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THE NOVEL REGULATION OF HISTONE MODIFICATION IN CANCER DEVELOPMENT

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A

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Dynamic changes in histone acetylation by various physiological cues play important roles in gene transcription and cancer. However, the cellular signaling underlying this regulation is not well understood. Here, we show that, in a glucose dependent manner, E3 ubiquitin ligase NEDD4 ubiquitinates histone H3 on previously unstudied lysine (K) 23/36/37 residues, which specifically recruits histone acetyltransferase (HAT) GCN5 for subsequent H3 acetylation. Genome-wide analysis of chromatin immunoprecipitation followed by sequencing (ChIP-seq) data sets reveals that NEDD4 regulates glucose-induced H3K9 acetylation at transcription starting site (TSS) and enhancer regions. Integrative analysis of ChIP-seq and microarray data sets also reveals a consistent role of H3 ubiquitination in transcription activation and H3 K9 acetylation in response to glucose. Functionally, we showed that NEDD4-mediated H3 ubiquitination is critical for tumorigenesis and that IL1A, IL1B and GCLM are important target genes to elicit the function of glucose-induced H3 ubiquitination in tumor sphere formation. Together, our study provides a

new model for glucose-induced transcriptome reprograming and epigenetic regulation in cancer through inducing NEDD4-dependent H3 ubiquitination.

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Chapter 1 Introduction

1.1 Chromatin dynamics

1.1.1 Chromatin structures

The term 'phenotype' in biology is used to describe an observable characteristics or traits of an organism. Advances in technology leads to the observation of many phenotypes beyond our bare eyes, including phenotypes that exists at molecular or atomic level. Generally, phenotypes of an organism are the reflection of the interaction between genotypes and environmental factors. Genotypes refers to the complete set of genes in an organism. Genes are coded and carried by DNA double helix, which is a cellular macromolecule that is composed of deoxyribonucleotide acids. In eukaryote, the number and size of genes are so large that the DNA molecule carries it becomes extremely long, which is many orders of magnitude longer than the diameter of the nucleus (1). To fit such an long string into the relative small size of the round shaped nucleus, eukaryotic genomes are packed into dense structures, known as chromatin (2, 3). The basic structure units of chromatin are the nucleosomes, which are formed by wrapping approximately every 147 base pairs of DNA double strand around a protein complex. The complex is composed of of eight core histone proteins, which are two copies of each histone H3, H4, H2A and H2B (4). The tandem repeat of nucleosome structures are connected by linker DNA, which is also called beads-on-a-string or 10nm fibre, is the euchromatin region of chromatin(5). With linker histone H1, multiple nucleosomes on the 'string' in some part of the chromatin are further packaged into 30nm fibers structures to form heterochromatin region of chromatin(5). During mitosis, 30nm fiber structure further packed into higher

level of structure is called metaphase chromosome(6). Thus, the changes in structure of chromatin are closely related to their biological function and will be discussed later.

1.1.2 Histone variants

There are five kinds of histone protein, which are H2A, H2B, H3, H4 and H1 as described above (4). Generally, DNA binds to core histone complex non-specifically (5). One of the notable characteristics of histone proteins is the large numbers and distinctive functions of their variants (7). Histone variants usually differ from their respective canonical ones from several amino acids or the addition of a large domain (7). Histone variants can be classified into replicative and replacement types according to their spatial and temporal involvement in the nucleosome structure (8). Replicative variants are transcribed in high amounts from tandemly arranged genes (different genes for the same protein) during S phase to ensure the replication of chromosome (9). Replacement histones are synthesized throughout the cell cycle and can be integrated into chromosome in a replication-independent manner (10). Each histone variant has a unique feature in sequence and thus enables it to interact with specific chromatin factors or DNA structures involved in various biological processes. For example, the incorporation or eviction of histone H3.3 by the histone chaperone, HIRA, occurs during cycle and is involved in the regulation of transcription, while H3.1 and H3.2 are only incorporated during replication (11, 12). Some of the histone variants are tissue specific or functional specific. CENPA, a large variant of H3 conveys special functions on the centromere, H3T is only expressed

in testes or sperm and H2AX is critical for the DNA damage response and DNA repair process (13-15).

1.1.3 Chromatin remodelers

As I have mentioned previously, environmental factors can also contribute to the changes of phenotypes. Mutations in gene sequence, that can be induced by mutagens, may result in the changes of amino acid sequence and the structure of target protein, leading to the alteration in phenotypes. More frequently, a relative milder environmental factor may affect the organism at the epigenetic level, including stable changes in DNA methylation or histone modifications (16). Epigenetic changes often result in the reprograming of gene expression patterns, which are also closely linked to biological characteristics (16). In eukaryotes, genes are first transcribed to RNA, the process of which involves the binding of transcription machinery to the DNA double strand, indicating that an open status of DNA double strand is required for the transcription machinery to work (17). Hence, the basal status of eukaryotic genome is transcription suppressive as DNA is packaged to chromatin. To activate gene transcription, chromatin regions containing target genes have to be locally opened up for the access of transcription machinery. In various model organisms, it has been found that the chromatin structure is dynamic, including the events of opening up of chromatin for active transcription and closing off of chromatin for transcription repression. These processes are achieved by a number of chromatin remodeling proteins, which often form larger complexes containing

functional specialized subunits (18, 19). Based on the functional definition, all chromatin remodelers share some basic properties, including the capability to bind to nucleosome, a DNA-dependent ATPase domain for trans-locating DNA from histone contact (DNA translocase), and the regulatory domains interacting with chromatin-associated factors and controlling the DNA translocase activity (20). Chromatin remodeler complexes are able to repositioning, ejecting, unwrapping, exchanging histone from nucleosome structures (20). Thereby, through regulating the direct accessibility of RNA polymerase II at specific chromatin sites, chromatin remodeler complexes could promote or inhibit transcription activity at multiple stages including transcription initiation, elongation and termination. Moreover, chromatin remodeler complexes also actively participate in other biological processes which require the access to DNA double strand within chromatin structure, including DNA replication, DNA repair and DNA recombination (21, 22). In human, the chromatin remodelers complexes are divided into four conserved families, namely SWI/SNF ISWI, CHD, INO80 family remodelers (Table 1). Each of them is composed of different ATPases and non-catalytic homologous subunits, which convey chromatin locus specificity and functional specialization (17).

SWI/SNF	**Complex**	**BAF**	**PBAF**	
	ATPase	hBRM/BRG1	BRG1	
	Non-catalytic subunits	BAF250/hOSA1	BAF180	
			BAF200	
		BAF155, BAF170 BAF60a, b, c hSNF5/BAF47/INI1 BAF57 BAF53a, b b-actin		
ISWI	**Complex**	**NURF**	**CHRAC**	**ACF**
	ATPase	SNF2L	SNF2H	
	Non-catalytic subunits	BPTF	hACF1/WCRF180	
		bAp46, 48	hCHRAC17	
			hCHRAC15	
CHD	**Complex**	**CHD1**	**NuRD**	
	ATPase	CHD1	CHD3, CHD4	
	Non-catalytic subunits		MBD3	
			MTA1, 2, 3	
			HDAC1, 2	
			RbAp46, 48	
			p66a, b	
INO80	**Complex**	**INO80**	**SRCAP**	**TRRAP/Tip60**
	ATPase	hIno80	SRCAP	p400
	Non-catalytic subunits	RUVBL1, 2/Tip49a, b		
		BAF53a		
		Arp5, 8	Arp6	Actin
		hles2, 6	GAS41	
			DMAP1	
			YL-1	
			H2AZ, H2B	Brd8/TRC/p120
			ZnF-HIT1	TRRAP/Tip60
				Tip60
				MRG15
				MRGX
				FLJ11730
				MRGBP
				EPC1
				ING3

Table 1 The classification of chromatin remodelers in human (17).

1.1.4 Histone chaperones.

Another group of critical factors in chromatin dynamics is the histone chaperones. They all bind histones and are broadly involved in histone transport, storage, nucleosome assembly and disassembly (23). Histone chaperones can be classified based on their binding substrates. Most of the histone chaperones specifically bind to H2A-H2B or H3-H4 oligomers, but a few of them recognize free histones, distinguishing the canonical ones and their variants (Table 2). Unlike the ATP dependent remodelers, histone chaperones utilize the spontaneous movement of the DNA around the dyad axis of nucleosome to destabilize the nucleosome, the process of which is slower than chromatin remodeling (20). Histone chaperones can also facilitate the histone exchange or regulate transcriptional activity by aiding the histone post translational modification (PTM) process at the desired locus. For instance, Rtt109-mediated H3K56 acetylation in the yeast is important for transcription initiation and elongation, but Rtt109 alone is not able to acetylate H3 within a nucleosome. Histone chaperone Asf1 mediated dissemble of nucleosome is required for Rtt109 to access H3 for acetylation (24). Similarly, histone methyltransferase Set2 also requires histone chaperone Spt6 for histone access and effective methylation (25). Thereby, histone chaperone mediated regulation of histone PTMs in turn alters the chromatin structures for transcription or other biological processes by its intrinsic chemical properties or by recruiting specific chromatin remodelers (20).

Histone Chaperone	Histone bound	Modulated event
HIRA	H3.3	H4S47 phosphorylation by PAK2
NAP1	H2A-H2B, H2A.Z-H2B	RSC remodeling
FACT	H2A-H2B	H2BK120 ubiquitination by Rad6, H3K36 methylation by Set2
ANP32E	H2A.Z-H2B	Unknown

Table 2 Substrate and function of histone chaperones in human (20).

1.2 Histone modification and its biological function

1.2.1 Histone acetylation

The regulation or specialization of chromatin remodelers or histone chaperones at targeted chromatin locus or biological processes are largely achieved by histone modification, which is the covalent PTM on the histone proteins. For example, histone chaperones are required for histone modification at normally inaccessible sites, in turn, specific histone modification also facilitates or inhibits the histone exchange by recruiting or repelling chromatin remodelers or histone chaperones. For example, the binding of histone chaperone HIRA and its specific histone substrate H3.3 is facilitated by PAK2-dependent H4 S47 phosphorylation, which disfavors its interaction with CAF1 (26). Rtt109 mediated H3 K56 acetylation facilitates histone exchange and transcription initiation, but Set2-mediated H3K36 methylation restricts histone exchange and transcription (25, 27). Thus to study the histone PTMs is important in understanding the regulation of chromatin structure and transcription.

Generally, histone proteins are heavily post-translationally modified by phosphorylation, acetylation, methylation, ubiquitination and etc., which exert various functions in regulating chromatin structure through various mechanisms (28, 29). Histone acetylation is one of the well-known histone modifications that are frequently decorated at K4, K9, K14, K18, K23, K27, K36 residues on histone H3 and K16 on H4 (30). Histone acetylation at multiple sites is important, but sometimes is redundant for opening chromatin and activation of gene transcription (31, 32). Histones acetylation at lysine

residues on the one hand neutralizes its positive charge, thereby reducing the affinity between histone and negatively charged DNA. On the other hand, it recruits specific chromatin remodeler complexes that recognize acetylated lysine residue on histone proteins (33). The recognition requires specialized protein domains, including bromo domain and tandem PHD domain, which are shared features in some chromatin remodelers (34, 35). Histone acetylation, in essence, is an outcome of transferring an acetyl group from acetyl-CoA to the lysine residues on histone protein by specific enzymes known as histone acetyltransferases (HATs) that are often resides in larger chromatin remodeling complexes. Distinct families of HATs, including GNATs, MYST HATs and others, have been independently discovered and are responsible for histone acetylation (36, 37). It is generally believed that a HAT could target multiple lysine residues on histone and a lysine residue on histone could also be modified by multiple HATs (37). There might be some extend of redundancy in HAT functions, but emerging studies have indicated the substrate or lysine residue preference of HATs and the differential activity of HATs under curtain cellular contexts (38, 39). In the future study, a related question would be whether there is a preference in the usage of HAT under curtain physiological cue and, if yes, what would be the underlining mechanism to convey the specificity.

1.2.2 Histone methylations.

Unlike acetylation that only occurs on lysine, histone methylation could be conjugated on arginine, lysine and histidine (40). A single lysine residue could be mono-methylated, dimethylated or trimethylated on their ε-amine group. Lysine methylation on H3K4, H3K9, H3K27, H3K36, H3K79 and H4K20 are relatively well studied in the chromatin biology field, however, arginine methylation on H3-H4 and many other methylation events on H2A-H2B have also been identified by recent quantitative mass spectrometry analysis and their functions remain to be determined (41, 42). Histone methylation is catalyzed by histone methyltransferases, which are composed of three families including SET domain containing proteins and DOT1-like proteins for lysine methylation and protein arginine N-methyl-transferase family for the arginine methylation (41). Traditionally, it is believed that histone methylations are relative stable modifications and their turn over rates are much slower than other histone modifications until the discovery of histone demethylases, which actively and site specifically remove histone methylation. There are two families of demethylases, including amine oxidases and jumonji C domain containing iron dependent dioxygenases for lysine demethylation (43, 44). Arginine demethylases remain to be discovered, although monomethyl arginine can be converted to citrulline by PADI4 (45). The precise recruitment of methyltransferases and demethylases to their target histones is an important area of study. Consensus DNA sequences have been identified as binding sites for several histone remodeling complexes containing methyltransferase and demethylase. For example,

polycomb group response elements directly recruit polycomb repressive complex 2 (PRC2), which could catalyze H3 K27 trimethylation, though DNA binding transcription factors that recognize consensus DNA elements (46, 47). Long non-coding RNAs (LncRNA) are known to interact with DNA sequences with high sequence specificity, and are reported to be adaptors in the targeted methylation and demethylation process. LncRNAs have been shown to interact with PRC2 complex and H3K4 methyltransferase complex member WD repeat domain 5 (WDR5) (48, 49). With the identification of large number of lncRNAs in human, it is possible that they may mediate a great many of gene specific regulations and may be the major mechanism for gene specific interaction between DNA and chromatin factors. Histone methylations are recognized by proteins with methyl-binding domains, including PHD finger, WD40 repeat, CW domain, PWWP domain, ankyrin repeat and royal superfamily, which further includes chromo domain, double chromo domain, chromobarrel, tudor domain, double or tandem tudor domain and MBT repeats (42). Since methylation generally adds a positive charge and hydrophobicity to lysine and arginine, any proteins with hydrophobic properties, aromatic cages and composite pockets could potentially bind to histone methylations (42). Unlike histone acetylation, it is more clear that the location and degree of methylation are both critical for its biological functions. For example, H3 K4 trimethylation is generally a marker for transcriptional activation, while H3 K27 trimethylation is a repressive marker for chromatin (50, 51). H3 K79 dimethylation is crucial in cell cycle regulation, but H3 K79 trimethylation is associated with the Wnt signaling pathway (52, 53). Interestingly, the

transcriptional activation marker H3K4 methylation, when binding to PHD domain containing co-repressor protein inhibitor of growth family member 2 (ING2), are associated with the repression of transcription (54). Hence, to study the dynamic changes of histone methylation is important to understand epigenetic regulations in various cellular processes.

1.2.4 Quantitative mass spectrometry and novel histone modifications.

Studies of histone modification often involve the generation of site specific antibodies. However, except for the issue of antibody specificity, antigen occlusion is a severe issue for such a densely post translationally modified protein, like histone proteins. Quantitative mass spectrometry (MS) is developed for PTM study and could simultaneously identify large numbers of PTMs on a large number of proteins in a much less biased way for biological samples under various conditions (55). Basically, total proteins were extracted and solubilized. Trypsin protease is commonly used for generating MS "friendly" peptides, while other proteases might also be used alone or in combination if the digestion pattern of trypsin is not ideal in some biological samples. Unlike traditional MS, quantitative PTM MS usually requires an additional enrichment step, including the usage of chemical, antibody or protein affinity to enrich the specific modification of interest. The best example would be the development of an antibody that specifically recognizes di-Glycine linked lysine, which is the left over modification of ubiquitinated lysine after trypsin digestion (56). Using this antibody, cellular

ubiquitinated proteins can be enriched before MS identification, which is used to be very inefficient in identification of protein ubiquitination. Enriched peptides were then sent for standard shot gun MS and subsequent computational analysis with optimized parameters for identifications of specific PTMs. Particularly, to generate data with high reliability, it is important to estimate the sufficient hits to cover the complete model peptides catalogs in the MS experiments. However, it is very difficult to generate the complete model catalogs for total cellular PTMs with current computational resources due to the great number of possible PTMs and PTM combinations on the same protein. Also, modern MS generated far less than sufficient hits for such a big model set of PTMs (57). Despite the robustness in study the global PTMs, it is relatively capable of discovering and quantifying the PTMs on limited protein targets, like histone proteins. Now, quantitative MS has broadened our view in histone modifications, since many novel and exotic PTMs were found, such as crotonylation, 2-hydroxysobutyrylation, malonylation, succinylation and etc. (58) Functional characterization of such modifications would be important follow up works for PTM MS and may create a new paradigm for chromatin biology.

1.3 Regulation of histone modification

1.3.1 Metabolism dependent regulation of histone modification.

Eukaryotic cells adapt to changes in its environment to survive and proliferate. As mentioned earlier, the most important adaptation is achieved by a precise reprogramming of gene expression to fine-tune the cellular functions in face of environmental conditions.

The dynamics of histone modification are critically responsible for the altered gene transcription. With regard to stress-induced changes in histone modification, there are basically two aspects. One is the stress sensing machinery in the cells to transduce environmental changes to cellular signaling pathways, the other one is the activated signaling cascades that eventually activate the histone modification enzymes to regulate gene transcription (59). Alternatively, environmental changes like metabolism related changes have been shown to regulate histone modification directly, as many metabolic intermediates are co-factors or substrates for the enzymatic reaction of histone modifications (60). Glucose deprivation, which causes a rapid decrease in cellular energy, is proposed to affect histone acetylation through depletion of cellular acetyl-CoA and/or elevating the NAD+/NADH ratio, which inhibits HATs but activates histone deacetylases (HDACs), such as Sirtuin (61).

Another important histone modification affected by metabolism is the histone methylation. All histone methyltransferases use S-adenosyl-methionine (SAM) as a methyl donor for methylation and also produce S-adenosyl-homocycsteine (SAH), which is an inhibitor of SAM on a similar reaction order (62). Thus fluctuation of cellular levels of SAM/SAH may affect histone methylation. SAM is synthesized from methionine and ATP. SAH is converted to homocysteine, which can be either degraded or re-enter into the SAM cycle through re-methylation to methionine. These reactions are all essentially dependent on vitamins, especially folate, vitamin B6 and vitamin B12 (63). Thus, histone methylation could be affected by the input of other metabolic pathways to the SAM cycle,

cellular availability of vitamins and any factors that affect the enzymes in the SAM cycle. There are a growing number of evidences that support this idea. For example, inhibition of threonine to SAM decreased the SAM/SAH ratio, H3 K4 methylation and restricted embryonic stem cell growth and differentiation (64). Histone demethylase LSD family is FAD-dependent and JmjC family is α-ketoglutarate dependent. These two metabolites are both closely linked to mitochondria oxidative metabolism and are component of TCA cycle (65). Alteration in the cellular level of FAD/FADH2 or a-KG affects histone methylation level, and gene transcription (66). Mutations in metabolic enzymes have been characterized in human cancer and affect histone methylation and affect cancer growth and metastasis (67). As metabolic intermediates globally affect enzymatic conjugation or removal of histone modifications, this mechanism alone is unlikely to achieve gene specific regulation for metabolic adaptation to a new environment. Hence, a more sophisticated cellular signaling network accounting for it remains to be characterized for the transcriptomic reprograming upon metabolic changes.

1.3.2 Studying the dynamics of histone modification.

With the development of next generation sequencing technology, chromatin immunoprecipitation followed by sequencing (ChIP-seq) method has been widely used to obtain the genome wide DNA binding site information of any protein of interest (68). To study how a specific transcription factor or histone modification linked to biological functions or phenotypes requires the identification of downstream effector genes. ChIP-

seq is mainly used to understand such regulatory mechanism both globally and gene specifically. ChIP-seq is performed by capturing protein binding DNA by traditional ChIP method first, followed by adding oligonucleotide adaptors to those small DNA fragments for massive parallel sequencing and computational analysis. Cancer cells or stem cells are known to be heterogenetic, thus the chromatin and transcription status are distinct in each cells. By utilizing the microfluidic droplet barcoding technology, DNA fragments from each cells were separated in each droplet and barcoded respectively for next generation sequencing (69). This technology enabled us to understand the heterogeneity of human cancers or stem cells and to identify small subpopulations with unique feature and functional importance.

Classical methods and sequencing technology have built the foundation of chromatin biology. However, the spatial and temporal resolution of such 'snapshot' methods are low to monitor the dynamic and rapid process of transcription initiation, elongation and termination. Recent advances in fluorescence microscopy and transfection of single chain antibody facilitate the development of technology that can visualize chromatin or transcription dynamics at a single site level (70). Single transcription factor or histone modification tracking within a single cell enabled us to 'see' how transcription factors are recruited by histone modification and assembled at their target sites. Super-resolution microscopy based technology enabled us to map the chromatin structures at a single nucleosome resolution (71). New technologies have helped us to either confirm or

challenge existing concepts in the chromatin biology field and may put great insights into the future study.

1.4 Cancer stem cell theory

1.4.1 The basic concept of cancer stem cells

Current concepts suggest that cancers are originated from abnormal genetic or epigenetic alterations (72). A small portion of cancer cells, displaying the capability of self-renew and generation of large number of more differentiated form of cancer cells, are called cancer stem cells (CSCs), which are likely responsible for tumor initiation, metastasis, relapse and drug resistance(73, 74). Cancer stem cell is initially a hypothesis proposed to explain the heterogeneity of cancer cells (75). Until mid 1990s, a subpopulation of leukemia with stem cell like properties was discovered and named 'cancer stem cells', mimicking the multipotency and self-renewal properties of adult stem cells (76). Later, cancer stem cell theory was extended to solid tumors and it has been identified in almost every cancer types today (77). According to the cancer stem cell hypothesis, cancer stem cell is capable of forming a new tumor in the immune compromised animal model, while differentiated cancer cells can not (78). However, inconsistent results showing that limited number of differentiated cancer cells can also form tumor in xenograft model have been observed (79, 80). Although this might be due to the inaccuracy of methodology and the fact that all cancer stem cells must be removed to eliminate cancer, development of other models may explain those observations and the

original cancer stem cell theory should be modified. In fact, cancer stem cell is not the only theory to explain the cancer heterogeneity. Stochastic model and the model emphasizing the importance of the unique environment of a given cell have also been proposed (81). Their basic ideas are about that intrinsic genetic or epigenetic variations or extrinsic microenvironment differences within a group of cancer cells caused the heterogeneity, which is interchangeable upon changes of intrinsic or extrinsic factors (81). In the field of tumor initiation, there is increasing support for a non-mutually exclusive model that combines all above three models to explain the heterogeneity of cancer and explain the tumorigenicity of differentiated cancer at a given time (82). Although the cancer stem cell theory is under debate, the study of this interesting subpopulation of cells remains critical due to its possible drug resistance feature in cancer therapy (83). Cancer stem cells in many types of cancers are known to be resistant to chemotherapy and radiation therapy. Treating cancer without taking cancer stem cells into account would enrich cancer stem cells and exacerbating the malignancy of many cancers (79). Thus the identification and study the drug resistance mechanism of cancer stem cells become an urgent task in the cancer stem cell field.

1.4.2 Cancer stem cell markers.

To identify and study cancer stem cells, the methods include the isolation of specific surface markers or the use of selective culture conditions that prefer the clonogenicity of cancer stem cells, including *in vitro* sphere forming assay, *in vivo* limited

dilution of xenograft engraftment assay, renal graft assay and etc (84). Live imaging and *in vivo* lineage tracking are also important methodology to monitor the self-renewal and multipotency of cancer stem cells (84). Various biomarkers have been identified for many cancer types as listed in the table 3. Cancer stem cell markers can be classified into several categories. The most common one is the cell surface marker category, which is selected by contrasting the cell surface markers of cancer cells grow under normal and cancer stem cell enrichment environment. Surface markers facilitate the researchers to use flow cytometry to analysis the population of cancer stem cells or use fluorescence activated cell sorting (FACS) or magnetic activated cell sorting (MACS) to isolate putative cancer stem cells for the functional study (84). The next category is drug resistance markers, especially include many chemo drug efflux transporters at cell surface to convey the drug resistant feature of cancer stem cells. However, controversial data showing that drug resistant markers does not enrich the tumorigenicity of cancer cells in xenograft were observed in some cancer types (83, 85), suggesting that using markers for drug resistance alone may not be ideal to distinguish cancer stem cells. Another important category of markers is the markers for pluripotency. Embryonic stem cell pluripotent factors, like Oct4, Sox2 and Nanog are important for cancer stem cells, as the embryonic stem cell gene expression signature was also observed for cancer stem cells in many cancer types (86). However, the presence of multiple splicing variants of those pluripotent factors complicated the marker detection, since most of the variants are inactive. Thus, simply checking transcription activity of pluripotent genes by GFP reporter or protein expression

level by immunofluorescence staining is not sufficient and it is risky to link such observations to the the evidence of cancer stem cells. Thus using multiple markers and functional analysis methods are crucial in studying the cancer stem cells. However, there are still some limitations of even combining those methods. First, cancer stem cell traits (markers or functions) may be context specific and depending on the specific tumor microenvironment, which is different from assay conditions. Moreover, it is not clear whether cancer stem cells or non-stem cancer cells can be converted mutually. This plasticity in phenotypes raises serious questions in the validity of marker isolated cancer stem cells in this field of study (82).

Breast	Lung	Melanoma	Ovarian	Pancreatic	Prostate
ALDH1	ABCG2	ABCB5	CD24	ABCG2	ALDH1
BMI-1	ALDH1	ALDH1	CD44	ALDH1	BMI-1
CD24	CD90	CD20	CD117	BMI-1	CD44
CD44	CD117	CD133	CD133	CD24	CD151
CD133	EpCAM	Nestin	Ovastacin	CD44	CD166
CXCR4		NGF R		CD133	Integrin a1, b1
DLL4				c-Met	Integrin a6
GLI				CXCR4	Trop2
Her2				PON1	
IL-1a					
Integrin a6					
PTEN					

Table 3 List of cancer stem cell markers in various solid tumors (87).

Accumulating evidences suggested that cancer stem cell markers are not mere cancer stem cell defining molecules. CD44+ is widely used cancer stem cell markers in various cancer types, pointing the important role of CD44 in tumors. CD44 is a transmembrane glycoprotein composed of a conserved extracellular hyaluronan (HA)-binding domain, variably spliced regions, transmembrane sequences and intracellular cytoskeletal-signaling domains (88). One of the interesting areas of research in cancer stem cells is to study interaction of CD44 with the extracellular matrix HA, which promotes adhesion, migration and invasion of cancer cells (89). Other studies also found that HA-bound CD44 interacted with and promotes the receptor downstream kinase signaling of human epidermal growth factor receptor 2 (HER) (90). HA-bound CD44 activates p300-dependent acetylation of beta-catenin and NF-κB (91). HA-CD44 complex also activates Nanog, which subsequently promotes stem cell factor SOX2 and drug efflux transporter MDR1 (92). Cancer stem cell marker CD133 is also a transmembrane glycoprotein, but its biological role is not yet well defined as CD44. However, the property of the CD133+ cancer cells has been studied and associated to many stemness factors, drug resistance transporters, receptor tyrosine kinase signaling and HIF hypoxia related signaling (93).

Aldehyde dehydrogenase (ALDH) activity is another marker widely used in variety of cancer types. Hematopoietic stem cells display high level of ALDH activity, mainly through high expression of ALDH1 gene (94). This concept was then adopted in the cancer stem cell field for many cancer types. ALDH converts retinol to retinoic acid,

which is important for stem cell differentiation (95). It has also been suggested that ALDH could mediate the oxidation of many toxic aldehydes, in turn promote the maintenance of cancer stem cells and possibly drug resistance (95). Similar to the role of cancer stem cell markers as a cancer stem cell defining molecules, there were controversial data reported for the function of those cancer stem cell markers in different cancers types (96), suggesting that the functions of those cancer stem cell markers are also context dependent.

1.4.3 Signaling pathways involved in the cancer stem cells.

Cancer stem cells have been shown to harbor altered cellular signaling pathways, especially the pathways that are involved in stem cell self-renew, development and differentiation, including Wnt/β-catenin, hedgehog, BMP, TGF-β and notch signaling (97-99). Wnt signaling pathway has been suggested in the maintenance of both normal stem cells and cancer cells. Wnt binds receptor Frizzled and activates Dvl, in turn inhibited Axin/APC/GSK3-beta complex mediated beta-catenin turn over and promote beta-catenin downstream target transcription (100). In the case of cancer stem cells, inhibition of Wnt signaling results in the increased chemo-sensitivity for the chemo resistant population of cancer cells and reduced expression of stemness factor OCT4 (101), suggesting a critical role for Wnt in cancer stem cell functions. Notch signaling pathway determines cell fate probably through its four different Notch isoforms, the function of which are non-overlapping and/or opposing on differentiation and

development (102). This fact explains the observation that Notch1/2 in SCLC is tumor suppressive, but Notch3 is up-regulated in NSCLC (103). ALDH+ population of NSCLC has been shown to be dependent on Notch activity for clonogenicity (104), indicating a role of Notch in cancer stem cell function. The hedgehog pathway is a extensively studied area in developmental and stem cell biology, which regulates stem cell maintenance, proliferation and differentiation. Hedgehog binds to receptor patched-1 and releases smoothened protein from the membrane. Activated smoothened further antagonizes Gli and its downstream target. Deregulated hedgehog pathway results in tumor proliferation and metastasis (105). It is also reported that Hedgehog pathway promotes mammosphere formation and cancer initiation through cancer stem cell factor BMI, and pharmacological inhibition of Hedgehog pathway reduced CD133 or CD44 cell population (106), indicating its involvement in the maintenance of cancer stem cells. Together, studying signaling pathways related to cancer stem cells extended our view of cancer development and may facilitate the drug design for resistant cancer cells.

1.5 Protein ubiquitination and its role in signaling

1.5.1 Basic concepts of ubiquitination

Ubiquitination is a one of the well-studied post-translational modifications that is critically involved in many cellular processes (107). Basically it is a reaction of covalent attachment of the ubiquitin protein to the lysine residues on the substrate proteins. Ubiquitin is a small protein found in eukaryotic and there are four fusion genes in human

that can produce the precursor of ubiquitin. The ubiquitination process is accomplished by a cascade of reactions that involves three enzymes, namely E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligase (107). Ubiquitin is first activated by a ATP dependent two-step reaction by E1. Initially, E1 binds to ATP and catalyzes the acyl-adenylation of the C-terminus of ubiquitin. Adenylated C-terminus then forms a thioester bond with the cysteine sulfhydryl group on E1. E2 enzyme catalyzes a transesterification reaction, which transfers the ubiquitin from E1 to the active site cysteine of E2. At last step, E3 ligase catalyzes the formation of the isopeptide bond between a lysine residue of the substrate protein and C-terminus of ubiquitin. Human cells possess hundreds of E3 ligases to catalyze the ubiquitination of various substrates (107). E3 ligases generally determine the substrate specificity of the ubiquitination. HECT domain and RING domain E3 ligases are two basic categories (108). HECT domain E3 ligase forms a transient binding with ubiquitin, while RING domain E3 ligases directly transfer ubiquitin from E2 to substrate. Other E3 ligases include Anaphase-promoting complex (APC) and Skp, Cullin, F-box containing complex (SCF), which are large E3 ligase complexes that recognize substrates and transfer ubiquitin through the cooperation of different subunits (109).

Ubiquitination chain can range from mono, poly or even multi-mono, multi-poly ubiquitination, which decide the distinct fate of the substrate proteins (107). Mono ubiquitination can be involved in protein trafficking, endocytosis and viral budding. The situation for poly ubiquitination is more complicated. Depending on the linkage residue

between ubiquitin molecules within poly ubiquitination chain, it can form K6, K11, K27, K29, K33, K48, K63 and M1 linkage (107). K48 linked poly ubiquitination is the most well-studied one and its conjugation on protein marks the proteins for proteasomal degradation. K48 linked ubiquitination is recognized by the 26S proteasome and substrate proteins are rapidly degraded by proteases inside the proteasome, while ubiquitin chain is cleaved before this process and recycled for further usage. K63 linked ubiquitination does not trigger proteasomal degradation, but is involved in the various cellular processes including endocytosis, inflammation, kinase activation and DNA repair (107). K63 linked ubiquitination can be recognized by the ubiquitin binding domains, including UIM, UBA, UBC and etc., allowing that K63 linked ubiquitination to serve as a molecular scaffold to transduce and amply the cellular signaling (110). Little is know about other types of linkage, since the observations and functional studies of those linkages are just emerging and their involvement in cellular processes is largely unclear.

1.5.2 Nedd4 family E3 ligase and their function.

As mentioned above, E3 ligases are mainly categorized into two groups, by either containing a zinc-binding RING finger E2-binding adaptor domain or a Homologous to the E6-AP Carboxyl Terminus (HECT) catalytic domain. Nedd4 family E3 ligases belong to the HECT domain class of E3 ligases. Nedd4 is initially found in a screen for genes developmentally down regulated in the early embryonic mouse central nervous system (111). Other E3 ligases with similar structures, which are now Nedd4 family

members are subsequently found and characterized. They are Nedd4L, ITCH, SMURF1, SMURF2, WWP1, WWP2, NEDL1 and NEDL2 in human (111). Structurally, they all contain a HECT domain, a C2 domain and several WW domains on the N terminal for substrate recognition (111). However, shared molecular structure does not result in a redundant biological function, since individual knockout of Nedd4 family gene often exhibits strong phenotypes or even causes embryonic lethality (111). Consistent with the general concept of E3 ligases, this observation suggests that they may have different substrate specificity.

Nedd4 activity is regulated by auto-inhibition, where its C2 domain binds to its HECT domain to form an inhibitory structure (111). This inhibition can be disrupted by calcium, or phosphorylation of NEDD4 at specific tyrosine residues to activate the E3 ligase activity (112, 113). The WW domains on Nedd4 is required to recognize PY motif (PPxY) on its substrate, while adaptor proteins NDFIP1 and NDFIP2 can assist the binding between Nedd4 and it substrates lacking PY motifs (114). The HECT domain on Nedd4 forms a thioester bond with ubiquitin before it is transferred from Nedd4 to its substrates. Nedd4 has been shown to ubiquitinate several ion channels and membrane transporters and receptors, which often leads to endocytosis and proteasomal degradation of its substrates (115). While the majority of Nedd4 substrates localizing in either cytoplasm or plasma membrane are identified, its nuclear substrate is less well-defined. *In vivo*, Nedd4 deficiency causes embryonic lethality and profound decrease in IGF-1 and insulin signaling (115-117). Deletion of Nedd4 also leads to a reduction in effector T-

cells due to the impairment in the conversion of naïve T-Cells to activated T-Cells (118). Nedd4 is required for the neuronal development, the formation of dendrites in neuron and neuron muscular junctions and keeping the normal number of motor and axon neurons (119). Nedd4 has also been shown to poly-ubiquitinate tumor suppressor PTEN and drive proteosomal degradation of PTEN (120). However, in certain models, Nedd4 seems does not target PTEN for degradation (121). Although Nedd4 has been reported to be frequently overexpressed in many cancer types, its decrease is also associated with some cancers, where Nedd4 suppresses cancer through targeting N-Myc or c-Myc for degradation (115, 122). However, whether Nedd4 has a role in CSC regulation is not clear.

1.5.3 Rationale and Hypothesis

HATs often reside in larger protein complexes for the gene-specific recruitment and targeted histone acetylation(37). However, the mechanism underlying the recruitment of HATs for global histone acetylation is unclear. As depleting subunits recognizing known motifs or histone modifications in HAT complex did not affect global histone H3 acetylation (123), we speculated that there are other previously unknown H3 modifications, which may be involved in the global recruitment of HATs. By analyzing the published large-scale quantitative mass spectrometry data sets (124-126), we found that H3 proteins were ubiquitinated on multiple lysine (K) residues. Interestingly, this ubiquitination is also induced by glucose, suggesting that H3 ubiquitination may be a potential prerequisite for HAT recruitment and H3 acetylation upon glucose stimulation.

Since cancer stem cells represent a therapeutically challenging population of cancer cells, we also seek to examine whether this glucose induced H3 acetylation and its downstream target genes are involved in the regulation of cancer stem cells.

Chapter 2 Materials and Methods

2.1 Cell culture, glucose treatment, transfection, viral transduction and reagents.

Hep3b, HEK293T, Du145 cell lines were purchased from ATCC and cultured in DME/F12 medium (GE Healthcare HyClone) in 10% Fetal bovine serum (FBS, Sigma Aldrich), Penicillin Streptomycin (Pen Strep) and L-glutamine. *WT* and *Nedd4*$^{-/-}$ MEFs cultured in DMEM medium containing 10% FBS were obtained from Dr. Xinjiang Wang. For the add-back of glucose procedure, 80% confluent cells were cultured in DMEM medium without glucose (Invitrogen) for 4 hours and resupplied with glucose (4.5g/L) for indicated times. 293T cells were transfected by standard calcium phosphate method. Other cell lines were transfected using Lipofectamine 3000 (Invitrogen) by the manufacturer's protocol and if indicated, cultured in medium containing Hygromycin B (100 ug/mL) to establish stable cell lines. For lentiviral infection, 293T cells were co-transfected with lentiviral plasmid (pLKO-puro), packing plasmid (deltaVPR8.9) and envelope plasmid (VSV-G). 2 days after transfection, medium containing virus particles were used to infect mammalian cell lines. All the infected cells were cultured in the medium containing 2μg/ml puromycin for 1 week before further analysis. Short interfering RNA (shRNA) for NEDD4, H3.3 and GCN5 were purchased from Sigma Aldrich. Flag-H3.3 plasmid and lentiviral Flag-H3.3 plasmid were obtained by inserting human H3.3 open reading frame into pCDNA3.1-hygro or pLKO-as3w-puro vectors respectively. All H3.3 mutation constructs (K4R, K9R, K14R, K18R, K23R, K27R, K36R, K37R, K36/37R, K23/36/37R, K56R, K64R, K79R, K115R and K122R) were

generated by site-directed mutagenic PCR according to the kit manual (Stratagene). His-ubiquitin plasmid was previously described(127). HA-BMI-1 and HA-RNF2 were obtained from Dr. Shiaw-Yih Lin. HA-NEDD4 and HA-UHRF-1 plasmids were from Drs. Pier Paolo Pandolfi and Hung-Ying Kao, respectively. HA-CBL-A, HA-CBL-B and HA-CBL-C were received from Dr. Stanely Lipkowitz. Flag-HA-RNF8 was kindly provided by Dr. Junjie Chen. V5-NEDD4 WT, Y43/585F and Y43/585E mutant are from Dr. Daniela Rotin. Flag-NEDD4L, NEDL1 and NEDL2 are from Dr. Wesley Sundquist. Flag-WWP1 and WWP2 are from Dr. Wenyi Wei. The following antibodies were used in this study: anti-H3 (Abcam, ab12079), anti-H3 pan-ac (Active Motif, 39139), anti-H3 K4ac (Active Motif, 39381), anti-H3 K9ac (Active Motif, 39917), anti-H3 K14ac (Active Motif, 39697), anti-H3 K18ac (Active Motif, 39755), anti-H3 K23ac (Active Motif, 39131), anti-H3 K27ac (Active Motif, 39133), anti-H3 K36ac (Active Motif, 39379), anti-H3 K56ac (Active Motif, 61061), anti-H3 K4me3 (39915), anti-H3 K9me3 (Active Motif, 39765), anti-H3 K27me3 (Active Motif, 39155), anti-H3 K4me2, K9me2, K27me2, K36me2, K79me2 (Cell Signaling Technology, 9847), anti-H3s10p (Abcam, ab5176), anti-H3.3 (EMD Millipore, 09-838), anti-NEDD4 (Novus, NBP1-40112), anti-GCN5 (Active Motif, 39975), anti-USP22 (Abcam, ab4812), anti-Flag (Sigma), anti-HA (Covance), anti-Actin (Sigma), anti-IL1α (Abcam, ab17281), anti-IL1β (Abcam, ab2105) and anti-IgG heavy chain HRP (Sigma Aldrich, a1949). The following recombinant proteins were used in this study: active NEDD4 (Millipore), histone octamer (EMD Millipore), E1 enzyme (Sigma Aldrich), UbcH4 (EMD Millipore), UbcH5a (EMD

Millipore), UbcH5b (EMD Millipore), UbcH5c (Boston Biochem), UbcH6 (EMD Millipore), UbcH7 (Boston Biochem), ubiquitin (Boston Biochem), GST-ubiquitin (Millipore), GCN5 (Novus), IL1β human (Sigma Aldrich). The following chemicals were used in this study: N-acetyl-L-cysteine (NAC) (Sigma Alrich), N-Ethylmaleimide (NEM) (Calbiochem) and DCFDA (Invitrogen).

2.2 *In vivo* ubiquitination assay.

In vivo ubiquitination assay was performed as previously described(127). Briefly, 293T cells were transfected with his-Ubiquitin and other indicated plasmids for 36 hours and harvested by denaturing buffer (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole). Ni-NTA Agarose beads (QIAGEN) were incubated with cell lysates for 3.5 h to pull-down his-Ubiquitin and washed beads were analyzed by Western blot.

2.3 *In vitro* ubiquitination assay.

3ug recombinant histone octamer and 5ug active form of NEDD4 were incubated with 0.5 μg E1 activating enzyme, 1.5 μg Ubiquitin, 0.5 μg various E2 enzymes UBCH4, UBCH5a, UBCH5b, UBCH5c, UBCH6 or UBCH7 and 2.5 mM ATP in reaction buffer (1.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 20 mM HEPES (pH7.4)) in a total 20ul reaction volume at 37°C for 3 hours. Ubiquitination on substrate was then detected by Western blot analysis.

2.4 Whole cell extracts for histone modification detection, cellular fractionation, chromatin fractionation and immunoprecipitation.

Whole cell extracts were prepared by boiling the cell pellets in SDS sample buffers for 10min. Cytosol and nucleus were purified using standard protocol. Briefly, cells were re-suspended in hypertonic buffer (10mM Tris pH 7.6, 10mM $MgCl_2$, 0.1% NP-40), then lysed using Dounce homogenizer and centrifuged at 1000g. Pellets containing nucleus were washed twice with hypertonic buffer. Supernatant containing cytosol was further cleared by centrifuging at 12000g. Chromatin fractionation was performed as described(128). Briefly, cells were first lysed with buffer A (50 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M Sucrose, 10% Glycerol, 1 mM DTT, 0.1% Triton X-100, NEM, protease inhibitor cocktail (Biotool) and phosphatase inhibitor cocktail (Biotool)) on ice for 30 min. After centrifuge at 1000g, pellets including the nucleus were further lysed with buffer B [3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, NEM, protease inhibitor cocktail (Biotool) and phosphatase inhibitor cocktail (Biotool)]. After centrifuge, pellets containing the chromatin were washed, and sonicated in SDS sample buffer for Western blot analysis. To immunoprecipitate proteins, cells were lysed and sonicated in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% NP-40, 1mM EDTA, protease inhibitor cocktail (Biotool), phosphatase inhibitor cocktail (Biotool) and NEM). Cell lysates were incubated at 4°C with antibody overnight

and agarose protein A/G beads for 3 hours, and then beads were washed with RIPA buffer for 5 times before eluting with SDS sample buffer.

2.5 Endogenous ubiquitination assay.

Briefly, cells were collected and boiled in SDS lysis buffer (2% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, protease inhibitor cocktail) for 10 min. Lysate was sonicated and diluted 10 times with dilution buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Sodium Deoxycholate, 1% NP-40, protease inhibitor cocktail and NEM). Samples were then incubated on ice for 30 min and cleared by centrifuging. Immunoprecipitation assay using anti-Ub (FK2) antibody was performed as described above and H3 ubiquitination was detected by Western blot using anti-H3 antibody.

2.6 Chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) assay.

ChIP assay was performed as described(129) with some modifications. Cells were cross-linked in culture media (with 1% formaldehyde) with gentle shaking for 10min at room temperature and stopped by adding glycine to a final concentration of 0.125 M. Cells were washed with PBS three times and nuclei were isolated. Nuclei were then lysed in RIPA buffer with proteinase inhibitor cocktails and sonicated using Bioruptor to shear genomic DNA to a range of 200-1000 bps. Lysates were cleared and blocked with BSA (final concentration of 1mg/mL) and salmon sperm DNA (final concentration of

0.3mg/mL). Pre-cleaned lysates were immunoprecipitated with various antibodies, followed by adding protein A/G beads. Beads were washed and eluted by 400uL elution buffer (with 0.1 M $NaHCO_3$, 1% SDS). The DNA was directly recovered by gel extraction kit (Omega) and analyzed by real-time qPCR.

2.7 Chromatin immunoprecipitation followed by sequencing (ChIP-seq).

Chromatin immunoprecipitation assay was performed using ChIP-IT High Sensitivity kit (Active motif) according to the manufacturer's manual. The Illumina compatible libraries were prepared using DNA Library preparation kit (KAPA, KK8232), as per the manufacturer's protocol. In brief, DNA was fragmented to a median size of 150bp by sonication. Fragmented DNA ends were polished and 5′-phosphorylated. After addition of 3′-A to the ends, indexed Y-adapters were ligated and the samples were PCR amplified. The resulting DNA libraries were quantified and validated by qPCR, and sequenced on Illumina's HiSeq 2000 in a single-read format for 36 cycles. The resulting BCL files containing the sequence data were converted into ".fastq.gz" files and individual sample libraries were demultiplexed using CASAVA 1.8.2 with no mismatches.

2.8 ChIP-seq data analysis.

Briefly, 36 nucleotides (nts) sequencing data (.fastq.gz) were unachieved and imported to local galaxy project instance(130-132). Sequences for each sample were concatenated

and 3nts from both 5' and 3' end were trimmed off. The processed data were aligned to the hg19 (human) assembly using Bowtie2(133). For each sample, the ChIP-seq peak profiles were obtained by normalizing ChIP data to input data (mappable reads were normalized to 1X genome coverage of hg19) using BamCompare tool in DeepTools(134). The data were visualized by preparing custom track hubs on the UCSC genome browser. Global average profiles at TSS or enhancer regions were calculated by ComputeMatrix tool in DeepTools(134) and visualized by Microsoft Excel. Known enhancer regions were defined according to Broad ChromHMM tracks for HepG2 cells. Differential ChIP-seq peaks between samples were identified by Diffreps using G-test(135). Genes, the TSS of which was located within differential peaks, were then listed for subsequent Venn diagram visualization. Reviewer private access link for ChIP-seq data: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=apehcoywnhsjxcr&acc=GSE66340

2.9 RNA extraction and cDNA synthesis.

Cells were lysed in TRIZOL reagent (Invitrogen) and extracted by chloroform. Total RNA were then precipitated by isopropanol and washed with 70% ethanol. cDNA were synthesized according to the standard M-MLV reverse transcriptase protocol. Briefly, total RNA were mixed with oligo(dT), dNTP, M-MLV reverse transcriptase (Invitrogen) in the M-MLV buffer and the reaction was performed using a thermo cycler.

2.10 Microarray, Gene set enrichment analysis (GSEA) and TCGA data analysis.

Total RNA was extracted and purified using RNeasy mini kit (QIAGEN) according to manufacturer's manual. Microarray analysis was performed for total RNA on Illumina HumanHT12v4 platform following Illumina's standard procedure. All data sets were normalized based on the mean value, and differentially expressed genes were ranked by T-test. Gene set enrichment analysis was performed using GSEA software with KEGG pathway gene sets and GO term pathway gene sets respectively. Significantly enriched gene sets ($p<0.05$, $q<0.25$) were visualized using Cytoscape(136). TCGA exon expression data sets (Illumina) for various cancer types were visualized in UCSC cancer genome browser. Cases with highest and lowest 30% expression of NEDD4 were included to evaluate the correlation between the expression of NEDD4 and other genes by Wilcoxon test (Bonferroni correction). Exons show significant ($p<0.05$) correlations with NEDD4 expression were shown either in red (positive correlation) or green (Negative correlation) in the histogram. Reviewer private access link for microarray data: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=evazwqokhlgtbwl&acc=GSE66341

2.11 *In vitro* binding and native gel analysis.

Recombinant full-length GCN5 and ubiquitin were mixed in the binding buffer (25 mM HEPES, pH 7.4, 125 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5% Triton X-100) and incubated on ice for 30min. Samples were then mixed with sample buffer (62.5 mM Tris-HCl pH6.8, 25% glycerol, 1% Bromophenol blue) or SDS sample buffer, and then separated by native Polyacrylamide gel electrophoresis (PAGE) or SDS-PAGE respectively. GCN5 and ubiquitin proteins were then detected by Western blot.

2.12 *In vitro tumor* sphere formation and *in vivo* tumor engraftment assay.

For *in vitro* tumor sphere forming assay**,** 5000 cells were seeded in the ultra-low attachment 6-well plate (Corning Life Science) and cultured in tumor sphere forming medium (DME/F12 supplemented with 5ug/mL Insulin, 0.05ug/mL hEGF and 0.5ug/mL Hydrocortisone). Cells were incubated at 37 °C for 13 days and spheres larger than 100um were counted. For *in vivo* tumor engraftment assay, cancer cells were first stained using ALDEFLUOR Kit (StemCell Technology) following manufacturer's manual. Cells with top 2.5% Aldh enzymatic activity were then isolated by cell sorting using flow cytometry. 1000 isolated cells were subcutaneously injected into each nude mouse (NCRNU-F/Homozygous, Taconic Farms) and monitored for tumor growth for 70 days. Mice died tumor free were excluded from final results.

2.13 ROS and GSH detection.

To detect cellular ROS, cells were collected and incubated with DCFDA at 37°C for 30min and then subjected to flow cytometry analysis. ROS levels were calculated as the mean fluorescence signal. Cellular GSH level was determined by Glutathione assay kit (Sigma Aldrich, CS0260).

Chapter 3 Results

3.1 NEDD4 ubiquitinates H3 upon glucose stimulation

By analyzing the published large-scale quantitative mass spectrometry data sets (124-126), we found that H3 proteins were ubiquitinated on multiple lysine (K) residues (Figure 1a). While these proteomic studies are mostly carried out under non-stimulus conditions, it is critical to know which physiological cues can drive this ubiquitination modification and what functions it may play. To address these questions, we challenged cells with various physiological stimuli and performed *in vivo* ubiquitination assay to detect ubiquitination of endogenous H3 proteins. Of these stimuli, H3 ubiquitination was drastically inhibited by glucose deprivation, but only slightly affected under glutamine deprivation or remained unaltered by other stresses, such as serum starvation, genotoxic stress and oxidative stress (Figure 1b and 1c). We also excluded the possibility that glucose starvation causes irreversible damages to the cells, thereby leading to the reduction of H3 ubiquitination indirectly, as add-back of glucose to cells under glucose deprivation readily recovered H3 ubiquitination (Figure 1d and 1e). Accordingly, these results suggest that glucose is a *bona fide* physiological activator for H3 ubiquitination.

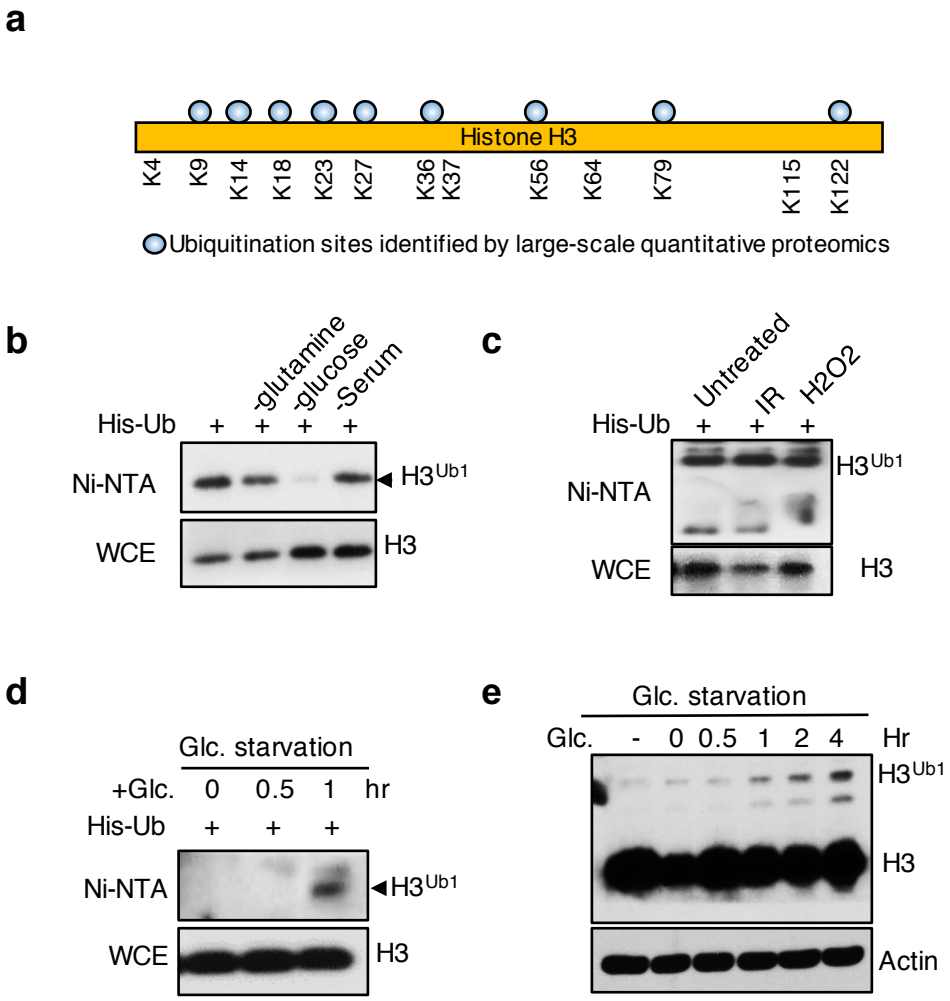

Figure 1 Glucose induces H3 ubiquitination.

a. Summary of H3 ubiquitination sites identified in various large-scale quantitative proteomics studies.

b, c. Glucose deprivation abolished H3 ubiquitination. 293T cells were transfected with his-ubiquitin plasmid (His-Ub) for 36 hours and treated with various stresses for 4 hours

before *in vivo* ubiquitination assay to access the H3 ubiquitination (See experimental procedures for details).

d. Feeding glucose recovered H3 ubiquitination. 293T cells were transfected with his-ubiquitin plasmid for 36 hours, then glucose-starved for 4 hours, and added-back glucose for indicated times before in vivo ubiquitination assay.

e. Glucose induces H3 ubiquitination in a time dependent manner. Hep3B cells were glucose starved for 4 hours and glucose was added for indicated time before chromatin fractionation assay.

We next determined which E3 ligase is responsible for glucose-induced H3 ubiquitination. By screening a panel of ubiquitin E3 ligases, NEDD4 (also known as neural precursor cell expressed developmentally down-regulated protein 4) was identified to be a potential E3 ligase for H3 ubiquitination, as wild-type (WT) NEDD4 could promote H3 ubiquitination, but not E3 ligase dead mutant NEDD4 (NEDD4-CS), (Figure 2a to 2c). In line with this possibility, NEDD4 knockdown abolished H3 ubiquitination (Figure 2d). To investigate whether NEDD4 is a direct E3 ligase for H3, we performed *in vitro* ubiquitination assay by mixing recombinant active form of NEDD4 with various E2 ubiquitin-conjugating enzymes and histone octamer. We found that NEDD4 in combination with UbcH7 effectively triggered *in vitro* H3 ubiquitination, although UbcH5 a/b/c mix or UbcH6 also exhibited enzymatic activity (Figure 2e).

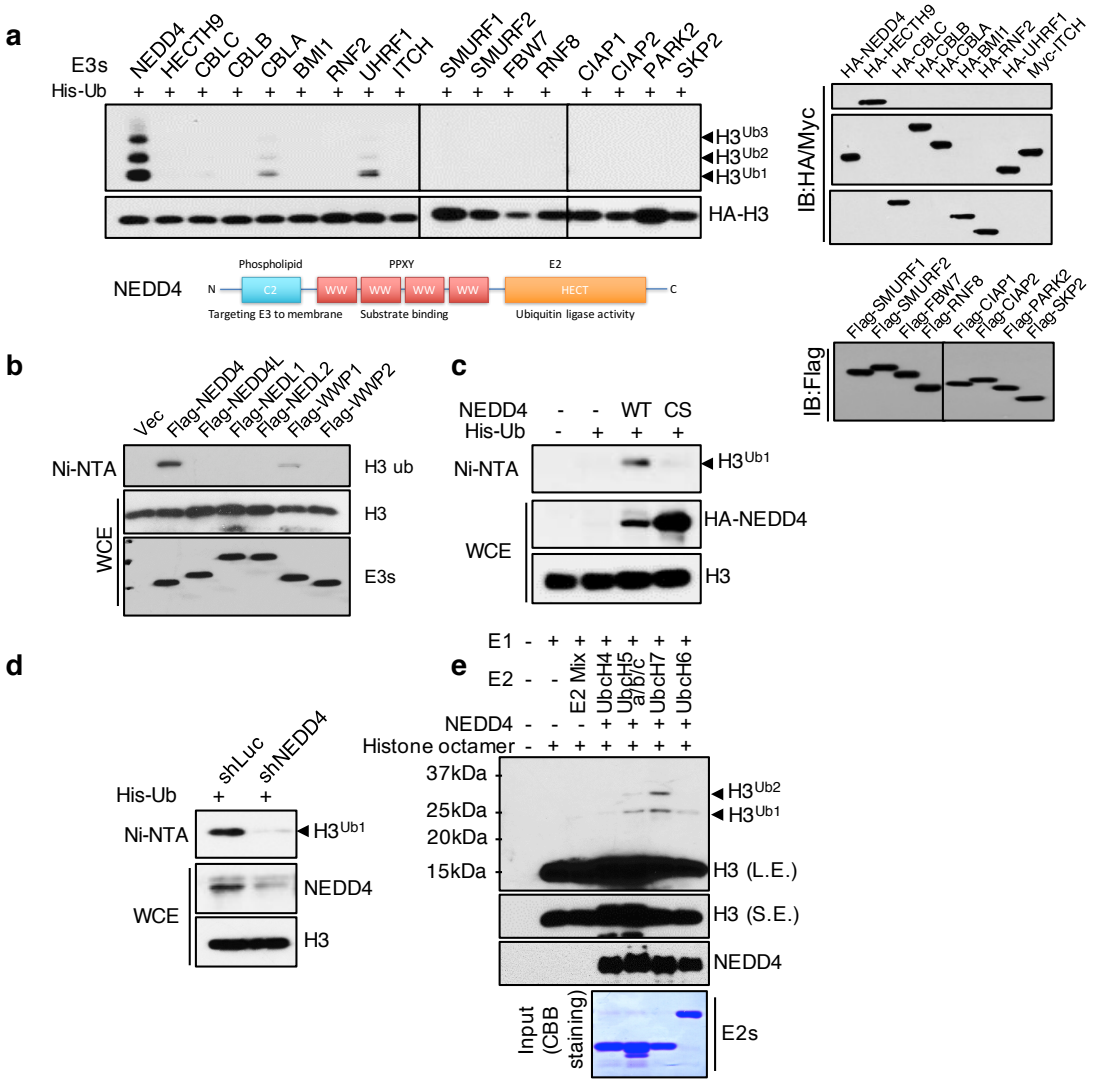

Figure 2 Nedd4 is the E3 ligase for H3.

a, b. Screening of E3 ligases for H3 ubiquitination. 293T cells were transfected with his-ubiquitin plasmid and various E3 ligases constructs for *in vivo* ubiquitination assay.

c. NEDD4 E3 ligase dead mutant (CS mutant) failed to trigger H3 ubiquitination. 293T cells were transfected with his-ubiquitin plasmid and WT NEDD4 or NEDD4 CS mutant construct for *in vivo* ubiquitination assay.

d. NEDD4 knockdown abolished H3 ubiquitination. Control and NEDD4 knockdown 293T cells were transfected with his-ubiquitin plasmid for *in vivo* ubiquitination assay.

e. NEDD4 ubiquitinated H3 *in vitro*. *In vitro* ubiquitination assay was performed for recombinant NEDD4 and histone octamer (See experimental procedures for details). Reaction products were then assessed by Western blot using anti H3 antibody. H3 mono- and di-ubiquitination have predicted molecular weights of ~25kDa and ~33kDa. S.E. and L.E. are short for shorter exposure time and longer exposure time respectively.

Next we investigated whether NEDD4 is required for glucose-induced H3 ubiquitination. By performing endogenous ubiquitination assay, we found that NEDD4 knockdown or knockout abolished endogenous H3 ubiquitination induced by glucose treatment (Figure 3a and 3b), indicating a physiological role of NEDD4 in mediating glucose-induced H3 ubiquitination. We further asked the question whether the glucose levels could orchestrate E3 ligase activity of NEDD4. Our data showed that NEDD4 overexpression failed to trigger H3 ubiquitination under glucose deprivation, whereas add-back of glucose readily rescued NEDD4 overexpression induced H3 ubiquitination (Figure 3c). As we have also noticed that H3 ubiquitination in some of our experiments displayed multiple ubiquitination bands at higher molecular weight, we sought to test whether H3 undergoes poly-ubiquitination or multi-mono-ubiquitination. We found that ubiquitin with its all K mutated to R (Ub K0) did not affect H3 ubiquitination pattern in the *in vivo* ubiquitination assay (Figure 3d), indicating that NEDD4 mono-ubiquitinates H3 at multiple sites. Moreover, while NEDD4 is previously shown to regulate protein mostly in the cytosol and plasma membrane, a few nuclear substrates for NEDD4 are found(137, 138). Consistently, we found that NEDD4 could be detected both in cytosol and nucleus by using biochemical fractionation and immunofluorescence assays (Figure 3e and 3f), suggesting that NEDD4 can target its protein substrates in the nucleus such as H3. Collectively, these data provide strong evidence that NEDD4 is a direct E3 ligase and responsible for glucose-induced H3 ubiquitination.

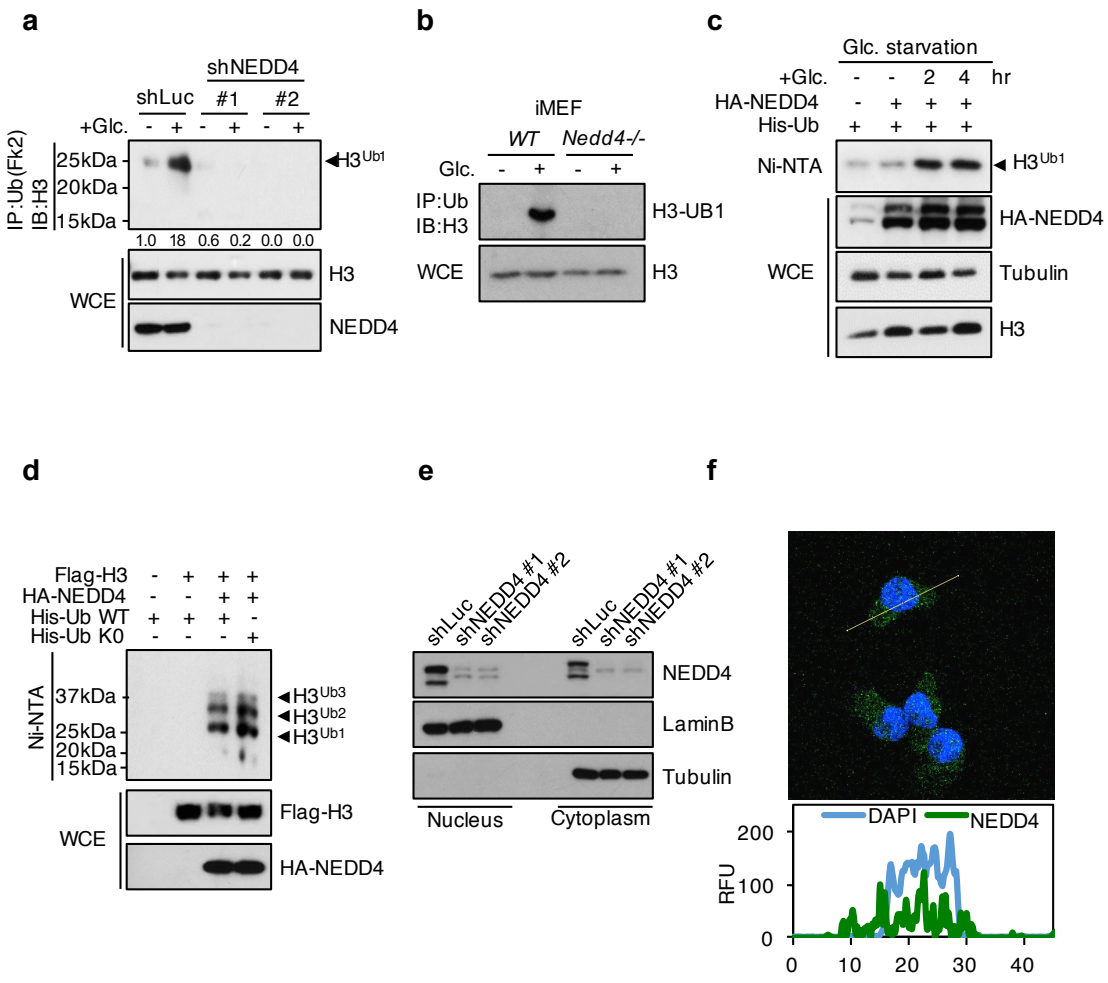

Figure 3 Glucose induced H3 ubiquitination is Nedd4 dependent.

a, b. NEDD4 knockdown or knockout abolished glucose-induced H3 ubiquitination. Hep3B cells were glucose starved for 4 hours and added-back glucose for 2 hours before endogenous ubiquitination assay (see experimental procedures for details). H3 ubiquitination was then visualized by Western blot.

c. Add-back of glucose recovered NEDD4 overexpression induced H3 ubiquitination. 293T cells were transfected with his-ubiquitin and NEDD4 plasmids for 36 hours, then glucose-starved for 4 hours, and added-back glucose for indicated times before *in vivo* ubiquitination assay.

d. NEDD4 triggered mono-ubiquitination on H3. 293T cells were transfected with Flag-H3, HA-NEDD4, His-Ub WT and His-Ub K0 as indicated before *in vivo* ubiquitination assay.

e. Cellular fractionation was performed for control and NEDD4 knockdown Hep3B cells.

f. Immunofluorescence assay was performed for Hep3B cells. The intensity of NEDD4 staining (Green) and DAPI staining (Blue) along the yellow line in the image was shown in the histogram.

To understand how glucose promoted NEDD4 mediated H3 ubiquitination, we speculated that glucose treatment might activate NEDD4 through altering the post-translational modification status of NEDD4. As glucose treatment could elevate the cellular level of ATP and acetyl-CoA, which are important cofactors for protein phosphorylation and acetylation, we evaluated overall phosphorylation and acetylation of NEDD4 protein. Interestingly, we found that tyrosine phosphorylation of NEDD4, but not NEDD4 serine/threonine and acetylation, is strongly induced by glucose treatment (Figure 4a). As a previous report shown that growth factor mediated NEDD4 phosphorylation at tyrosine (Y) 43 and Y585 promotes NEDD4 E3 ligase activity (112), we examined whether glucose also induced NEDD4 phosphorylation at tyrosine (Y) 43 and Y585 and found that mutating Y43/585 to phenylalanine (F) blocked glucose induced NEDD4 tyrosine phosphorylation (Figure 4b), indicating that glucose induces NEDD4 tyrosine phosphorylation at Y43 and Y585, which may then activate NEDD4 through Y43/585 phosphorylation. In support of this notion, NEDD4 Y43/585F mutant failed to trigger H3 ubiquitination, but phosphorylation mimetic mutant Y43/585E enhanced H3 ubiquitination more efficiently than WT NEDD4 (Figure 4c). Importantly, Y43/585E mutant rescued H3 ubiquitination under glucose deprivation to the level similar to that triggered by WT NEDD4 in glucose-stimulated conditions (Figure 4d). Collectively, our results suggest that glucose induces NEDD4 activation by inducing NEDD4 tyrosine phosphorylation at Y43 ad Y585, in turn leading to H3 ubiquitination.

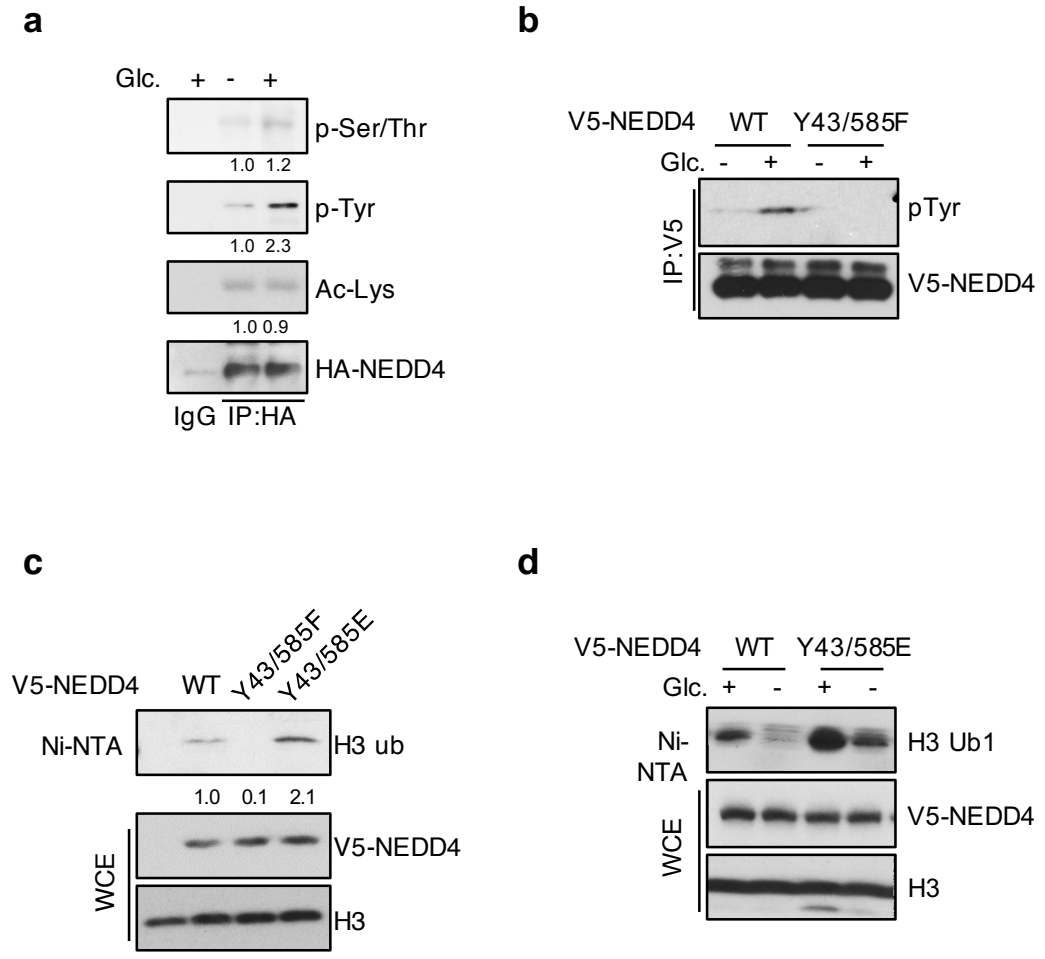

Figure 4 Glucose induces the tyrosine phosphorylation of Nedd4.

a. 293T cells transfected with HA-NEDD4 was treated with glucose and harvested for immunoprecipitation assay.

b. Glucose induced NEDD4 phosphorylation at Y43 and Y585. 293T cells transfected with WT or Y43/585F NEDD4 plasmids were treated with glucose and harvested for IP.

c. NEDD4 phosphorylation is required for H3 ubiquitination. 293T cells transfected with WT, Y43585F or Y43/585E NEDD4 plasmids were harvested for in vivo ubiquitination assay.

d. 293T cells were transfected with His-Ub and indicated plasmids and treated with glucose before in vivo ubiquitination assay.

3.2 Glucose-induced H3 K23/36/37 ubiquitination by NEDD4 is required for H3 K9 and K14 acetylation

Next we determined whether NEDD4 mediated H3 ubiquitination regulates H3 acetylation. Similar to H3 ubiquitination, we found that glucose treatment readily induced H3 acetylation on K9, K14, K27 and K56 sites, but had no effect on H3 phosphorylation, H3 di- and tri-methylation or H3 acetylation at other lysine sites (Figure 5a to 5e). Notably, NEDD4 knockdown or knockout specifically impaired glucose-induced H3 acetylation, but not other H3 modifications (Figure 5a to 5e), suggesting that there is a link between H3 ubiquitination and H3 acetylation under glucose stimulation.

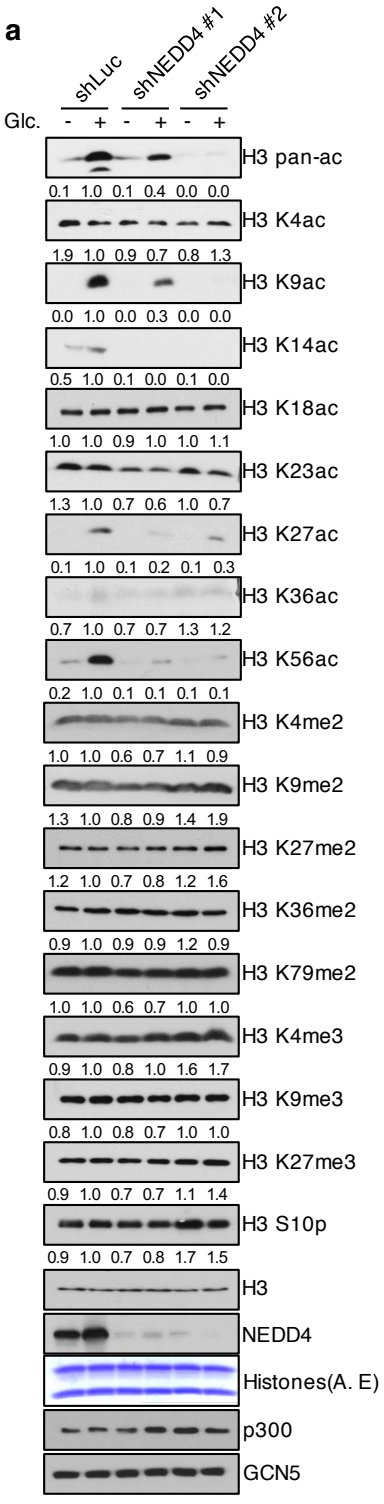
a
shLuc
shNEDD4 #1
shNEDD4 #2
Glc. - + - + - +
H3 pan-ac
0.1 1.0 0.1 0.4 0.0 0.0
H3 K4ac
1.9 1.0 0.9 0.7 0.8 1.3
H3 K9ac
0.0 1.0 0.0 0.3 0.0 0.0
H3 K14ac
0.5 1.0 0.1 0.0 0.1 0.0
H3 K18ac
1.0 1.0 0.9 1.0 1.0 1.1
H3 K23ac
1.3 1.0 0.7 0.6 1.0 0.7
H3 K27ac
0.1 1.0 0.1 0.2 0.1 0.3
H3 K36ac
0.7 1.0 0.7 0.7 1.3 1.2
H3 K56ac
0.2 1.0 0.1 0.1 0.1 0.1
H3 K4me2
1.0 1.0 0.6 0.7 1.1 0.9
H3 K9me2
1.3 1.0 0.8 0.9 1.4 1.9
H3 K27me2
1.2 1.0 0.7 0.8 1.2 1.6
H3 K36me2
0.9 1.0 0.9 0.9 1.2 0.9
H3 K79me2
1.0 1.0 0.6 0.7 1.0 1.0
H3 K4me3
0.9 1.0 0.8 1.0 1.6 1.7
H3 K9me3
0.8 1.0 0.8 0.7 1.0 1.0
H3 K27me3
0.9 1.0 0.7 0.7 1.1 1.4
H3 S10p
0.9 1.0 0.7 0.8 1.7 1.5
H3
NEDD4
Histones(A. E)
p300
GCN5
b
Normalized Signal
2
1.5
1
0.5
0
H3 K9ac
+Glc. - + - + - +
shLuc
#1
#2
shNEDD4
c
Normalized Signal
1.5
1
0.5
0
H3 K14ac
+Glc. - + - + - +
shLuc
#1
#2
shNEDD4
d
iMEF
WT
Nedd4 -/-
Glc. - + - +
H3 K9ac
H3 K14ac
H3 K27ac
H3
e
MDA231
shLuc shNEDD4
Glc. - + - +
H3 K9ac
H3 K14ac
H3

Figure 5 Nedd4 is required for glucose induced H3 acetylation.

a. NEDD4 knockdown abolished glucose-induced H3 K9, K14, K27 and K56 acetylation. Control and NEDD4 knockdown Hep3B cells were glucose-starved for 4 hours, and added-back glucose for 3 hours before whole cell extraction for Western blot analysis (See experimental procedures for details). A. E. is short for acid extraction.

b, c. Quantification of Western blot data (n=3, mean ± S.D.).

d, e. NEDD4 deficient iMEF cells or MDA231 cells were treated with glucose and harvested for Western blot analysis.

While mammalian H3 contains three isoforms (H3.1, H3.2 and H3.3), H3.3 is found to be the major isoform decorated by H3 acetylation(139). Consistent with the previous findings, we demonstrated that H3.3 knockdown abolished H3 acetylation on K4, K9, K14, K23, K27 and K56, but not K18 and K36 (Figure 6a). Similar to NEDD4 knockdown and glucose stimulation, H3.3 knockdown also did not alter H3 methylation (Figure 6a). We then used H3.3 to determine NEDD4 dependent ubiquitination sites on H3. After serial mutagenesis on all H3.3 lysine residues, we found that mutation of K23 or K36/37 effectively blocked glucose- and NEDD4-mediated H3.3 ubiquitination (Figure 6b to 6d), suggesting that NEDD4 specifically ubiquitinates H3 on K23, K36 and K37 residues. The existence of endogenous ubiquitination modification on these sites was also supported by previous large-scale quantitative mass spectrometry analysis(125, 126).

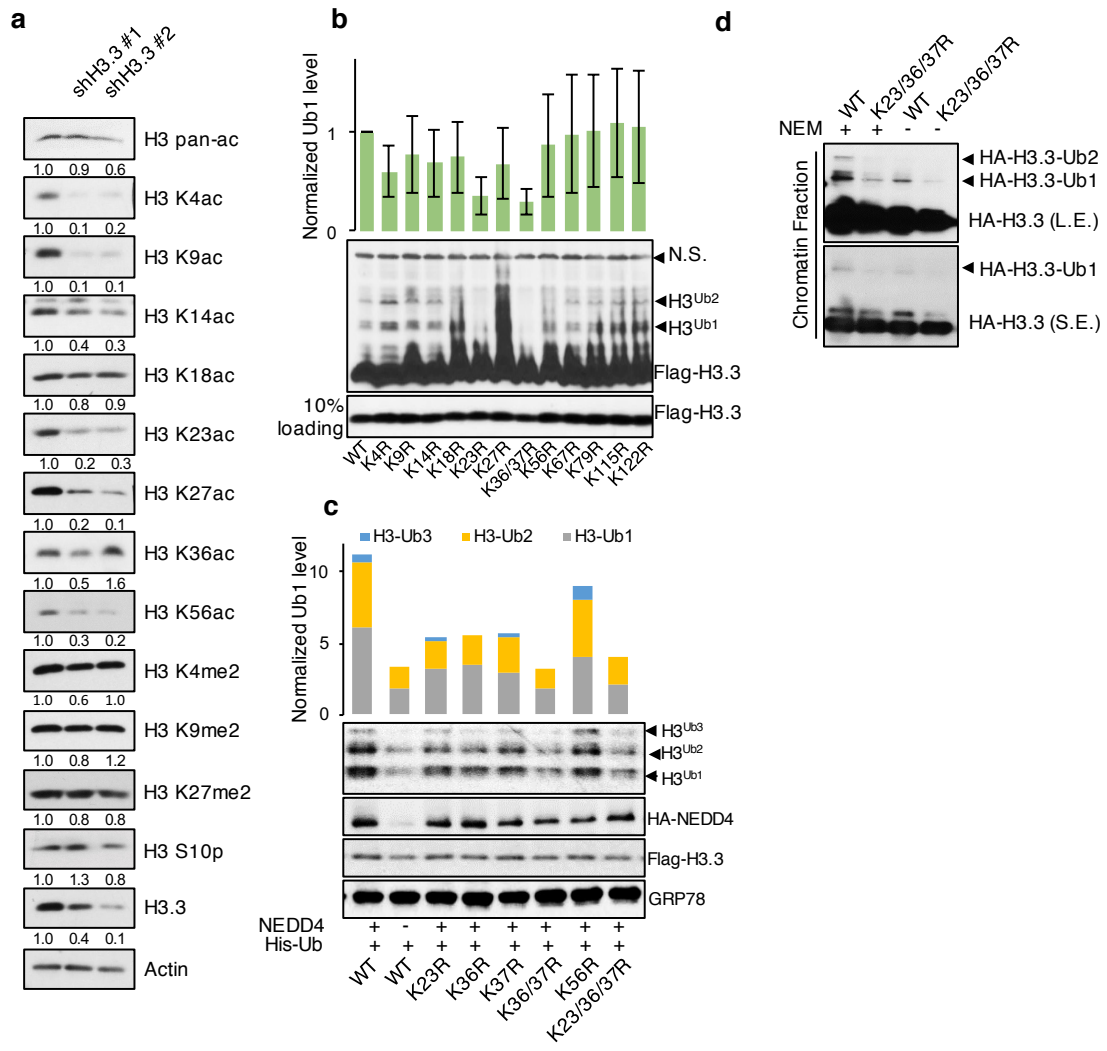

Figure 6 Nedd4 ubiquitinates H3 at K23/36/37 residues.

a. H3.3 knockdown abolished H3 K9, K14, K27 and K56 acetylation. Control and H3.3 knockdown Hep3B cells were lysed for Western blot analysis.

b. H3 K23R and K36/37R mutant abolished glucose induced H3 ubiquitination. Hep3B cells expressing various Flag-H3.3 constructs were glucose-starved for 4 hours and

added-back glucose for 2 hours before chromatin fractionation assay. Ubiquitination levels were normalized to input (n=5, mean ± S.E.M.). N.S. is short for non-specific band.

c. H3 K23 and K36/37R mutant abolished NEDD4 overexpression induced H3 ubiquitination. 293T cells were transfected with his-ubiquitin, NEDD4 plasmids and various Flag-H3.3 constructs for 36 hours before in vivo ubiquitination assay. Ubiquitination levels were normalized to input.

d. Chromatin fractionation was performed for Hep3B cells stably expressing Flag-H3.3 WT or K23/36/37R. NEM was added to preserve ubiquitination.

To determine the causal relationship between H3 ubiquitination and acetylation, we stably expressed WT H3.3 and H3.3 ubiquitination-deficient mutant in Hep3b cells to examine H3 acetylation. Consistent with the effect of glucose and NEDD4 on H3 acetylation, H3.3 K23/36/37R mutant displayed impaired H3 acetylation on K9 and K14 (Figure 7a to 7d), while single or double mutation also displayed partial effect on H3 acetylation (Figure 7e).

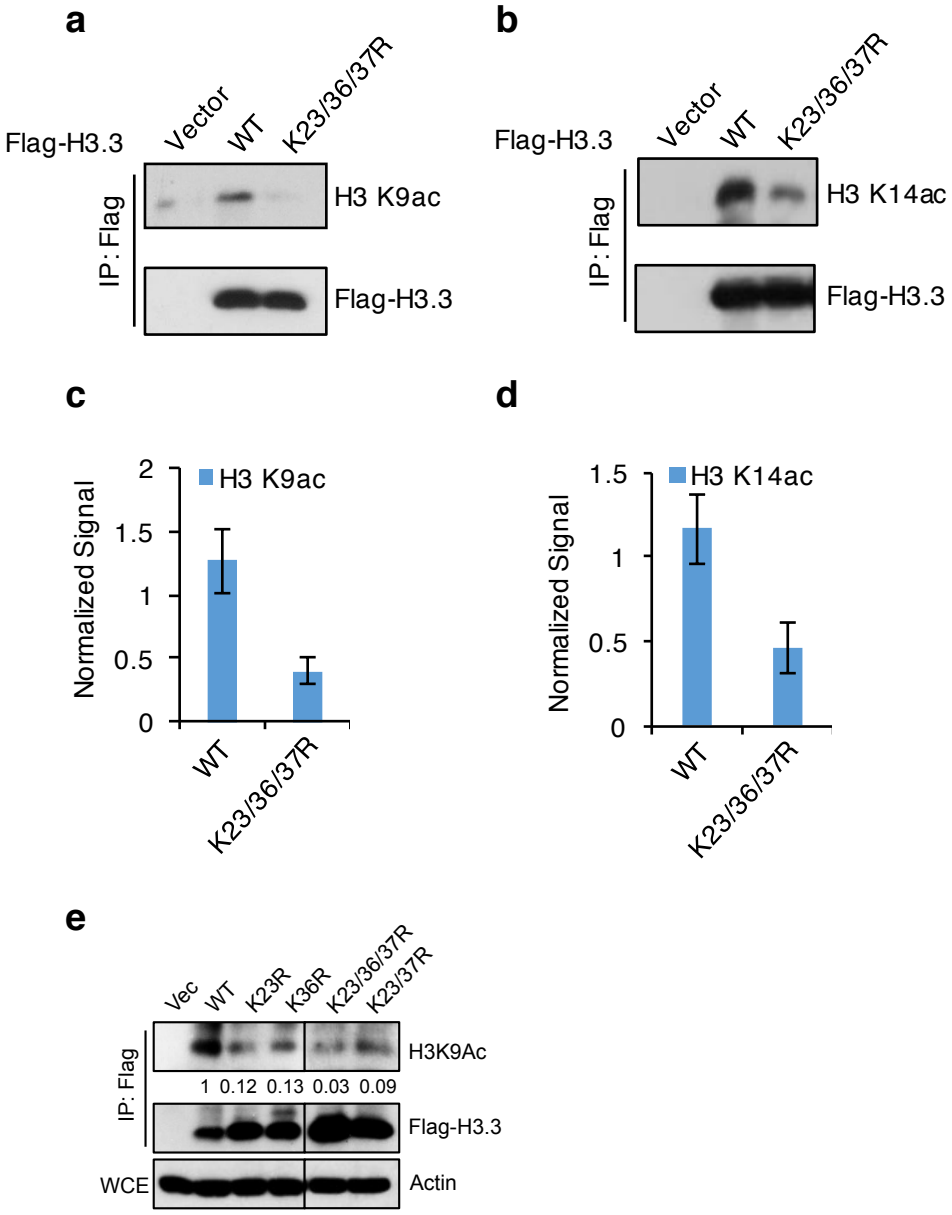

Figure 7 H3 ubiquitination is required for H3 acetylation.

a, b. H3 K23/36/37R is defect in H3 K9/K14 acetylation. WT or K23/36/37R Flag-H3.3 was stably expressed in Hep3B cells and immunoprecipitated for Western blot analysis.

c, d. Quantification of Western blot data (n=3, mean ± S.D.).

e. Various mutants of Flag-H3.3 were restored in shH3.3 Hep3B cells. Immunoprecipitation assay was used to access the H3 K9ac.

Additionally, we performed a kinetic study for H3 ubiquitination and K9 acetylation to further elucidate their regulation. Adding back glucose readily induced H3 ubiquitination within 1 hour, while acetylation of H3 at K9 occurred around 2 hours (Figure 8a), indicating H3 ubiquitination occurs earlier than H3 K9 acetylation and could be upstream of H3 acetylation. To test whether NEDD4 tyrosine phosphorylation at Y43 and Y583 plays a critical role in H3 acetylation, we restored NEDD4 WT, Y43/585F and Y43/585E mutant in the NEDD4 knockdown cells to examine their effect on H3 acetylation. We found that NEDD4 Y43/585F mutant failed to rescue glucose induced H3 K9 acetylation, but NEDD4 Y43/585E mutant fully rescued glucose-induced H3 K9 acetylation and executed such effect even more efficiently than NEDD4 WT (Figure 8b), confirming that glucose activated NEDD4 through NEDD4 tyrosine phosphorylation. Taken together, our data suggest that glucose-induced H3 ubiquitination by NEDD4 selectively regulates H3 acetylation.

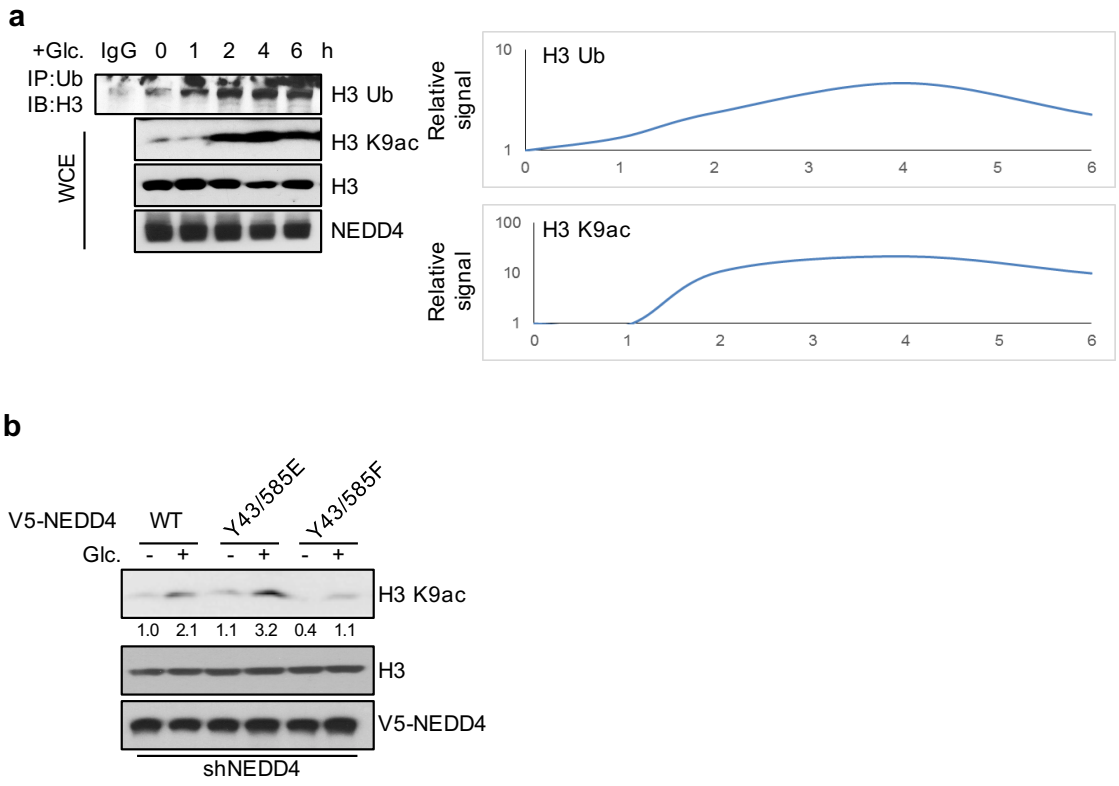

Figure 8 Nedd4 activity is important for H3 acetylation.

a. Hep3B cells treated with glucose for various times were harvested for immunoprecipitation assay. Kinetics of H3 ubiquitination and K9ac were shown in log scale.

b. NEDD4 knockdown Hep3B cells transfected with WT, Y43/585E or Y43/585F mutant were treated with glucose and harvested for Western blot analysis.

As Acetyl-CoA, which is a downstream metabolite of glucose, is important for glucose induced H3 acetylation(60), we then asked which downstream metabolite or

whether glucose per se is critical for NEDD4 mediated H3 ubiquitination. We treated cells with ATP, pyruvate or NADH, which can be uptake by cells, and found that none of them can rescue the impact of glucose deprivation on H3 ubiquitination, H3 K9 acetylation and NEDD4 phosphorylation (Figure 9a and 9b), indicating that metabolites upstream of pyruvate or glucose per se may promote NEDD4 phosphorylation and activity through an unknown mechanism. Given the fact that acetyl-CoA is crucial for H3 acetylation and pyruvate can be converted to acetyl-CoA, our surprising observation that pyruvate cannot fully activate H3 K9 acetylation further demonstrated the necessity of H3 ubiquitination to trigger glucose-induced H3 acetylation (Figure 9a). Of note, ATP (or pyruvate/NADH, which can also generate ATP) could partially enhance H3 ubiquitination but not NEDD4 phosphorylation (Figure 9b), suggesting that this ATP dependent ubiquitination reaction may also be highly sensitive to cellular ATP level. In line with this observation, glucose treatment, which can strongly induce cellular ATP level, further enhances H3 ubiquitination triggered by active NEDD4 Y43/585E mutant (Figure 4d), implying that both ATP level and E3 ligase activity of NEDD4 could regulate H3 ubiquitination. However, without Acetyl-CoA generated by glucose, ATP, NADH or active NEDD4 mutant alone could not rescue H3 acetylation (Figure 9a). These results collectively depict a previously unexpected model of glucose action, which regulates H3 acetylation through not only acetyl-CoA, but also H3 ubiquitination driven by glucose mediated ATP production and NEDD4 activation.

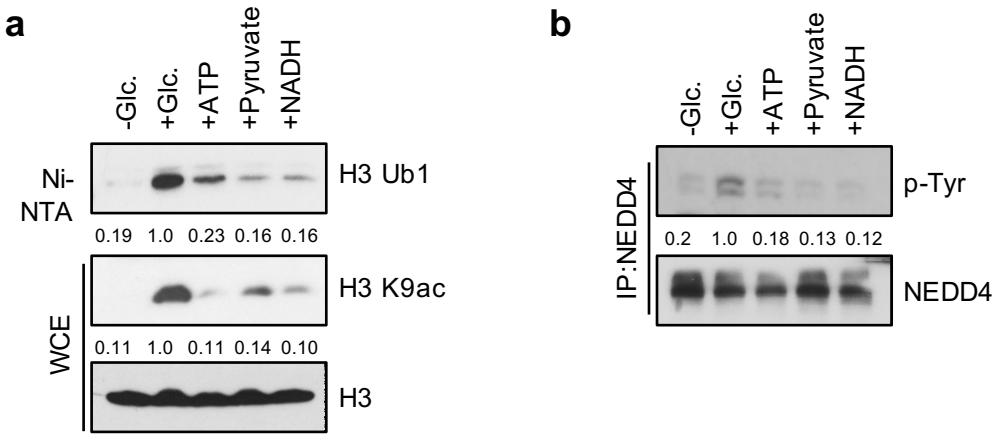

Figure 9 Nedd4 is activated by glucose but not ATP, pyruvate or NADH.

a. 293T cells transfected with his-Ub and treated with various metabolites were harvested for *in vivo* ubiquitination assay.

b. 293T cells treated with various metabolites were harvested for immunoprecipitation assay.

3.3 NEDD4 is required for glucose-induced H3 K9 acetylation at TSS and enhancers

H3 K9 acetylation is known to localize at the TSS and generally regulates gene transcription in mammalian cells(140). Having shown that glucose treatment increases total or cellular level of H3 K9 acetylation in a NEDD4 dependent manner, we asked whether this process is gene-specific. Therefore, genome-wide pattern of H3K9 acetylation was determined by ChIP-seq assay. Consistent with Western blot result, we found that glucose globally enhanced H3 K9 acetylation at TSS of genes and such effect was impaired upon NEDD4 knockdown by ChIP-seq assay (Figure 10a). Our results also indicated that glucose specifically induces H3 K9 acetylation at TSS of around 2000 genes, and 40% of those acetylation events were NEDD4 dependent (Figure 10b and 10c), highlighting the critical role of NEDD4 in glucose-induced H3 K9 acetylation at TSS. Meta-analysis also revealed that glucose promoted H3 K9 acetylation on known enhancers, and this event was also NEDD4-dependent (Figure 10d), suggesting that NEDD4 may also participate in the activation of enhancers in response to glucose stimulation. Together, these data provide further evidence to support that NEDD4 regulates glucose-induced H3 K9 acetylation at TSS and enhancers.

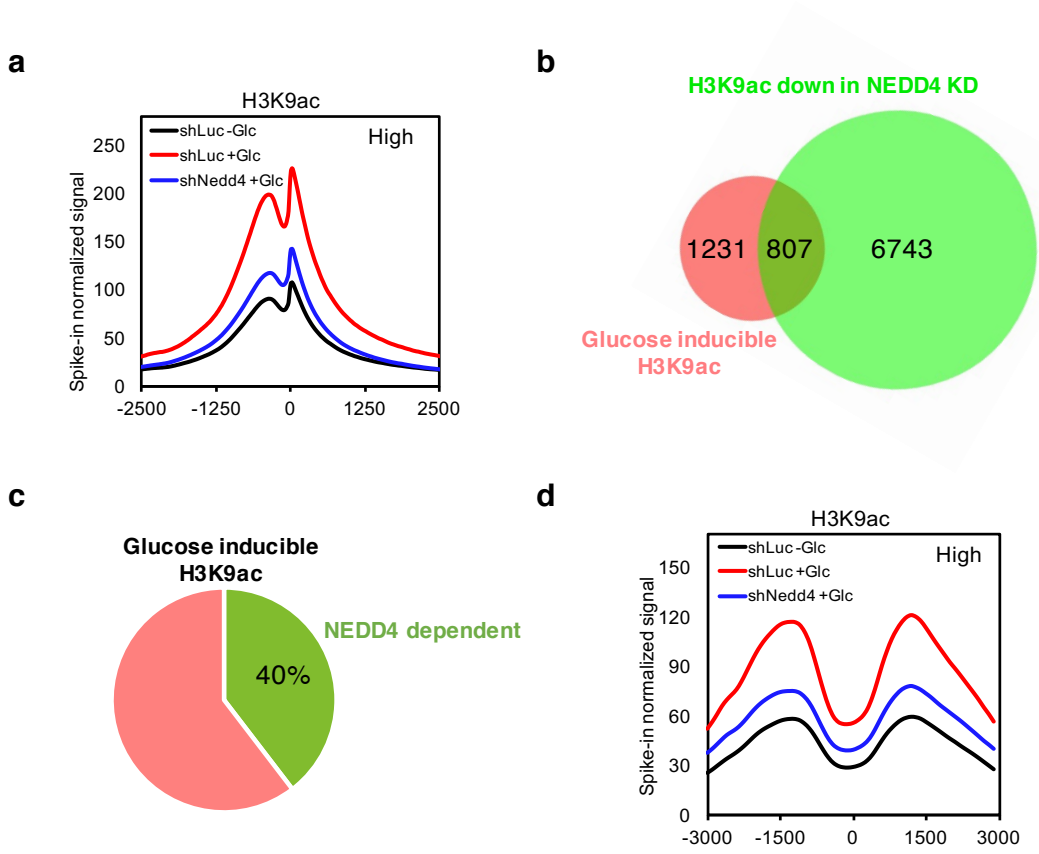

Figure 10 Nedd4 is required for glucose induced H3 acetylation at TSS and enhancers.

NEDD4 knockdown impaired glucose-induced genome-wide H3 K9 acetylation at TSS and enhancer regions. ChIP-seq was performed for control and NEDD4 knockdown Hep3B cells before or after adding-back of glucose for 3 hours. Shown were global H3 K9ac profiles at TSS (a), Venn diagram of genes with differential H3 K9ac peaks at TSS under various conditions (b and c) and global H3 K9ac profiles at known enhancers (d). See experimental procedures for details.

3.4 Glucose-induced H3 ubiquitination by NEDD4 regulates gene transcription

We next determined whether glucose-induced H3 ubiquitination by NEDD4 regulates transcription, and if so, whether NEDD4 regulates gene transcription through H3 K9 acetylation. To this end, genome-wide differential gene expression pattern was determined by gene expression microarray. Integrative analysis of microarray and ChIP-seq data sets revealed that NEDD4 knockdown caused down-regulation of around 5,000 genes, and 50% of these genes showed reduced H3 K9 acetylation at TSS upon NEDD4 knockdown (Figure 11a and 11b). Additionally, we applied GSEA (141, 142) (gene set enrichment analysis) using modified Kolmogorov–Smirnov (K-S) test to evaluate the statistical relationship between H3 K9 acetylation and gene expression regulated by NEDD4. The K-S test showed a significant positive correlation ($P<0.05$) between NEDD4-dependent H3 K9 acetylation at TSS and NEDD4-regulated gene transcription (Figure 11c), suggesting that NEDD4 orchestrates gene transcription by regulating H3 K9 acetylation at TSS. We then used qPCR to validate the expression of top list genes from microarray and showed that NEDD4 deficiency indeed inhibited their expression (Figure 11d and 11e).

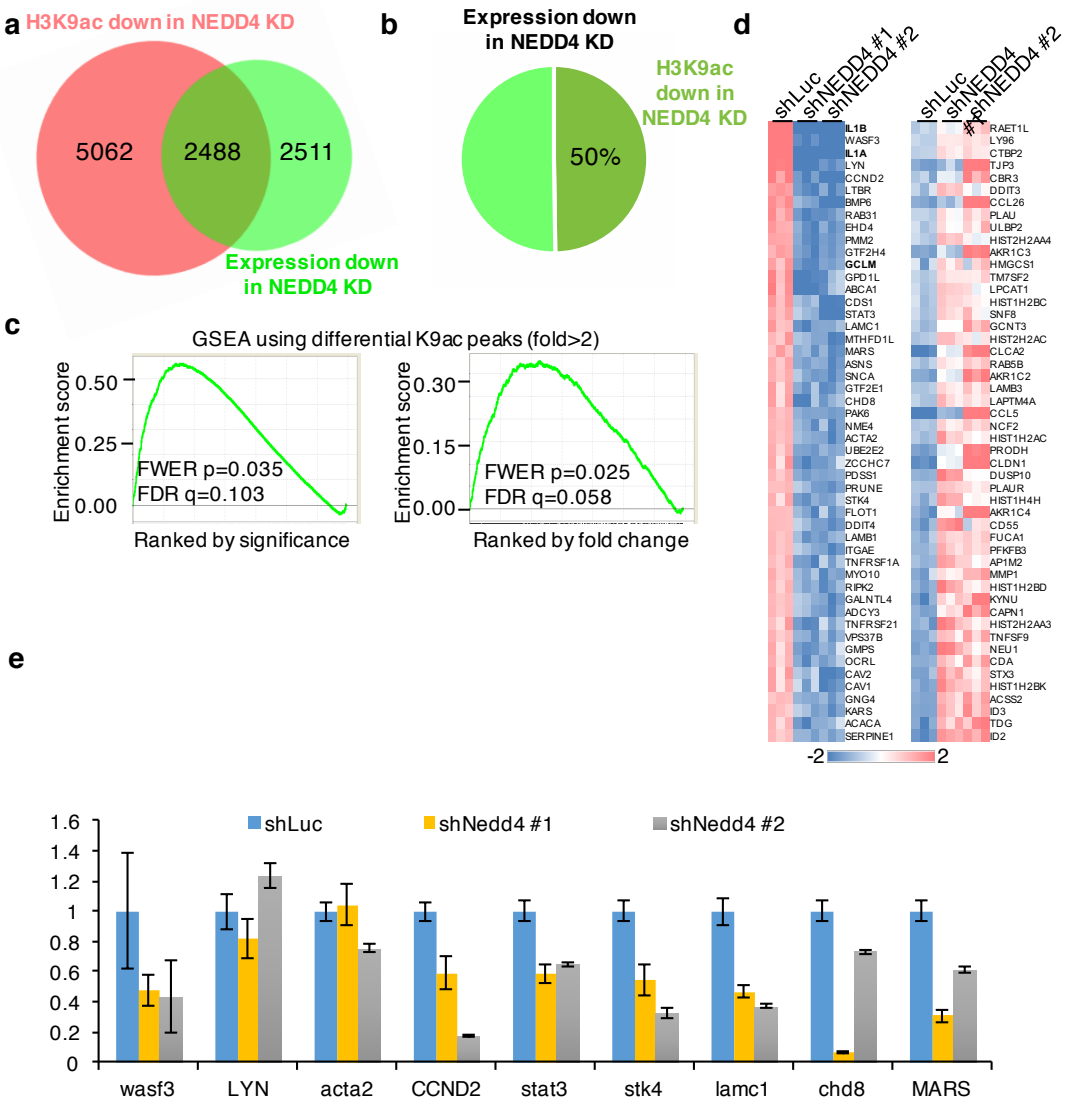

Figure 11Nedd4 mediated H3 acetylation is required for gene expression.

a-c. NEDD4 regulates H3 K9ac at TSS of NEDD4 target genes. Shown were Venn diagram of genes with differential expression or differential H3 K9ac at TSS. GSEA was performed to evaluate the distribution of genes that show down-regulation of H3K9ac at

TSS in NEDD4 knockdown cells in microarray-derived gene list, which is rank ordered either by T-test or fold change.

d. Heat map view of top and bottom gene list of microarray data sets. Microarray analysis for total RNA was performed for control and NEDD4 knockdown Hep3B cells.

e. qPCR was performed to analyze the mRNA level in NEDD4 deficient iMEFs or Hep3B cells (n=3, mean ± S.E.M.).

Among those tested genes, *IL1α, IL1β* and *GCLM* were most affected and regulated by glucose (Figure 12a to 12c). ChIP-seq assay revealed that H3 K9 acetylation at TSS of *IL1α, IL1β* and *GCLM* was induced by glucose in a NEDD4-dependent manner (Figure 12d and 12i). Consistently, ChIP-qPCR analysis confirmed this result in both NEDD4 knockdown and *Nedd4*$^{-/-}$ MEFs (Figure 11e to 11g). In addition, we found that RNA polymerase II binding at TSS of IL1α and IL1β was induced by glucose in a NEDD4-dependent manner (Figure 11h). As H3.3 ubiquitination affects H3 K9/K14 acetylation, we determined whether H3.3 and its ubiquitination are required for the transcription of IL1α, IL1β and GCLM.

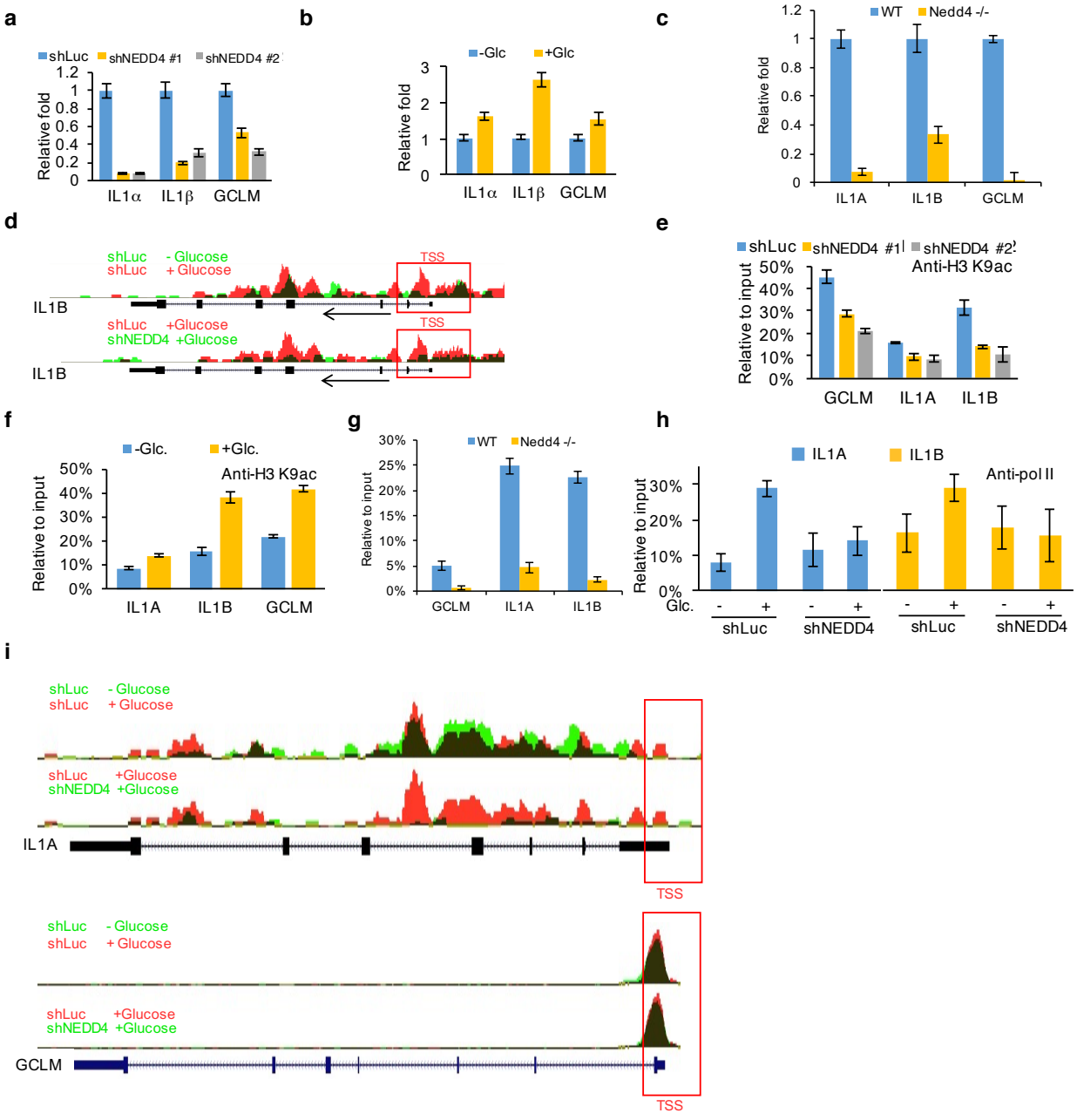

Figure 12 IL1A, IL1B and GCLM are glucose inducible target genes of Nedd4.

a. NEDD4 knockdown impaired IL1α, IL1β and GCLM expression. qPCR was performed to analyze the mRNA level in control and NEDD4 knockdown Hep3B cells (n=3, mean ± S.E.M.).

b. IL1α, IL1β and GCLM were induced by glucose. Hep3B cells were glucose-starved for 4 hours and added-back glucose for 6 hours before qPCR analysis (n=3, mean ± S.E.M.).

c. qPCR was performed to analyze the mRNA level in NEDD4 deficient iMEFs or Hep3B cells (n=3, mean ± S.E.M.).

d. UCSC genome browser view of ChIP-seq H3 K9ac signals along IL1B gene.

e. NEDD4 knockdown impaired H3 K9ac at TSS of IL1 ✔ IL1 🚲 and GCLM genes. ChIP-qPCR using anti-H3 K9ac antibody was performed for control and NEDD4 knockdown Hep3B cells (n=3, mean ± S.E.M.).

f. H3 K9ac was induced at TSS of IL1 ✔ IL1 🚲 and GCLM genes by glucose. Hep3B cells were glucose-starved for 4 hours and added-back glucose for 6 hours before ChIP-qPCR analysis using anti-H3 K9ac antibody (n=3, mean ± S.E.M.).

g. ChIP-qPCR was performed for WT and Nedd4-/- iMEFs.

h. NEDD4 knockdown impaired glucose-induced polymerase II (pol II) binding at TSS of IL1Aand IL1B genes. Control and NEDD4 knockdown Hep3B cells were glucose-starved for 4 hours and added-back glucose for 6 hours before ChIP-qPCR analysis using anti-pol II antibody (n=3, mean ± S.E.M.).

i. UCSC genome browser view of ChIP-seq H3 K9ac signals along IL1A and GCLM genes.

Knockdown of H3.3 decreased the mRNA level of IL1α, IL1β and GCLM (Figure 13a), and such defects could be rescued by the restoration of WT H3.3, but not H3.3 K23/36/37R mutants (Figure 13b to 13d). We also found that restoration of single or double mutations of H3.3 partially rescued gene expression (Figure 13e and 13f). There data underpin the function of H3 ubiquitination by NEDD4 in transcription activation.

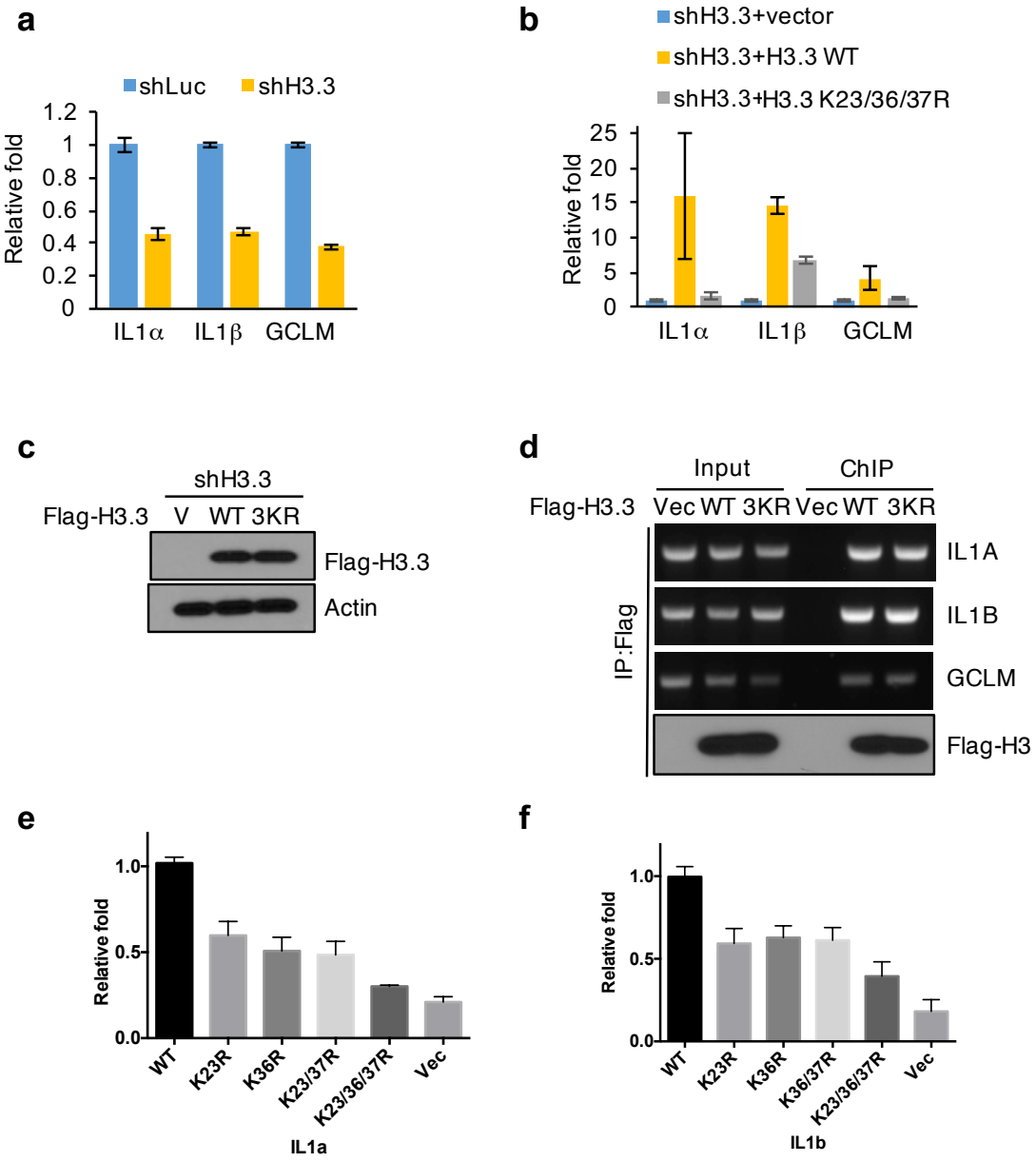

Figure 13 H3 ubiquitination is required for gene expression.

a. H3.3 knockdown impaired IL1α, IL1β and GCLM expression. qPCR was performed to analyze the mRNA level in control and H3.3 knockdown Hep3B cells (n=3, mean ± S.E.M.).

b. H3 ubiquitination deficiency impaired IL1α, IL1β and GCLM expression. qPCR was performed to analyze the mRNA level in vector, H3.3 WT and H3.3 K23/36/37R mutant restored H3.3 knockdown Hep3B cells (n=3, mean ± S.E.M.).

c, d. Shown are Flag-H3.3 WT and K23/36/37R restoration efficiency (a) and chromatin integration efficiency (b) in H3.3 knockdown Hep3B cells.

e, f. qPCR was used to analyze IL1a and IL1b mRNA level in Hep3B cells restored with various H3.3 constructs.

3.5 H3 ubiquitination specifically recruits GCN5 for H3 acetylation

To decipher the underlying mechanism by which H3 ubiquitination regulates H3 acetylation, we determined whether H3 ubiquitination is crucial for the recruitment of chromatin remodeling complexes containing acetyltransferase activity. By analyzing microarray data sets against published ChIP-seq tracks for various chromatin binding factors(143), we identified GCN5, a histone acetyltransferase that preferentially catalyzes H3 K9 and K14 acetylation in mammalian cells, may be a potential candidate to mediate the unidirectional crosstalk between H3 ubiquitination and acetylation (Figure 14a). We then provided a series of experimental evidence to further confirm this notion. First, we found that the mRNA level of IL1α, IL1β and GCLM was reduced upon GCN5 knockdown (Figure 14b), similar to the effect of glucose deprivation, NEDD4 knockdown and H3 ubiquitination deficiency. Second, knockdown of GCN5 selectively impaired H3 acetylation on K9, K14, K27 and K56 (Figure 14c to 14e), but failed to affect H3 acetylation on other sites and H3 methylation, phenocopying the effect of NEDD4 knockdown.

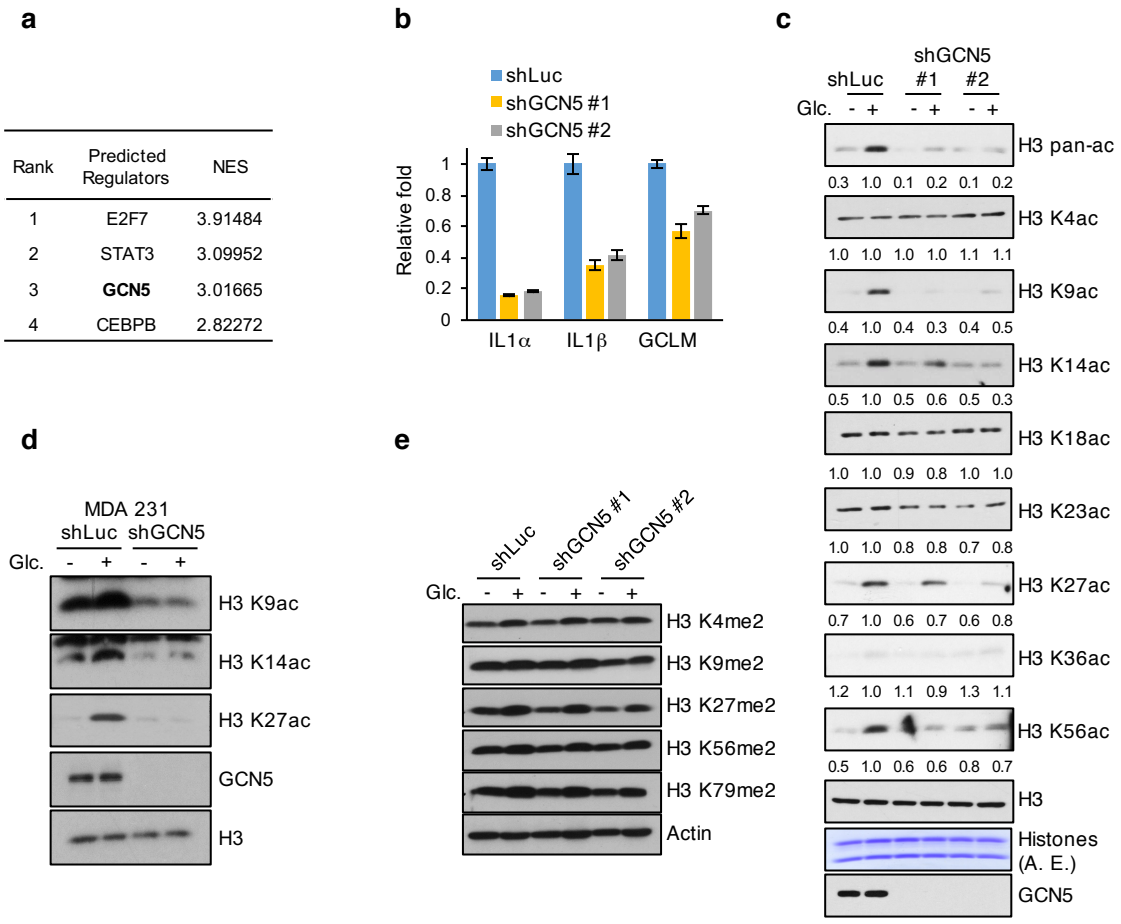

Figure 14 Gcn5 is the potential HAT regulated by Nedd4.

a. GCN5 is predicted as one of the potential regulator for NEDD4 target genes. Top 5000 up-regulated genes from microarray data sets in Fig. 4D were included to predict potential regulators by using iRegulon software. See experimental procedures for details.

b. GCN5 knockdown impaired IL1α, IL1β and GCLM expression. qPCR was performed to analyze the mRNA level in control and GCN5 knockdown cells (n=3, mean ± S.E.M.).

c. GCN5 knockdown abolished glucose-induced H3 K9, K14, K27 and K56 acetylation. Control and GCN5 knockdown Hep3B cells were glucose-starved for 4 hours and added-back glucose for 2 hours before whole cell extraction for Western blot analysis.

d. Control and GCN5 knockdown MDA 231 cells were treated with glucose and harvested for Western blot analysis.

e. GCN5 is not required for H3 di-methylation. Control and GCN5 knockdown Hep3B cells were glucose-starved for 4 hours and added-back glucose for 2 hours before whole cell extraction for Western blot analysis.

Third, we found that NEDD4 knockdown impaired the interaction between H3 and GCN5 by using the reciprocal immunoprecipitation assays (Figure 15a and 15b). Notably, GCN5 preferentially binds to mono-ubiquitinated H3 *in vivo*, indicating that H3 ubiquitination facilitates the recruitment of GCN5 to H3 (Figure 15a). Fourth, we demonstrated that glucose induced the interaction between H3 and GCN5 in a H3 ubiquitination-dependent manner (Figure 15c). Moreover, we showed that recombinant GCN5, when mixing with ubiquitin *in vitro*, could interact with ubiquitin efficiently (Figure 15e), demonstrating that H3 ubiquitination serves as a molecular scaffold to recruit GCN5 to H3. Finally, by sequential purification assay, we demonstrated that GCN5 and ubiquitinated H3 formed complex *in vivo* (Figure 15d), further supporting the interaction between GCN5 and ubiquitinated H3. To determine whether there is a putative ubiquitin binding motif on Gcn5, we have created different truncated Gcn5 mutants (Δ aa1-485, Δ aa485-650, Δ aa650-837) and purified them for in vitro binding assay by mixing with recombinant GST-ubiquitin proteins. Our data showed that Δ aa485-650 of Gcn5 did not bind to ubiquitin, while other mutants did bind to ubiquitin, suggesting that aa485-650 of Gcn5 is required for ubiquitin binding (Figure 15f and 15g). These data indicate that aa485-650 may contain a putative ubiquitin-binding region.

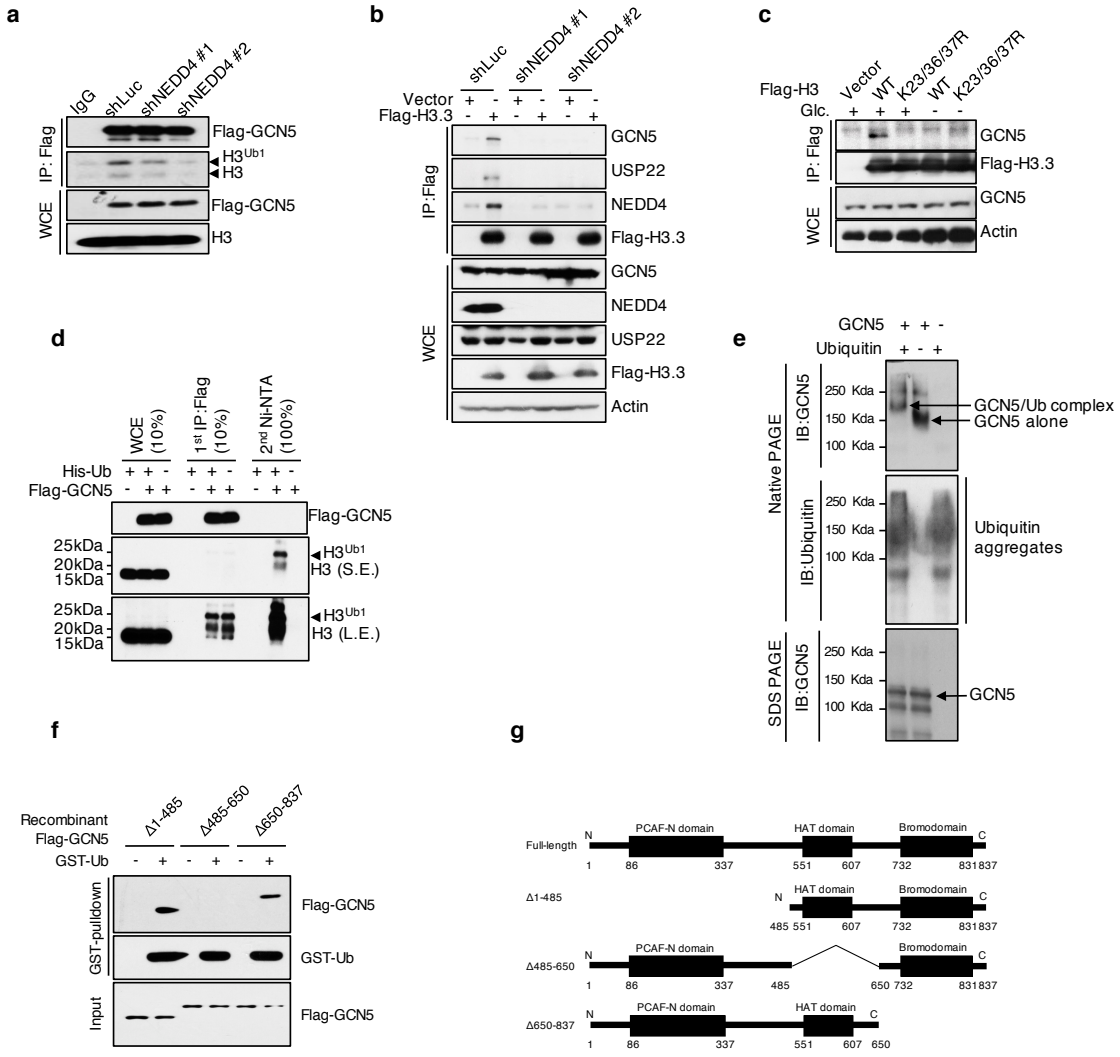

Figure 15 H3 ubiquitination is required for the recruitment of Gcn5.

a, b. NEDD4 knockdown impaired the interaction between GCN5 and H3.3. Transfected Flag-H3.3 or Flag-GCN5 in Hep3B cells was immunoprecipitated to analyze its co-immunoprecipitates by Western blot.

c. H3 ubiquitination deficiency impaired the interaction between GCN5 and H3.3. Hep3B cells were transfected with Flag-H3.3 WT or K23/36/37R for 36 hours, then glucose-starved for 4 hours and added-back glucose for 2 hours. Cells were then lysed for co-immunoprecipitation assay using anti-Flag antibody and subsequent Western blot analysis.

d. GCN5 and ubiquitinated H3 form complex *in vivo*. 293T cells were transfected with Flag-GCN5 and His-Ub as indicated. Briefly, sequential purification is done by first IP with Flag antibody from whole cell extracts in RIPA buffer. Immunoprecipitates were then released from antibody/beads by buffer A and followed by *in vivo* ubiquitination assay for endogenous H3.

e. GCN5 formed complex with ubiquitin *in vitro*. See experimental procedures for details.

f, g. Various truncated Flag-GCN5 were purified from mammalian cells for the *in vitro* binding assay with GST-Ub.

To comprehensively understand the dynamic recruitment of acetyltransferase by H3 ubiquitination, we also examined the recruitment of other known H3 acetyltransferases, p300 and PCAF, by co-immunoprecipitation assay. However, we found there is no obvious difference between the interaction of p300 or PCAF with WT H3 and H3 K23/36/37R mutant (Figure 16). In addition, we did not find that NEDD4 knockdown or H3.3 K23/36/37R mutation reduces H3.3 localization on the TSS of IL1A, IL1B and GCLM genes, which might affect H3 acetylation and gene transcription (Figure 17a to 17c). Also, GCN5 knockdown did not affect H3 ubiquitination (Figure 17d). Collectively, these results suggest that glucose-induced H3 ubiquitination by NEDD4 specifically regulates the recruitment of GCN5 to H3 for acetylation during transcription.

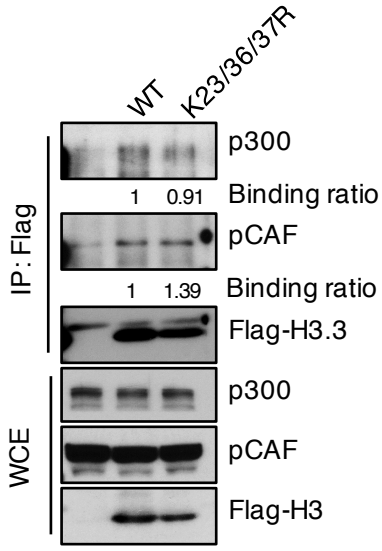

Figure 16 H3 ubiquitination on K23/36/37 sites is not required to recruit p300 or pCAF to histone H3.3.

Stably expressed Flag-H3.3 WT or K23/36/37R was immunoprecipitated from Hep3B cells to analyze its co-immunoprecipitates by Western blot.

Gene-specific regulation is usually achieved by the recognition of cis-regulatory elements on the genome, like glucose responsive elements by trans-regulatory elements, including glucose activated transcription factors and chromatin remodeling factors. To know whether NEDD4 conveys gene-specific effect for H3 acetylation, we determined whether NEDD4 resides in the TSS of its target genes. Chromatin fractionation and ChIP-seq assays revealed that NEDD4 does not display chromatin binding (data not shown). Since H3 ubiquitination is also enriched in nucleoplasm (Figure 17e), H3 ubiquitination by NEDD4 likely occurs outside of chromatin and gene specificity is likely not conveyed by the binding of NEDD4 to specific genomic locus. We also asked whether histone chaperones, which are involved in the nucleosome assembly in the nucleoplasm, regulate H3 ubiquitination and acetylation. Knockdown of HIRA, the major histone chaperone for H3.3, did not alter glucose induced H3 ubiquitination and H3 K9 acetylation (Figure 17f), ruling out the possibility that glucose induced H3 ubiquitination regulates H3 acetylation and gene transcription through affecting nucleosome assembly and histone deposition. Of note, disabling H3.3 deposition by HIRA knockdown did not affect glucose induced H3 K9 acetylation (Figure 17f), suggesting that glucose driven H3 acetylation may also occur mainly in the nucleoplasm. Moreover, NEDD4 knockdown did not affect the binding between H3.3 and HIRA (Figure 17g). These observations well support our model that NEDD4 ubiquitinates H3 in the nucleoplasm and in turn regulates H3 acetylation, as glucose induced H3 ubiquitination is upstream of glucose induced H3 acetylation occurred in the nucleoplasm.

Although NEDD4 ubiquitinates H3 in the nucleoplasm, we could detect H3 ubiquitination on chromatin and the localization of H3 ubiquitination at TSS of IL1A, IL1B and GCLM by sequential ChIP assay (Figure 17h), indicating that ubiquitinated H3 could be deposited into chromatin. Knockdown of NEDD4, which abolished H3 ubiquitination, also blocked glucose induced GCN5 recruitment to chromatin (Figure 17i), implying that H3 ubiquitination, once deposited into chromatin, may also regulate GCN5 recruitment for glucose induced H3 acetylation at chromatin level.

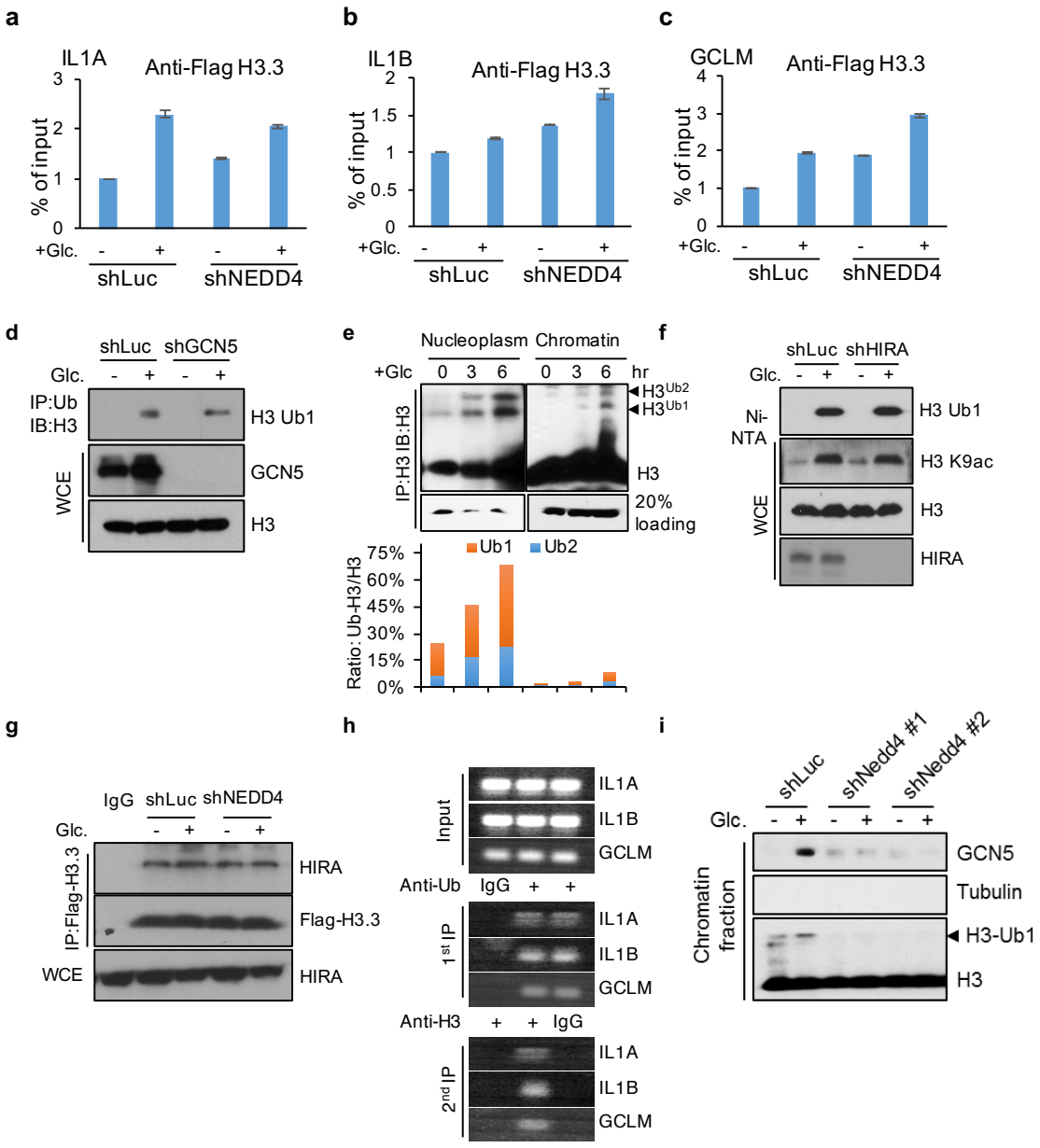

Figure 17 H3 ubiquitination occurs at nucleoplasm and does not affect the incorporation of H3.3.

a-c. ChIP-qPCR was performed for control and NEDD4 knockdown Hep3B cells.

d. GCN5 knockdown Hep3B cells were subjected to endogenous ubiquitination assay.

e. Immunoprecipitation assay was performed for the Hep3B cells with glucose treatment.

f. *In vivo* ubiquitination assay was performed for control and HIRA knockdown Hep3B cells treated with glucose.

g. Immunoprecipitation assay was performed for control and NEDD4 knockdown Hep3B cells.

h. Sequential ChIP assay was performed using Re-ChIP-IT→ kit (Active Motif, anti-H3 (Abcam, ab12079), anti-Ub (Thermo scientific, 10H4L21)).

i. Hep3B cells were treated with glucose for indicated times before chromatin fractionation.

3.6 Glucose induced H3 ubiquitination by NEDD4 is required for the tumor sphere formation and tumor engraftment

To understand the biological significance of glucose-induced H3 ubiquitination by NEDD4, we applied Gene Set Enrichment Analysis (GSEA) to discover enriched gene sets in microarray data sets. We found that knockdown of NEDD4 profoundly impaired multiple cancer-related pathways(136) (Figure 18a to 18c), indicating the potential role of NEDD4 in cancer(115). To evaluate the clinical relevance of this finding, we analyzed the TCGA exon expression data sets for NEDD4 and top-ranked genes in cancer-related pathways from microarray (Figure 19a). We found that in multiple cancer types their expressions were significantly ($p<0.05$) positively correlated (Figure 19b).

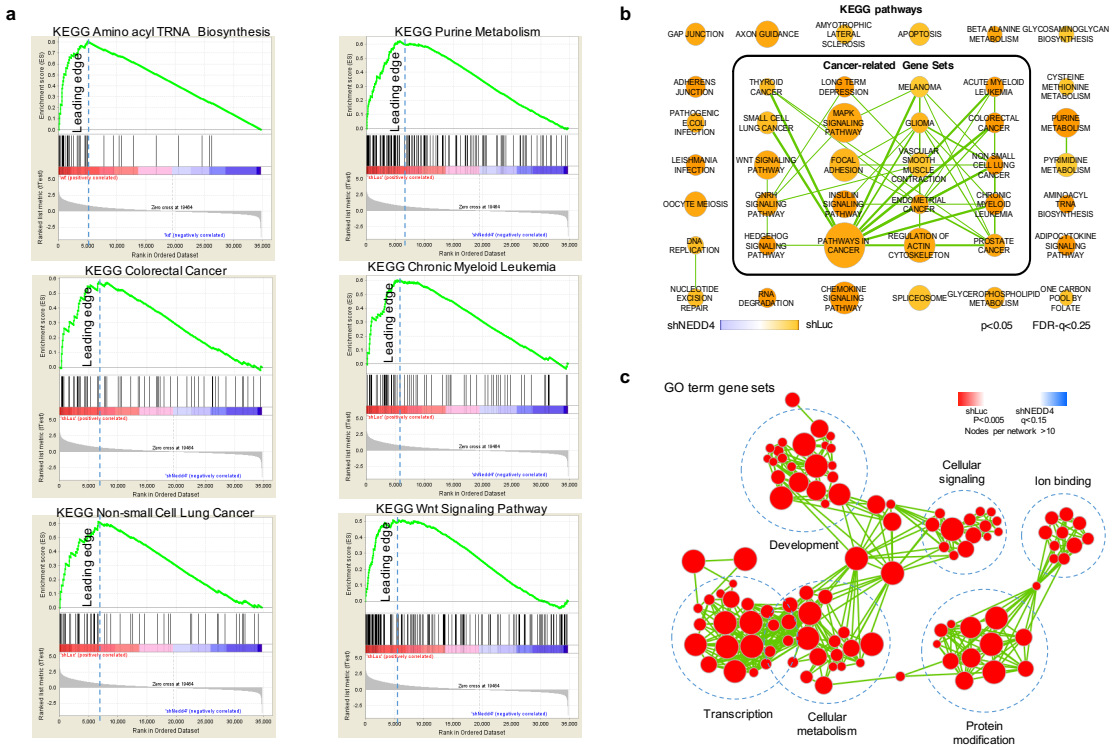

Figure 18 Nedd4 target genes are enriched for cancer pathways.

a. Multiple functional gene sets are enriched in control versus NEDD4 knockdown Hep3B cells.

b, c. Enrichment map view of gene set enrichment analysis results.

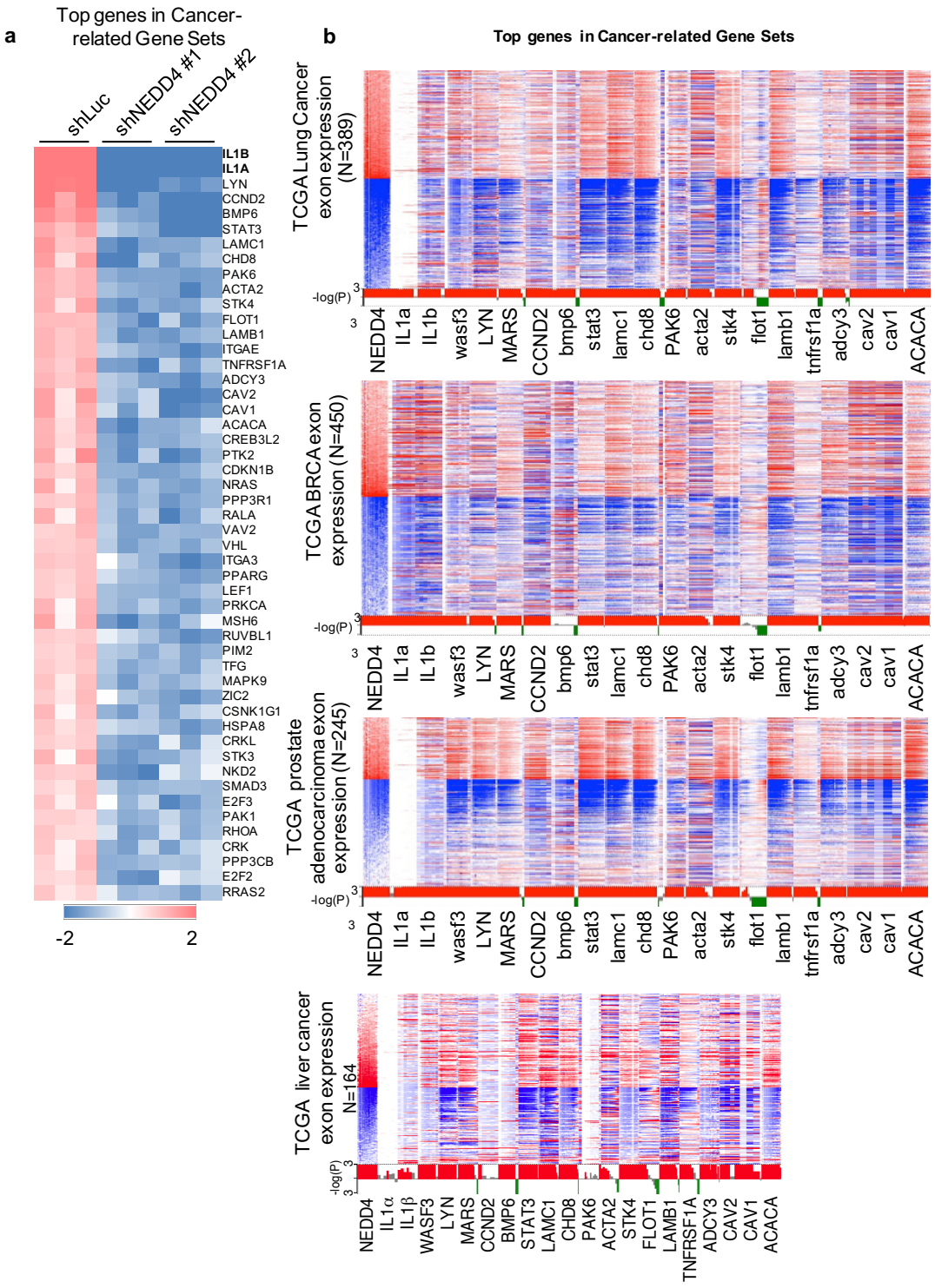
a
Top genes in Cancer-related Gene Sets
shLuc
shNEDD4 #1
shNEDD4 #2
IL1B
IL1A
LYN
CCND2
BMP6
STAT3
LAMC1
CHD8
PAK6
ACTA2
STK4
FLOT1
LAMB1
ITGAE
TNFRSF1A
ADCY3
CAV2
CAV1
ACACA
CREB3L2
PTK2
CDKN1B
NRAS
PPP3R1
RALA
VAV2
VHL
ITGA3
PPARG
LEF1
PRKCA
MSH6
RUVBL1
PIM2
TFG
MAPK9
ZIC2
CSNK1G1
HSPA8
CRKL
STK3
NKD2
SMAD3
E2F3
PAK1
RHOA
CRK
PPP3CB
E2F2
RRAS2
-2
2
b
Top genes in Cancer-related Gene Sets
TCGA Lung Cancer exon expression (N=389)
TCGA BRCA exon expression (N=450)
TCGA prostate adenocarcinoma exon expression (N=245)
TCGA liver cancer exon expression N=164
-log(P)
NEDD4
IL1a
IL1b
wasf3
LYN
MARS
CCND2
bmp6
stat3
lamc1
chd8
PAK6
acta2
stk4
flot1
lamb1
tnfrsf1a
adcy3
cav2
cav1
ACACA
NEDD4
IL1α
IL1β
WASF3
LYN
MARS
CCND2
BMP6
STAT3
LAMC1
CHD8
PAK6
ACTA2
STK4
FLOT1
LAMB1
TNFRSF1A
ADCY3
CAV2
CAV1
ACACA

Figure 19 Nedd4 target genes is correlated with Nedd4 in the TCGA data sets.

a. Heat map view of top gene list of cancer-related gene sets.

b. Heat map view of the TCGA exon expression profile for the NEDD4 and NEDD4 target genes from cancer-related gene sets. Patient samples ranked tops or bottoms 30% for NEDD4 expression were included and rank listed. Wilcoxon test was performed to evaluate the correlation of gene expression between NEDD4 and each gene. Significant ($p<0.01$) positive correlations were shown in red and negative correlations were show in green.

This observation led us to hypothesize that glucose-induced H3 ubiquitination by NEDD4 may have a critical role in cancer regulation. To test this hypothesis, we determined whether $Aldh^+$ population and sphere forming ability are affected by glucose-induced H3 ubiquitination by NEDD4. Notably, we found that *in vitro* tumor sphere numbers and $Aldh^+$ cell population were reduced upon glucose depletion, NEDD4 knockdown, NEDD4 tyrosine phosphorylation deficient mutation, GCN5 knockdown, H3.3 knockdown and deficiency of H3 ubiquitination (Figure 20a to 20p).

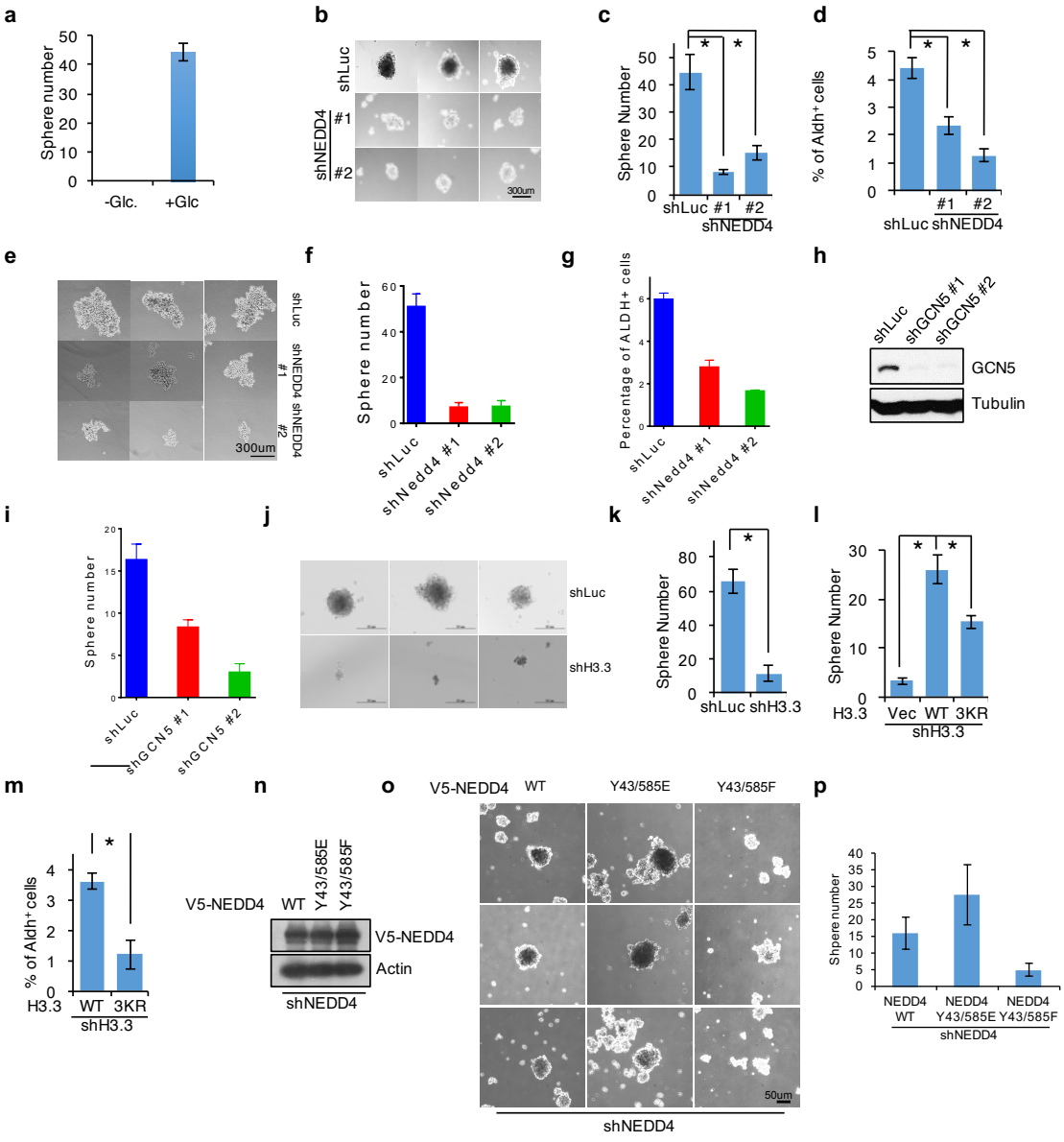

Figure 20 H3 ubiquitination is required for the maintenance of cancer stem cells.

a. Glucose deprivation abolished *in vitro* tumor sphere formation for Hep3B cells.

b-p. NEDD4, H3.3, Gcn5, H3 ubiquitination and Nedd4 phosphorylation are required for *in vitro* tumor sphere formation and maintaining $Aldh^+$ cell population. Cells were analyzed by *in vitro* tumor sphere forming assay (See experimental procedures for details). Data were presented as the mean number of three biological replicates ± S.E.M. Cells were stained for Aldh enzymatic activity and analyzed by flow cytometry. Data were presented as the mean percentage of three biological replicates ± S.E.M.

To further examine the *in vivo* function, 1000 $Aldh^+$ cells were subcutaneously injected into nude mice to evaluate the *in vivo* tumor engraftment frequency. We found that knockdown of NEDD4 or H3 K23/36/37R mutation reduced the tumor incidence and average tumor size, suggesting that NEDD4-mediated H3 ubiquitination is also required for the tumorigenecity of $Aldh^+$ cells *in vivo* (Figure 21a to 21h). Together, these studies reveal an important role of glucose-induced H3 ubiquitination by NEDD4 in cancer development.

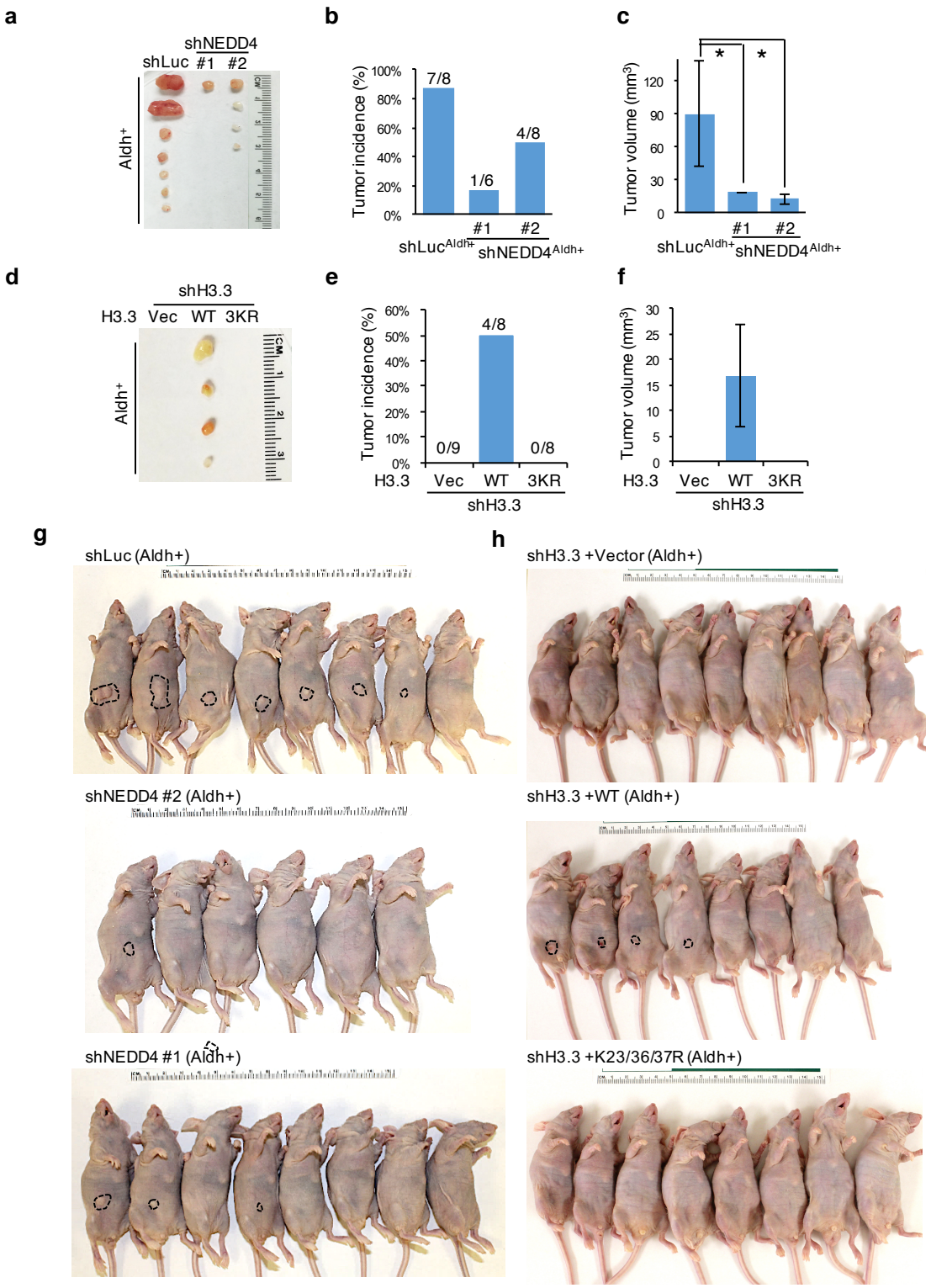
a
shNEDD4
shLuc #1 #2
Aldh+
b
Tumor incidence (%)
100%
80%
60%
40%
20%
0%
7/8
1/6
4/8
#1
#2
shLuc Aldh+
shNEDD4 Aldh+
c
Tumor volume (mm3)
120
90
60
30
0
*
*
#1
#2
shLuc Aldh+
shNEDD4 Aldh+
d
shH3.3
H3.3 Vec WT 3KR
Aldh+
e
Tumor incidence (%)
60%
50%
40%
30%
20%
10%
0%
0/9
4/8
0/8
H3.3 Vec WT 3KR
shH3.3
f
Tumor volume (mm3)
30
25
20
15
10
5
0
H3.3 Vec WT 3KR
shH3.3
g
shLuc (Aldh+)
shNEDD4 #2 (Aldh+)
shNEDD4 #1 (Aldh+)
h
shH3.3 +Vector (Aldh+)
shH3.3 +WT (Aldh+)
shH3.3 +K23/36/37R (Aldh+)

Figure 21 H3 ubiquitination is required for the in vivo engraftment of cancer stem cells.

a-c. NEDD4 knockdown reduced *in vivo* tumor engraftment frequency. Shown were tumor image, tumor incidence and tumor size, which was presented as the mean volume of tumors ((L x W x W)/2) ± S.E.M. See experimental procedures for details.

d-f. K23/36/37R mutation reduced in vivo tumor engraftment frequency. Shown were tumor image, tumor incidence and tumor size, which was presented as the mean volume of tumors ((L x W x W)/2) ± S.E.M. See experimental procedures for details.

g, h. Shown are the mice image of xenograft model.

3.7 IL1α/IL1β and reaction oxygen species (ROS) homeostasis are critical for tumor sphere formation

We next determined which set of target genes is involved in tumor sphere formation. As we have already identified IL1α, IL1β and GCLM as transcriptional targets (Fig. 4e and 4l), we investigated whether any of these genes may be involved in tumor sphere formation. While neutralizing extracellular IL1α and IL1β individually had little impact on tumor sphere formation *in vitro*, simultaneous sequestering of IL1α and IL1β resulted in a drastic reduction of tumor sphere numbers (Figure 22a and 22c), suggesting that IL1α and IL1β are required, but likely compensate each other, for maintaining tumor sphere formation ability. Interestingly, we also found that the treatment of IL1β could not fully rescue the defect in tumor sphere formation upon NEDD4 knockdown, despite that IL1β alone readily increased the tumor sphere numbers in control cells (Figure 22b and 22d), indicating that there are other important factors involved. As excess of ROS has deleterious impact on cancer cells(144), we rationalized that loss of GCLM, which is a key enzyme subunit in the biosynthesis of anti-oxidant glutathione (GSH), may lead to ROS elevation and subsequent inhibition of tumor sphere formation. In line with this assumption, we found that the knockdown of NEDD4 or deficiency of H3 ubiquitination, which impaired GCLM expression, increased cellular ROS level and decreased GSH level (Figure 22e to 22g). Treatment of NAC, a widely used ROS scavenger, not only increased tumor sphere number in control cells, but also partially rescued NEDD4

knockdown phenotype (Figure 22b and 22d). Remarkably, co-administration of NAC and IL1β completely rescued the defect in tumor sphere formation upon NEDD4 knockdown (Figure 22b and 22d), suggesting both IL1β and anti-oxidant are critical for tumor sphere formation. Together, these results reveal that IL1α, IL1β and GCLM are critical downstream mediators of H3 ubiquitination signaling in cancer regulation.

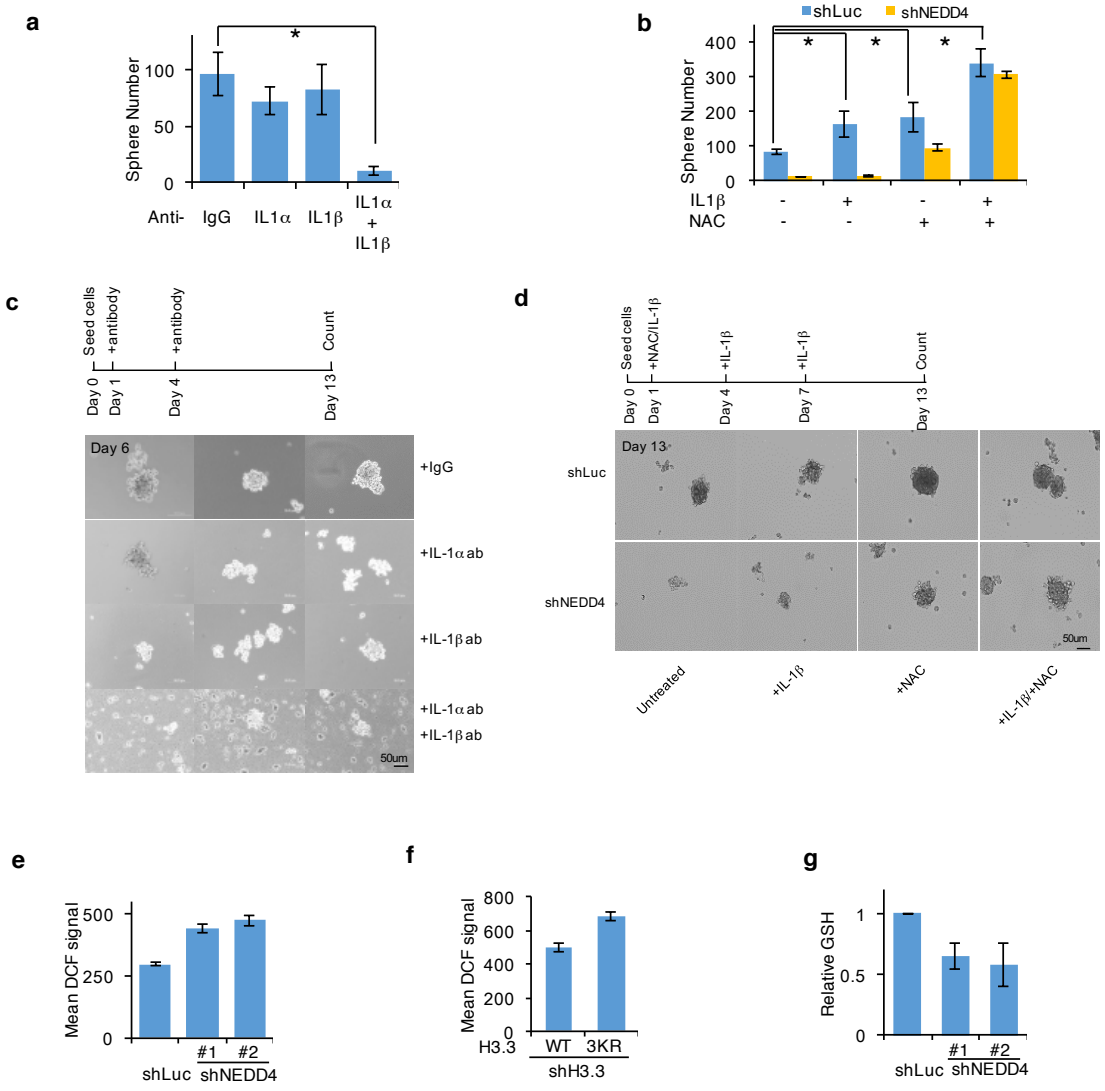

Figure 22 IL1A, IL1B and GCLM are important cancer stem cell factors.

a. Simultaneous neutralization of IL1α and IL1β abolished *in vitro* tumor sphere formation. Anti-IL1α (1:200) and anti-IL1β (1:200) neutralizing antibodies were added to medium on day 1 and day 4 after seeding Hep3B cells for *in vitro* tumor sphere-forming

assay. Data are presented as the mean number of three biological replicates ± S.E.M. See Fig. S7A and experimental procedures for details.

b. IL1β and NAC co-treatment rescued the defect of NEDD4 knockdown in the *in vitro* tumor sphere formation. Control and NEDD4 knockdown Hep3B cells were treated with recombinant IL1β and/or NAC (0.5mM) in the *in vitro* tumor sphere-forming assay. Data were presented as the mean number of three biological replicates ± S.E.M. See Fig. S7B and experimental procedures for details.

c, d. Treatment schemes and images of tumor sphere formation assay.

e, f. NEDD4 and H3 ubiquitination are required for the maintenance of cellular ROS. Control and NEDD4 knockdown or H3.3 WT or K23/36/37R restored Hep3B cells were stained by DCFDA for cellular ROS and subjected to flow cytometry analysis. Data were presented as the mean DCFDA signals of three biological replicates ± S.E.M.

g. GSH level is reduced in NEDD4 knockdown cells. Control and NEDD4 knockdown Hep3B cells were collected and GSH levels were measured by a colorimetric enzymatic reaction. Data were presented as the mean value of three biological replicates ± S.E.M.

Chapter 4 Discussion and Future Plans

Histone acetylation is generally a mark for active transcription. In eukaryote, glucose generally enhances H3 acetylation through the glucose downstream metabolite acetyl-CoA. However, it is unclear whether this metabolite-driven H3 acetylation is also under the control of glucose mediated cellular signaling. In this study, we uncover that glucose-induced H3 ubiquitination by NEDD4 specifically recruits GCN5 for H3 acetylation, which is required for the transcription of important tumor sphere forming factors (Figure 23). This discovery inspired us to study which downstream metabolites are required for the full activation of H3 acetylation induced by glucose. Notably, our data suggest that not only acetyl-CoA, but also H3 ubiquitination driven by glucose mediated ATP production and NEDD4 activation is required for H3 acetylation, extending our concepts on how glucose precisely induced H3 acetylation. Although we found that glucose induced H3 acetylation should mainly occur in the nucleoplasm, we surprisingly found that glucose-induced H3 acetylation is still enriched at TSS or enhancers by ChIP-seq and largely correlated with glucose induced gene expression pattern, suggesting that glucose may just amplify the existing basal level of acetylation at specific loci. Based on these observations, we speculate that the global induction of H3 acetylation by glucose shown in our western blot analysis may likely reflect the summation of gene/genomic locus specific decoration of histone H3 acetylation marks, which are first conjugated to H3 in the nucleoplasm and then deposited into chromatin at designated loci with the help of chromatin remodelers or histone chaperones.

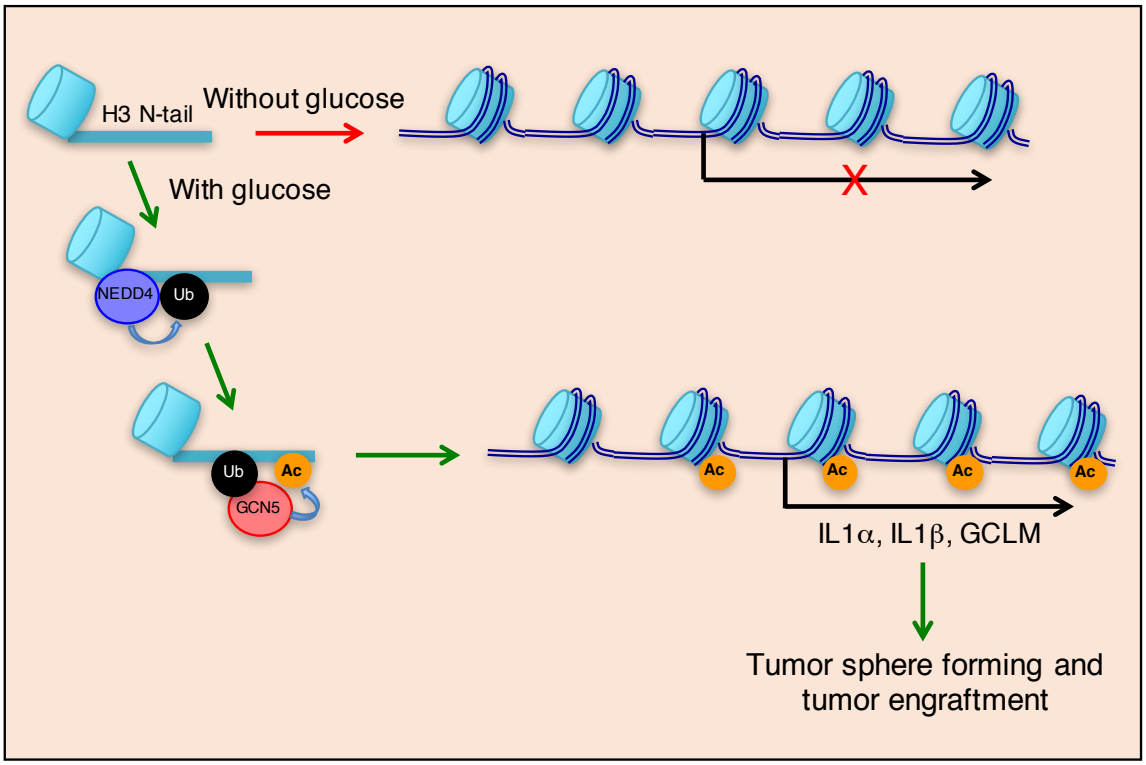

Figure 23 Schematic model.

Model of glucose-induced H3 mono-ubiquitination by NEDD4 and subsequent GCN5-mediated H3 acetylation, which regulates tumor sphere forming and tumor engraftment through transcription activation of genes, such as IL1α, IL1β and GCLM. N-tail represents histone H3 N-terminal tail.

NEDD4 in many occasions is identified as a cytosolic E3 ligase, which targets its protein substrates mostly for ubiquitination and proteasome degradation(115). Our study reveals for the first time the non-proteolytic role of NEDD4 in nucleus. We show that NEDD4 is presented in both cytoplasm and nucleus under normal conditions. Glucose treatment on glucose-starved cells triggers NEDD4 Y43/585 phosphorylation for its E3 ligase activation, providing an explanation as to why H3 ubiquitination is enhanced by glucose treatment. Moreover, we also identify that NEDD4 mono-ubiquitinates H3 at multiple sites, consistent with the capability of NEDD4 in triggering mono-ubiquitination on its substrates as IGF1R and IRS2(117, 145). We also found that NEDD4 did not display chromatin binding, which is consistent with the data indicating that glucose induced H3 acetylation may also occur mainly in the nucleoplasm. Although NEDD4 has been implicated in regulating AKT signaling(120, 121) and AKT signaling may also participate in the regulation of H3 acetylation(146), we did not find that AKT signaling is affected by NEDD4 knockdown in our glucose treatment and sphere forming experiments (Figure 24a and 24b). Interestingly, AKT phosphorylation is negatively correlated with the glucose action on H3 acetylation (Fig. S24a), which opposes the previous finding(146), revealing that the correlation between AKT phosphorylation and H3 acetylation could be altered under distinct stimuli(146). Thus, our finding showing NEDD4 regulates H3 acetylation and tumor sphere is unlikely through AKT signaling pathway. These results further extended the versatile role of NEDD4 in cellular signaling.

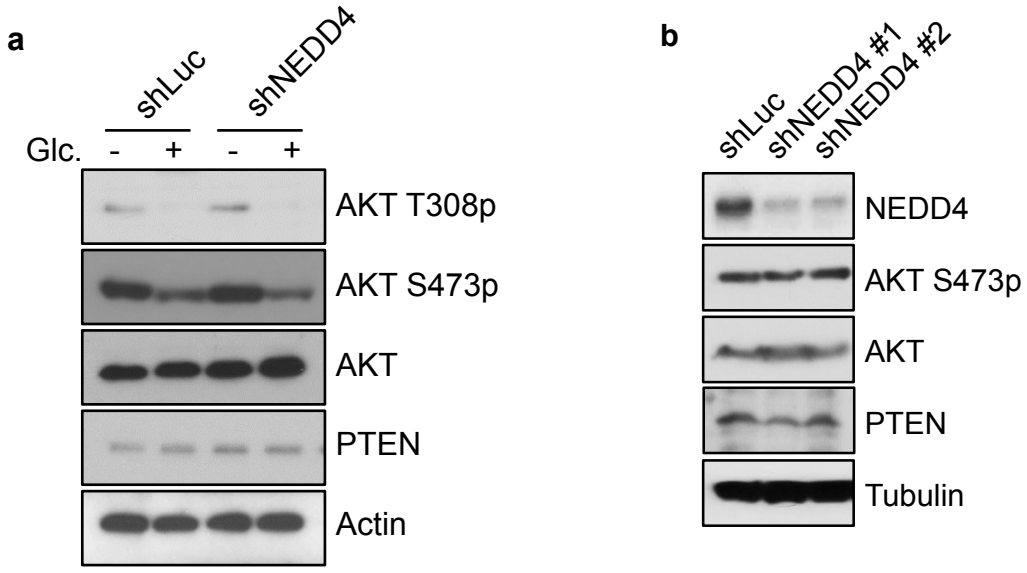

Figure 24 Akt is not involved in the glucose induced h3 acetylation by Nedd4.

a. Hep3B cells treated with or without glucose were analyzed by Western blot.

b. Cancer spheres from fig. 6c were analyzed by Western blot.

Many HATs, such as GCN5, PCAF, p300/CBP, and RTT109, are shown to catalyze H3 acetylation on the N-terminal tail(36, 37). Each of these HATs has its own preferred acetylation sites on H3. Although histone acetylation by HATs generally promotes transcriptional activation, it is unclear how those HATs are differentially utilized and how differential H3 acetylation patterns are established upon various stimulations. In this study, we found that under glucose stimulation conditions GCN5 specifically acetylates K9, K14 and K27 on H3. More importantly, H3 ubiquitination by NEDD4 specifically recruits GCN5, but not PCAF and p300 for H3 acetylation upon glucose stimulation. Of note, H3 acetylation on K23 and K36, which are H3 ubiquitination sites, are not triggered by glucose, supporting our model that these lysine sites are reserved for H3 ubiquitination under glucose stimulation condition to convey specificity for HAT recruitment. While it is thus far unclear why cells prefer to utilize GCN5 or its H3 acetylation sites for glucose-induced gene transcription activation, warranting for further explorations for HAT specificity under various stresses and/or stimuli, our findings collectively provide great insights into the glucose-induced histone acetylation.

Tumor sphere can be formed by a subgroup of cancer cells that possesses normal stem cells like characteristic and likely responsible for tumor initiation, drug resistance, relapse and metastasis(72, 73, 97-99). These traits are generally originated from abnormal genetic or epigenetic alteration, including histone modification. Our study provides evidences that glucose-induced H3 ubiquitination as well as other components in this

pathway, namely NEDD4, H3.3 and GCN5, play critical roles in maintaining such population of cancer cells. Moreover, by ChIP-seq and microarray analysis we found that IL1α, IL1β and GCLM are target genes of glucose-induced H3 ubiquitination. Notably, we experimentally demonstrate that maintenance of tumor sphere forming cells requires both IL1α/IL1β and cellular ROS homeostasis. Thus in the glucose context, our results indicate that increased glucose demand in cancer cells is a prerequisite not only for producing more anti-oxidants, energy and cellular building blocks, but also for activating important cellular signaling to maintain H3 ubiquitination and acetylation, thereby driving transcription activation for crucial tumor sphere forming factors. Our findings further emphasize the important role of glucose in tumor sphere forming cells and provide a renovated view for glucose in the maintenance of tumor sphere forming cells.

In summary, our study established a new role of glucose in transcriptome reprogramming, tumor sphere forming and tumor engraftment through NEDD4-dependent H3 ubiquitination and subsequent GCN5-mediated H3 acetylation (Figure 23). Hence, targeting NEDD4 and GCN5 or their transcriptional targets such as IL1α, IL1β and GCLM may be promising approaches for cancer therapy.

Since there are still some unsolved mysteries left in my study, I'd like to point out the future direction. NEDD4 deficiency affects H3 acetylation and transcription of selected genes, but NEDD4 does not bind to chromatin. Thus the chromatin localized H3 ubiquitination may convey the gene specificity of NEDD4. Of note, sequential ChIP of ubiquitinated H3 shown its localization at the TSS of IL1A, IL1B and GCLM, indicating

that H3 ubiquitination may be localized at glucose target genes. However, to provide convincing evidence, it is better to generate specific antibody for H3 ubiquitination at either K23, 36 or 37 site. Using this antibody, we could perform ChIP-seq analysis to examine the genome wide localization of H3 ubiquitination and examine whether it is induced by glucose and colocalized with H3 acetylation at specific genes on chromatin. If this is the case, it remains to be answered that how H3 ubiquitination is deposited into chromatin in a gene specific manner. This might be achieved by transcription factor/histone chaperone coupled deposition of H3.3. To identify this mechanism, there might be two screening methods. First, we could use bioinformatics to search for the potential transcription factors that have similar binding genes with glucose induced H3 ubiquitination and/or H3 acetylation. Secondly, we could pull down ubiquitinated H3 using specific antibody that we generated and identify its binding proteins, which may be potential chaperones or transcription factors that mediate the gene specific deposition. Since LncRNA is reported to mediated gene specific regulation of chromatin factors, we should also test whether LncRNA may regulate gene specific deposition of H3 ubiquitination and provide molecular adapters for ubiquitin recognition. Together, this may provide more insights into how glucose induces H3 acetylation at specific loci and answer the major remaining question left in my thesis project.

Another important question is that how exactly glucose induces Nedd4 activation, which may be through tyrosine phosphorylation of Nedd4. Since pyruvate or ATP is not responsible for this, we would like to test which downstream metabolites of glucose or

glucose per se is important for the activation of Nedd4. There might be several possibilities. It has been reported that glucose elicited signaling from hexokinase when binding to it (147), the pathway of which is still not been identified and may convey kinase activity towards Nedd4. It would be also interesting to test whether glucose directly binds to any other proteins to activate Nedd4 E3 ligase activity, which is an interesting area to explore, since the direct sensing mechanism of glucose is not well understood in the human cells. As a well-established method in the plant biology and microbiology, we could screen glucose binding proteins by sepharose beads, the free saccharide ends of which are the immobilized glucose molecules. It is also possible that Nedd4 activation is mediated by the downstream metabolite of glucose that is upstream of pyruvate. However, those metabolites are mostly not permeable to plasma membrane, and could not be simply tested by treating the cells in the medium. Thus developing a membrane free *in vitro* system to test H3 acetylation is important for testing which metabolite is critical. In addition, since the E3 ligase activity of Nedd4 Y43/585E could be further enhanced by glucose, it is also possible that other mechanisms are involved in the glucose induced Nedd4 activation. Nedd4 is known to be activated by cellular calcium, which opens up the auto inhibitory formation of Nedd4. It is also implicated that glucose could increase calcium level in the pancreatic β-cells, thus a universal mechanism whether glucose increases calcium level in cancer cells should be determined and how glucose elevates cellular calcium level should be further identified.

In the future, it would be also interesting to systematically study how glucose induced H3 acetylation is regulated. CRISPR/Cas9 is a new technology that enabled the researchers to knockout genes in almost any given cell lines. The CRISPR knockout library covering all known human genes represents a powerful tool for functional screening, since its capability to suppress gene expression is many fold higher than traditional shRNA. By coupling FACS and next generation sequencing (NGS), we could first sort the cells with decreased H3 acetylation signal induced by glucose in the pooled library infected cells and then distinguish the enriched knockout Cas9 clones with NGS. Candidates from this screening would provide global information about how H3 acetylation is regulated by the input from any other cellular signaling.

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