescence (Maul et al., 1972). Other studies have investigated how the nuclear pore complex is formed, broken down and re-formed during the cell cycle (reviewed in (Rabut et al., 2004b)). More recent studies have shown the detailed three dimensional structure and composition of the (yeast) nuclear pore complex (Alber et al., 2007), which is comprised of multiple copies of 30 different nucleoporins (NUP), totalling at least 456 proteins (Rout et al., 2000). The “scaffolding” NUP proteins of the Nup107-Nup160 and Nup93-Nup205 complexes (Debler et al., 2008) form an annulus through which macromolecules are transported. The Nup107-Nup160 complex forms an important part of the annulus and is required for assembly of the NPC (Walther et al., 2003a). This sub-complex is composed of one third of the proteins of the NPC and we identified six of the nine main components, Nup37, Nup43, Nup85, Nup107 and Nup160, as well as the WD-repeat protein, Sec13. The Nup107-Nup160 complex plays a key role in mediating mRNA export (Aitchison et al., 1995; Dockendorff et al., 1997; Vasu et al., 2001) and a component of this sub-complex, Nup96, is regulated during the cell cycle. It is most highly expressed in the S and G2 phases and although it was identified in our mass spectrometry analyses, it did not increase in binding to C/NM during the G0→G1 transition. It is possible that it is induced later in the first cell cycle. Nup96 controls cell cycle progression by regulating the export of specific mRNAs involved in particular functions. In T cells, these include mRNAs encoding proteins required for progression through G1 (Chakraborty et al., 2008). Therefore, the increase in C/NM-bound NUP proteins we observed in our study are consistent with both establishment of the capacity to increase nuclear/cytoplasmic transport, but also to mediate the regulated transport of specific mRNAs involved in particular functions. Thus the ordered production of proteins is not only controlled by co-ordinated gene expression programmes, but classes of mRNA may also be transported to the cytoplasm, facilitating orchestrated translation.

In addition to being part of the Nup107-Nup160 complex involved in mRNA export, Sec13 and the Nup88 proteins identified in our study regulate transcription (Capelson et al., 2010). In Drosophila, Sec13 (and Nup98) bind to developmentally regulated genes and are required for their transcription, whereas Nup88 binds to silent loci. Genome-wide binding studies in
mammalian cells show that NUPs bind predominantly to repressed gene loci (Brown et al., 2008). Some peripheral NUPs that are not part of the scaffold (core) are more loosely bound to the NPC in mammalian cells (Rabut et al., 2004a) and it is not clear to what extent genome-wide binding occurs as part of the NPC complex or as separate intra-nuclear proteins.

Sub-network analysis of the NPC proteins which we identified increase in binding to C/NM in G1 are shown in Figure S4B and listed in Table S6B. This analysis predicts that the NPC associates with a number of proteins, 55.3% of which were identified in our mass spectrometry screen (shown as triangles in Figure S4). These include Ran and RanBP proteins that control macromolecular transport (traffic) through the NPC.

The NPC has a transport channel of approximately 300Å diameter, but it is packed with proteins containing phenylalanine/glycine (FG) repeats that limit diffusion of proteins larger than 40kDa. Nuclear trafficking of molecules larger than this occurs because transporters (carrier proteins) facilitate the transport of macromolecule cargoes, such as proteins or RNA, through the NPC. Transport is directional and the direction of transport is dictated by the disassembly of the cargo-carrier complex once it has reached the other side of the NPC. The carrier is then recycled, thus preventing the cargo from re-crossing through the NPC. The RanGTP/RanGDP cycle (reviewed in (Weis, 2003)) controls the nuclear traffic of macromolecules through the NPC and it does so in association with Karyopherin-β proteins. It is important in regulating transport throughout the cell cycle but it also has functions in nuclear envelope changes in mitosis. There are 20 members of the Karyopherin-β family. Ten are involved in nuclear import, including Ipo4, 5, 7, 8, 9, 11 and 13 and six in nuclear export, including Xpo1 (Crm1), Xpo4, 5, 7. Initially, the formation of the nuclear pore complex is thought to be initiated by the Karyopherin and RanGTP complex (Harel et al., 2003; Lusk et al., 2002; Ryan et al., 2003; Walther et al., 2003b). This complex, together with KPNB1, which was also identified in our mass spectrometry analyses, is involved in the nuclear import of proteins with an NLS. We also identified a number of Importins, including Ipo4 & 7, RanBP1 and RanBP2. RanBP2 binds RanGTP and has SUMO1 E3 ligase activity (Pichler et al., 2002). RanGTP is also associated with chromatin (Hinkle et al., 2002), which may provide the connection between the nuclear pore complex and nuclear “factories”, described earlier.

In contrast to protein transport, mRNAs are exported from the nucleus by a mechanisms involving NXF1-NXT1 (reviewed in (Stewart, 2010)). A large number of proteins bind to hnRNA and to the mature, spliced mRNAs that will be transported out of the nucleus (reviewed in (Kelly and Corbett, 2009)). The RNA-binding proteins that mediate these
processes that we identified in our study are discussed above and include splicing factors, PABPs, hnRNPs, components of the TREX complex such as ALY/THOC4 and the mRNA export complex protein, NXF1. The NXF1-mRNA interaction facilitates export through the NPC, but the nucleus to cytoplasm directionality is mediated by the DDX19/GLE/Nup214 complex (DDX19 and GLE were identified as up-regulated in G\textsubscript{i}). This complex causes disassembly of the mRNA export complex at the cytoplasmic side of the NPC, depositing mRNAs in the cytoplasm for sequestration or translation.

**C. Proteasome proteins**

A small number of proteasomal proteins that form part of the 26S proteasome (Goldberg, 1995) were detected in the C/NM-bound fraction and decreased during the G\textsubscript{0}→G\textsubscript{1} transition (Figure S4C and Table S6C). Two (PSME1 and PSME2) are implicated in immunoproteasome assembly whilst the other three (PSMD7, PSMD1 and PSMC1) have roles in 26S regulation. Nearest neighbour network analyses (Figure S4C) indicate they interact with many proteins required for cell cycle entry that are regulated by proteasomal degradation, such as p27\textsuperscript{kip1}, Cdt1 and Cyclin E (reviewed in (Thomas, 2004)). In lymphocytes, the catalytic subunits of the canonical proteasome are replaced totally or in part by inducible counterparts β\textsubscript{1i}, β\textsubscript{2i} and β\textsubscript{5i} to form the immunoproteasome or intermediate proteasomes that are involved in antigen processing (Chen et al., 2001; Guillaume et al., 2010; Rock and Goldberg, 1999). We detected these subunits, but our mass spectrometry analyses indicate that they are not significantly induced during the G\textsubscript{0}→G\textsubscript{1} transition.

The proteasome is known to be active in the nucleus (Coux et al., 1996), and there is an increase in the nuclear localization of proteasomes during cell cycle progression (Palmer et al., 1994). Many proteasomal proteins have a putative nuclear localisation signal which may control their nuclear import (Wojcik and DeMartino, 2003). Our analyses show a possible interaction between PSMD1 and IPO4 (Importin 4/RanBP4), which is involved in importing proteins into the nucleus (see Subnetwork B), indicating that components of the proteasome are actively transported into the nucleus. The proteasome is involved in a number of processes within the nucleus including response to DNA damage, ribosome biogenesis and transcriptional regulation. A role for the proteasome in nucleotide excision repair (NER) is suggested by the predicted interactions between XPC (RAD4) and PSMC1, PSMD1 and PSMD7. PSMC1 and PSMD1 also interact with RAD23a and RAD23b, which are involved with NER. Our analyses predict interactions between PSMD1 and the ribosomal proteins RPL10, RPSA, RPS5 and RPS14. Ribosomes are assembled in the nucleolus and ribosomal proteins have been shown to accumulate at a higher rate than nucleolar proteins. Only a subset of these ribosomal proteins are assembled into ribosomes and exported to the
cytoplasm. Lam et al. showed that inhibition of the proteasome caused an accumulation of ribosomal proteins in the nucleus but not in the cytoplasm (Lam et al., 2007). They concluded that ribosomal proteins are synthesised at a faster rate than is required for normal rates of ribosome biogenesis and this excess is balanced by a continual degradation of unassembled ribosome proteins in the nucleolus. This ensures that the synthesis of ribosomal proteins is not rate-limiting for ribosome biogenesis. This is an energy-inefficient process, but may allow the cell to increase the rate of ribosome biogenesis very rapidly if required. This may occur to translate the large increase in mRNAs synthesised during entry into G\textsubscript{1} from G\textsubscript{0} in response to CD3/CD28 stimulation. A role for the 26S proteasome in regulating gene transcription also occurs, which is illustrated by the observation that inhibiting proteasomal activity increases the transcriptional activity of many steroid hormone receptors (Kinyamu and Archer, 2007). Also, both ATPase and non-ATPase components of the 19S regulatory particle (which forms part of the 26S proteasome) have been shown to be involved in gene transcription by regulating the loading of the thyroid hormone receptor (TR) on to thyroid hormone response elements (Satoh et al., 2009). A role for the 26S proteasome in regulating immune responses has also been shown by the observation that the absence of PSMC5 (Sug1) leads to reduced HLA-DR promoter activity and decrease in MHC Class II transcription (Bhat et al., 2008). A role for the proteasome in regulating protein synthesis via the mTOR pathway has also been shown. HT-29 colon cancer cells treated with the proteasome inhibitor, MG-132, showed reduced proliferation, rate of protein synthesis, phosphorylation of mTOR at S\textsuperscript{2448} and S\textsuperscript{2481} and the phosphorylation of the downstream targets 4E-BP1 and p70/p85 S6 kinases (Wu et al., 2009).

The components of the proteasome associate with proteins of the ubiquitin system, such as USP14 and HIP2, described in more detail in E.

**D. Solute carriers**

The superfamily of 55 SLC gene families encode more than 362 membrane-bound transporters (He et al., 2009). These solute carriers are present in the membranes of most cellular organelles and are necessary to transport amino acids and short peptides, glucose and other sugars, cations and anions, organic anions, essential metals, lipid components and many other essential cellular components (ibid). The sub-networks of the solute carriers identified in our study that are up- or down-regulated during the G\textsubscript{0}\rightarrow\textsubscript{G} transition, and their predicted partners, are shown in Figure S4D and Table S6D. Specific proteins identified in our study include the glucose transporters (GLUT1/SLC2A1 (we observe protein fold change from G\textsubscript{0}\rightarrow\textsubscript{G} =16.26) & GLUT3/SLC2A3 (FC=2.22)), discussed below. We also observe an increase in the amino acid transporters SLC3A2 (FC=15.05) and SLC1A5 (FC=9.21). Also a
decrease in the choline-like transporter SLC44A2 (FC = -6.53). There are decreases in a number of C/NM-bound proteins, including the organic ion transporter SLC22A23 (FC = -9.80) and SLC30A9 involved in Zn²⁺ efflux (FC = -4.98).

The transition from quiescence to a proliferative state requires a T cell to increase in mass as well as to replicate the genome (Coller, 2007; Lea, 2003; Thomas, 2004). This requires a huge production of ATP to fuel these processes. Non-activated, quiescent T cells require energy for maintaining “house-keeping” functions and ATP is generated by catabolic metabolism that fuels mitochondrial oxidative phosphorylation (Fox et al., 2005b; Krauss et al., 2001). However, when stimulated, T cells do not simply increase the production of ATP by this process, rather there is a well-documented change form catabolic to anabolic metabolism (aerobic glycolysis; known as the Warburg effect) (Fox et al., 2005b; Frauwirth et al., 2002; Jones and Thompson, 2007). Glucose is the principal substrate for ATP production in proliferating cells and two molecules of ATP, NADH and pyrovate are produced from each molecule of glucose. The energetic yield is lower than that derived from oxidative phosphorylation and only approximately 3% of the glucose is oxidised by the TCA cycle, the remainder being excreted as lactate (Brand et al., 1988). However, production of ATP by aerobic glycolysis occurs faster than that produced by oxidative phosphorylation, as long as glucose is not limiting. This is achieved in part by the CD28-dependent induction of glucose transporters such as SLC2A1/GLUT1 and the localisation of GLUT1 to the plasma membrane, increasing glucose uptake by 20-fold in the first hour (Greiner et al., 1994). Glucose uptake is required to maintain cell proliferation and loss of stimulation results in GLUT1 down-regulation, atrophy and apoptosis (Rathmell et al., 2000). However, a high ATP/AMP ratio in a cell inhibits phospho fructo kinase activity and glycolysis. ATP levels may be regulated by an enzyme in the endoplasmic reticulum called ectonucleoside triphosphate diphosphohydrolase 5 (ENTPD5), which was identified in proliferating prostate cancer cells (Fang et al., 2010).

**E. Ubiquitin system**

The ubiquitin pathway is the major mechanism by which proteins are degraded or modified for specific cellular functions. Ubiquitin is a highly conserved protein that is attached to proteins covalently and poly-ubiquitination (K48) labels them for degradation. Four significant components of the Ubiquitin modifying system (UBE2K/HIP2, USP10, USP15 and UBE2V1) were identified to be up-regulated in the free fraction in G₁ and to interact with other ubiquitin-associated proteins, such as UBAP2, UBAP2L. Ubiquitination involves at least three classes of enzymes (Herrmann et al., 2007; Welchman et al., 2005): ubiquitin-
activating enzymes, or E1 proteins (we identified UBE1DC1/UBA5, UBA3 (Catalytic subunit of the dimeric UBA3-NAE1)), ubiquitin-conjugating enzymes, or E2 proteins (we identified UBE2M/UBC12), and E3 ubiquitin-protein ligases (we identified RNF2 and TRIM3). RNF2 mediates monoubiquitination of Lys-119 of histone H2A, thereby playing a central role in histone code and gene regulation. This protein is predicted to interact with HIP2 (described in more detail below), based on a yeast two-hybrid screen as well as cell-based pull-down and functional assays (Lee et al., 2001).

There are also a number of different ubiquitin-like proteins (UBLs), which were identified to interact with the seed-set. Those UBLs represent known classes of the UBL such as neuronal-precursor-cell-expressed developmentally down-regulated protein-8 (NEDD8). Covalent attachment to its substrates requires prior activation by the E1 complex UBE1C-APPBP1 and linkage to the E2 enzyme UBE2M/UBC12 (Huang et al., 2009; Walden et al., 2003). Attachment of NEDD8 to cullins activates their associated E3 ubiquitin ligase activity and thus promotes poly-ubiquitination and proteasomal degradation of cyclins (Bornstein et al., 2006), which is required for cell cycle progression. In the sub-network UBE1C, was predicted to interact with NEDD8 and UBE2M.

Another important ubiquitin-like modifier is SAE1, which acts as an E1 ligase for the small Ubiquitin modifiers SUMO1, SUMO2, SUMO3 and probably SUMO4. Posttranslational modification of proteins by the addition of the small protein SUMO regulates protein structure and intracellular localization. SAE1 (identified) and UBA2/SAE2 (predicted in the sub-network) form a heterodimer that functions as a SUMO-activating enzyme for the sumoylation of proteins (Okuma et al., 1999). SAE1 was identified in our mass spectrometry screen and is predicted to interact with UBE1DC1/UBA5 and UBE1C (Dou et al., 2005). Interestingly, SAE2 is predicted in the network to bind to UBE1C and USP14.

Other modifiers involved in the ubiquitin system and identified are the Ubiquitin-like protein-5 (UBL5) and the Ubiquitin-binding protein LOC51035/UBXN1. Interaction of LOC51035 with auto-ubiquitinated BRCA1, inhibits the E3 ubiquitin-protein ligase activity of the BRCA1-BARD1 heterodimer (Wu-Baer et al., 2010). UBL5 is a component of a complex required to couple deglycosylation and proteasome-mediated degradation of misfolded proteins in the endoplasmic reticulum that are retrotranslocated in the cytosol.

RAD23A and RAD23B were identified in our mass spectrometry dataset and are predicted to bind to HIP2. These proteins contain an N-terminal ubiquitin domain (Grabbe and Dikic, 2009), interact with the 26S proteasome (Katiyar et al., 2004) and thus may be involved in
the ubiquitin-mediated proteolytic pathway in cells. RAD23A and RAD23B are also involved in nucleotide excision repair (NER: see sub-network A).

The ubiquitin tag is capable of directing proteins to the proteasome. USP14 and HIP2/UBE2K were identified and predicted to interact with a number of proteasome (PSMA) proteins. USP14 is a deubiquitinase associated with the proteasome that releases ubiquitin from proteins that are targeted to the proteasome for degradation. USP14 was reported to be involved in the deubiquitination of CXCR4, which is physically associated with the T cell receptor to signal in T cells (Kumar et al., 2006; Mines et al., 2009). The proteasome is a large multi-catalytic protein complex essential in all eukaryotes for degradation of proteins that are not required, or are misfolded or damaged. The most common form of the PSMA is the 26S proteasome, consisting of a 20S proteasome core and two 19S regulatory subunits (Peters et al., 1994). We identified PSMC1, PSMC2, PSMC6, PSMD3, PSMD4, PSMD6, PSMD7 and PSMD8 of the 26S proteasome and specifically PSMA1, PSMA4, PSMA5, PSMA6 and PSMB2 of the 20S core (see sub-network C).

The proteasomal degradation pathway is essential for many cellular processes, including the regulation of gene expression and the cell cycle. NFκB regulates transcription of many genes involved in immune responses (including T cells). Ubiquitination of NFκB (p105) requires among others E2-25k/HIP2 (Coux and Goldberg, 1998). NFκB was identified to interact with HIP2. Further, HIP2 was identified to interact with NEDD8 and p53. Also, NEDD8 plays an important role in cell cycle control (Tateishi et al., 2001). The NEDD8 pathway is essential for SCF (beta-TRCP) mediated ubiquitination and processing of the NF-kappaB precursor p105 (Amir et al., 2002).

NOL1/NOP2 was identified to be induced in G1 and predicted to interact with USP10 and UBAP2L. NOL1 is suggested to play a role in the regulation of the cell cycle and the increased nucleolar activity that is associated with the cell proliferation. The protein encoded by this gene is a nucleolar antigen expressed in proliferating cells. It is not detectable in non-proliferating normal tissue but is detectable in many human tumours. The p120 protein displays a dramatic increase in expression at the G1/S transition, suggesting that p120 regulates the cell cycle and nucleolar activity that is required for cell proliferation.
SUPPLEMENTARY FIGURES

A. Cell cycle status of T cell samples analyzed by mass spectrometry. Non-activated human primary quiescent T cells were isolated from peripheral blood by negative selection and stimulated with PMA/ionomycin for 40 hours. The percentage of cells in each cell cycle phase was determined by PI (DNA content) and FITC (protein content) staining and flow cytometric analysis (mean±SEM; n=8 experiments).

B. Activation state of T cell samples. Isolates of quiescent T cells were stained for cell surface CD69, an early marker of T cell activation and a negative gate was set using an isotype-matched control antibody. The level of staining was compared with that obtained with the same T cells 16h post-stimulation with PMA/ionomycin.

C. Comparison of protein content and forward scatter measurements. T cells stimulated for 24h with PMA/ionomycin were stained with PI and FITC, as for panel A. Electronic gates were set for cells with 2n DNA content and low, medium and high FITC (protein) staining (left panel). The forward scatter (FS) corresponding to each are shown in the right panel in the same colours. The lower panel is a plot of FITC and FS for all the cells in G₁.

Figure S1
Figure S2

A. Functional annotation of proteins that bind to C/NM post stimulation with PMA/ionomycin. The functions of proteins identified in an initial study with a DecaXP+ mass spectrometer that increase in C/NM binding in G₁ as compared with G₀ were assigned...
to functional groups by reading the literature and with reference to the Entrez database (www.ncbi.nlm.nih.gov). The percentage of proteins in each group is shown.

**B. Protein interaction networks of proteins that bind to C/NM post stimulation with PMA/ionomycin.** Proteins identified in the initial mass spectrometry screen as increasing in binding to C/NM during cell cycle entry were mapped to the human interactome on the basis of co-citations in the literature filtered for experimentally confirmed interactions (Marcotte et al., 2001; Ramani et al., 2005) and visualised using Cytoscape software (www.cytoscape.org). Proteins are represented as nodes with colours assigned according to their function. The distance between nodes is inversely proportional to the (predicted) likelihood of interaction between proteins. MCODE analysis (http://cbio.mskcc.org/~bader/software/mcode/index.html) of the network reveals clusters of proteins involved in RNA splicing, structure and transport (1), also ribosome proteins (2) and nucleolar proteins including proteins involved in ribosome biogenesis (3). The components of these sub-networks are listed in Table S2.

**C. Protein interaction networks of proteins that bind to C/NM post stimulation with PMA/ionomycin: DecaXP+ analyses.** A file containing the high-resolution image of the main network shown in B is attached. Zoom in to see the names of the proteins. The .cys file of the network that can be opened in Cytoscape is in cys File 1 (attached).

**D. Protein interaction networks of proteins that bind to C/NM post stimulation with PMA/ionomycin: Orbitrap analysis.** A file containing a high-resolution image of the main network shown in Figure 3B (see main text) is attached. Zoom in to see the names of the proteins. The .cys file of the network that can be opened in Cytoscape is in cys File 2 (attached).
Figure S3. Effect of detergent removal on detection of proteins. (A) Chromatin/nuclear matrix bound (Chrom.) and unbound (Free) protein extracts of T cells were analysed by western blotting both before (Pre) and after (Extract) detergent removal with Extractigel D resin. The resin was boiled in SDS lysis buffer to elute proteins to bound to the resin (Gel). (B) The effect of micelle formation was determined by performing the detergent removal procedure in a small (50µl) or large (250µl) volume. Samples were analysed as for (A).
Separate files are appended for the following sub-networks:

A. DNA damage pathways
B. Nuclear pore proteins
C. Proteasome
D. Solute carriers
E. Ubiquitin pathway

Figure S4. Protein sub-networks. Protein interaction sub-networks involved in: (A). DNA damage pathways, (B). Nuclear pore proteins, (C). Proteasome, (D). Solute carriers and (E). Ubiquitin pathways were predicted using a “seed-set” of proteins identified by the mass spectrometry screen (nodes, depicted as triangles) and identifying their nearest neighbours using HumanNet (Lee, 2011). Predicted nearest neighbours that were identified by the mass spectrometry screen are shown as triangles and colours indicate the functions of any that are C/NM-bound and induced in cells cultured for 40h with PMA/ionomycin (see Figure 3B). The proteins in each sub-network are listed in Tables S6A-E, together with the HumanNet evidence codes for each protein:protein interaction. Tables S6F has a list of the seed sets used to generate the networks and S6G a list of the predicted interacting proteins.
Figure S5. Entry into cell cycle and growth cycle. Quiescent T cells were stimulated with PMA/Ionomycin and total protein extracts were prepared at the time points shown. The expression and phosphorylation of the proteins indicated were analysed by western blotting. GAPDH was used as a loading control. The phosphorylated forms of proteins analysed were mTOR$^{S2448}$, eIF4E$^{S209}$ and 4EBP1$^{T37/46}$. Representative of n=3 independent experiments.
SUPPLEMENTARY TABLES

All tables are provided in separate files.

Table S1. Proteins bound to C/NM in T cells. Proteins bound to C/NM were identified in the mass spectrometry screen (Surveyor/DecaXP+, ThermoFinnigan). Those that increase in C/NM binding 40h post stimulation with PMA/ionomycin as compared with G0 are shown and they were assigned to functional groups by reading the literature and GO ontology database searches (www.geneontology.org). The fold change in mRNA for each protein was calculated by comparing gene expression array data (U133A-arrays; Affymetrix) for T cell samples pre- and 24h post-stimulation with PMA/ionomycin. The proteins in the top three MCODE sub-networks are indicated, together with their functions.

Table S2. C/NM-bound and free proteins. Proteins that are bound to C/NM and unbound (free) proteins in all T cell samples in G0 and 40h post stimulation with PMA/ionomycin (G1) were identified using an LTQ-Orbitrap mass spectrometer (Thermo Scientific). The fold change in abundance of each protein between G0 and G1 was calculated for those observations with a z-score cut-off of >1.96. Changes in mRNA expression for each protein as well as functional annotations were as for Table S1.

Table S3. C/NM-bound proteins that increase during entry into the first cell cycle. Proteins identified as described for Table S2 that increase in binding to C/NM are shown, together with corresponding functional annotations, sub-cellular locations and protein and mRNA expression fold changes. The master protein interaction network of these proteins that changes post stimulation with PMA/ionomycin was compiled (see Figure 1B) and the proteins in the three most significant sub-networks determined by MCODE analyses are shown, along with their functions.

Table S4. Free proteins that increase during entry into the first cell cycle. As for Table S3 sheet 1, but for proteins that are not C/NM-bound.

Table S5. mRNA expression during entry into the first cell cycle.
Table S6. Protein sub-networks that increase during entry into the first cell cycle. Protein interaction sub-networks involved in: (A). DNA damage pathways, (B). Nuclear pore proteins, (C). Proteasome, (D). Solute carriers and (E). Ubiquitin pathways, shown in Figure S4, were derived as described in Supplementary Results and Discussion. The proteins in each sub-network and the HumanNet evidence codes for the interactions are shown in Tables S5A-E and a list of the seed-set proteins and the predicted partners are shown in Tables S5F and G.

Table S7. mRNA expression in G0.

Table S8. Fold change of C/NM binding as determined by mass spectrometry.
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