A novel role for GSK3β as a modulator of Drosha microprocessor activity and MicroRNA biogenesis

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Received September 17, 2015; Revised September 13, 2016; Editorial Decision October 04, 2016; Accepted October 19, 2016

ABSTRACT

Regulation of microRNA (miR) biogenesis is complex and stringently controlled. Here, we identify the kinase GSK3β as an important modulator of miR biogenesis at Microprocessor level. Repression of GSK3β activity reduces Drosha activity toward pri-miRs, leading to accumulation of unprocessed pri-miRs and reduction of pre-miRs and mature miRs without altering levels or cellular localisation of miRs and reduction of pre-miRs and mature miRs, leading to accumulation of unprocessed pri-

INTRODUCTION

MicroRNAs, first identified in 1993, are 18–22 nucleotide non-coding RNAs. The accepted dogma is that they negatively regulate gene expression through association with complementary sequences within target gene 3′ UTRs, leading to transcript degradation and/or translational inhibition (1,2). A single transcript can be targeted by hundreds of miRs, and individual miRs can target hundreds of genes, hence the regulatory activity of miRs is being increasingly accepted as a complex network of tissue- and disease-specific interactions (3,4). MiRs are transcribed by RNA polymerase II, generating a primary microRNA transcript (pri-miR), which is then 5′ capped and adenyalted (5). The majority of pri-miRs are polycistrionic and generate several functional mature miRs. The pri-miR is cleaved into one or more ~70 nt hairpin-structured precursor miRs (pre-miRs), by the Drosha-containing Microprocessor (MP) complex (6). Drosha, an RNase III enzyme, is stabilised by association with double-stranded RNA binding domain protein DiGeorge Critical Region 8 (DGCR8)/Partner of Drosha (Pasha) (7). Other cofactors such as p72, p68, FUS and hnRNPA1 modulate fidelity, efficiency and specificity of cleavage or act as scaffold proteins to aid complex formation (8). Some cofactors alter biogenesis of the entire miRNAome, others demonstrate activity against a defined miR subset. Thus, the MP is very large multi-protein complex (>650 kD in human cells (9)) containing at least 20 different polypeptides. Drosha cleavage generates a 2 nt 3′ overhang, vital both for recognition by Exportin-5, which facilitates Ran-GTP-dependent export of the pre-miR to the cytoplasm, and for cleavage of the stem-loop by a second RNase III enzyme, Dicer (10,11). Optimal Dicer activity requires the accessory dsRBD protein TRBP/PACT, and yields a ~22 nt miR duplex. The two strands separate and one strand associates with Argonaute-2 (AGO2), a protein component of the RNA-induced silencing complex (RISC). The mature miR guides RISC to complementary sequences within the 3′ UTR of target mRNAs, resulting in translational repression and/or transcript degradation.

MiR biogenesis is emerging as a stringently controlled and remarkably complex pathway, about which much remains to be learnt. Coordinated regulation, including...
feedback from miR targets, likely serves to prevent mis-expression of miRs both spatially and temporally, safeguarding sophisticated transcriptional processes. MiR processing is thought to be particularly important in development and tumourigenesis. For example, in early development many pri-miRs are expressed but not efficiently converted into their mature forms (12). Equally, reduced processing has been shown to contribute to widespread down-regulation of many miRs in human cancers (11,13,14).

Glycogen synthase kinase 3β (GSK3β) is a serine/threonine protein kinase, initially identified as a regulator of glycogen metabolism, that has been shown to perform vital roles in a number of essential cellular signalling pathways, including Wnt/β-catenin, Hedgehog, Notch and Insulin signalling (15). It plays a key role in signal transduction during processes such as cell cycle progression, proliferation and inflammation. GSK3β phosphorolyses diverse target proteins, and is itself regulated by phosphorylation. Its activity is decreased by Ser7 phosphorylation, mediated by the serine/threonine kinase Akt (a component of the PI3K/MAPK signalling pathway). MAPK-activated protein kinase-1 or p70 ribosomal S6 kinase-1. In contrast, phosphorylation at Tyrβ results in activation of GSK3β and is believed to be vital for signal transduction in resting cells (16). It has been established that more than 40 proteins are substrates for GSK3β (17), including cyclin D1 (18) and the transcription factors AP-1, NFκB, c-Jun, GR and Notch (19–21), permitting highly sensitive regulation of cell cycle progression in response to extracellular stimuli.

GSK3β initially gained prominence as a drug target in treatment of diabetes mellitus and obesity (22,23). It also plays important roles in signal transduction in several key neurotransmitter pathways so is linked to mood disorders, bipolar disorder, depression and schizophrenia, and the GSK3β inhibitors lithium and valproate are currently used to treat such conditions (24,25). In cancer, GSK3β has been implicated in development and progression of breast (26), brain (27), pancreatic (28), colon (29) and prostate tumours (30), although it displays disparate activity in differing tissues and tumour types. In breast cancer cells, activation of GSK3β by rapamycin induces downregulation of cyclin D1, cell cycle arrest and inhibition of anchorage-dependent growth (26). GSK3β may also play a role in preventing epithelial to mesenchymal transition (EMT) in tumourigenesis, as its inhibition promotes EMT in cultured epithelial cells (31). In contrast, GSK3β overexpression has been observed in ovarian, colon and pancreatic tumours (21) resulting in enhanced proliferation and survival of ovarian cancer cells in vivo and in vitro (32). Additionally, GSK3β inhibition suppressed ovarian cancer cell proliferation in vitro (32) and decreased growth and survival of colon cancer cells in vivo (29).

It was recently shown that inhibition of GSK3β using small molecule inhibitors decreases levels of the majority of mature miRs in mouse embryonic stem cells, the data suggesting a reduction in nuclear Drosha levels in such cells may be responsible (33). It has also been reported that GSK3β phosphorolyses Drosha at residues S300 and S302 (34). Impacts of such modifications, and of GSK3β inhibition, on Drosha’s essential ribonuclease activity and miR biogenesis were, until now, unknown. The data presented here are the first to describe a mechanism for GSK3β regulation of miR biogenesis as a regulatory component of the MP. We demonstrate that GSK3β enhances Drosha association with its cofactors, DGCR8 and p72 and increases Drosha:pr-miR binding to enhance pri-miR cleavage. This is achieved through direct binding of GSK3β to DGCR8 and p72 within the Microprocessor in an RNA-dependent manner. In addition, we have shown that GSK3β-mediated phosphorylation of Drosha at S300 and S302 increases miR biogenesis not through altered Drosha localisation, but by enhancing Drosha:DGCR8 interaction. We hypothesise that GSK3β constitutes a ‘missing link’ between essential mitogenic signalling pathways and miR biogenesis.

**MATERIALS AND METHODS**

**Mammalian cell culture**

Cells were maintained at 37°C in 5% CO₂. HeLa, HEK293T and COS-1 cells were maintained in Dulbecco’s Modified Eagle’s Medium (Sigma). LNCaP and PC3 cells were maintained and passaged in RPMI-1640 (Sigma). All media supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Sigma).

**Cell lysis, Western blotting and antibodies**

Cells were lysed and protein extracted as described (35). Proteins were resolved by 8–12% SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose membrane (Bio-Rad). After blocking (5% non-fat dried milk powder in 0.05% Tween-20 in 1xPBS, or 5% BSA in TBST for phospho-proteins) for 40 min, membranes were incubated with rabbit anti-Drosha pAb (Sigma, SAB4200151), mouse anti-GSK3β mAb (Abcam, ab93926), rat anti-AGO2 Ab (gift from Geok Tan, Imperial College London), mouse anti-FUS mAb (SantaCruz, sc-47711), mouse anti-Flag epitope tag M2 mAb (Sigma, F1804), rabbit anti-DGCR8 pAb (Sigma, SAB4200089), rabbit anti-DGCR8 pAb (Abcam ab36865), mouse anti-HA mAb (Covance, 16B12), mouse anti-phospho-serine (Sigma P5747), rabbit anti-PTEN pAb (R+D systems, AF847), rabbit anti-FOXO1 mAb (Abcam, ab52857), rabbit anti-ZEB1 mAb (Cell Signalling, 3396), mouse anti-β-tubulin mAb (Sigma, T4126) or mouse anti-β-actin mAb (Abcam, ab6276) for 1 h and visualised using goat anti-mouse, goat anti-rabbit or goat anti-rat IgG-HRP as appropriate. Detection was by Luminata Forte HRP substrate (Millipore).

**Plasmid stocks**

pCK-Flag-Drosha construct was a kind gift from Prof. V. Narry Kim, Seoul University. pCK-Flag-Drosha-S300A,S302A and pCK-Flag-Drosha-S300,E302D plasmids were generated by site-directed mutagenesis of the pCK-Flag-Drosha vector using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene). pMT23-c-Myc-GSK3β-S/A and pMT23-HA-GSK3β-K5/R plasmids were generously gifted by Dr Robert Kypta, Imperial College London. pMT23-HA-GSK3β (wild-type)
and pMT23-HA-GSK3β-S9A plasmids were generated by site-directed mutagenesis of the pMT23-HA-GSK3β-K85R vector as above. CMV-pri-miR-23a27a24-2-GL4.18 Drosha activity reporter vector was constructed as previously described (36). pGEMT Easy-pri-miR-23a27a24-2 was generated by insertion of the genomic miR-23a27a24-2 sequence into the multiple cloning site. pMiRTarget-ACLY 3′ UTR was a kind gift of Dr Hector Keun (Imperial College London), generated by insertion of the full-length ACLY 3′ UTR 3′ of the luciferase gene in the pMiRTarget vector. pMiRTarget-ACLY 3′ UTR miR-27a binding site (BS) mutant was generated by G→U point mutation of the pMiRTarget-ACLY 3′ UTR vector at nucleotide 700 of the ACLY 3′ UTR. This nucleotide is located in the centre of the miR-27a seed region binding site (Supplementary Figure S9).

**Drosha cleavage activity reporter luciferase assays**

The CMV-pri-miR-23a27a24-2-GL4.18 Drosha activity reporter vector was cotransfected into Cos-1 or HEK293T cells alongside pMT23-HA-GSK3β-S9A or pMT23-HA-GSK3β-K85R mutant GSK3β expression vectors, or pCK-Flag-Drosha or pCK-Flag-Drosha-S300E,S302D Drosha expression vectors as appropriate using the calcium phosphate method (37). Twenty-four hours post-transfection, cells were treated with the GSK3 inhibitor, 99021, if required and harvested after a further 24 h. Luciferase assays were performed using the Lucite assay (Packard, USA) and activity normalised for transfection efficiency using the Galacton kit (Tropix) as previously described (38).

**MiR, pre-miR, pri-miR and mRNA quantitative real-time PCR**

Matute miR expression was quantified by quantitative real-time RT-PCR using TaqMan microRNA assays and TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocol. For detection of pri-miRs, Drosha coding region and ACLY, ZEB1, PTEN or FOXO1 3′UTRs, cDNA was prepared from 500 ng total RNA using Precision qScript Reverse Transcription kit (PrimerDesign) and oligo d(T) primers. cDNAs were amplified using either (i) 2x Fast SYBR Green Master Mix (Applied Biosystems) and 250 nM forward and reverse primers (see Supplementary Table S1), or (ii) TaqMan pri-miR assays (Applied Biosystems) for pri-miR-23a27a24-2, pri-miR-141/200c and pri-miR-182 and 2x Taqman Fast Universal PCR Master Mix No AmpErase UNG as per manufacturer’s instructions. For detection of pre-miRs, small RNAs (<200 nt) were isolated using the miRVana miRNA Isolation Kit (Ambion). A total of 500 ng RNA was reverse transcribed using PrimerDesign (PrimerDesign) and random nonamers. cDNAs were amplified using 2x Fast SYBR Green Master Mix (Applied Biosystems) and 250 nM forward and reverse primers to pre-miR-27a (see Supplementary Table S1). All data were analysed using the ΔΔCt method, with U18 and L19 as endogenous references for miR and mRNA/pre-miR/pri-miR levels respectively, using DMSO/ethanol-treated samples as calibrators where appropriate.

**RNA-immunoprecipitation**

Full protocol is described in Supplementary Methods. Briefly, lysates were generated from HEK293T cells expressing exogenous GSK3β mutants and Flag-Drosha and incubated with anti-Flag affinity gel (Sigma) overnight at 4°C with rotation. Beads were washed, reconstituted with DNase solution (Qiagen) and treated with proteinase K. RNA was then extracted from beads using Trizol LS (Life Technologies) according to manufacturer’s instructions and qRT-PCR performed for pri-miRs.

**Immunofluorescent cell staining**

Cos-1, PC3 or LNCaP cells on coverslips were fixed with 1% formaldehyde in PBS at room temperature for 10 min, washed in PBS and blocked with 10% goat serum in PBS for 1 h. Primary antibodies (Rb anti-Drosha, Rb anti-DGCR8, Ms anti-HA and Ms anti-GSK3β) were diluted 1/100-1/200 in 10% goat serum and added to cells for 1 h at room temperature, as appropriate. Following washing with PBS, secondary antibodies (Alexa Fluor 488 Goat anti-Mouse and 594 Goat anti-Rabbit SFX kits, Invitrogen) were diluted 1/200 in 10% goat serum and added to cells for 1 h at room temperature in the dark. Cells were washed briefly in PBS and coverslips were mounted onto glass slides using DAPI-containing Vectashield mounting solution. Staining was visualised using a Zeiss LSM510 confocal microscope.

**Flag-Drosha subcellular fractionation**

Subcellular fractionation was performed as described (39) to isolate cytoplasmic, soluble nuclear and chromatin-bound protein fractions. See Supplementary Methods.

**Immunoprecipitation**

Immunoprecipitation was performed as described in the Supplementary Methods. Briefly, lysates were generated from HEK293T cells expressing exogenous pMT23-GSK3β-WT/ S9A/ K85R/ K85A,K86A and/or Flag-Drosha-WT/S300A,S302A/S300E,S302D as appropriate, pre-cleared and incubated with anti-Flag M2 or EZView anti-HA agarose beads (Sigma) as appropriate at 4°C overnight with rotation. Beads were washed in TBS, followed by RNase A treatment (200 μg/ml) for 15 min at 4°C, as appropriate. Beads were boiled in IP sample loading buffer, pelleted and supernatant subjected to Western blotting. For immunoprecipitation of endogenous proteins, lysates were generated from 99021-treated HEK293T cells and incubated with rabbit anti-Drosha (Cell Signalling, 3364) or mouse anti-GSK3β (Abcam, ab93926) antibody-bound Protein G Dynabeads (ThermoFisher) or anti-phosphoserine-agarose (Sigma-Aldrich, A8076) as appropriate at 4°C overnight with rotation. Beads were washed x3 with Wash Buffer (Dynabeads® Protein G Immunoprecipitation Kit, ThermoFisher), eluted as above and subjected to Western blotting.

**In vitro pri-miR processing assay**

In vitro processing assays were performed as described (40) with modifications. Briefly, pGEMT Easy vector contain-
ing 652 bp pri-miR-23a27a24-2 sequence was linearised at a Sall restriction site ∼30 bp 3’ of the end of the pri-miR sequence. The pri-miR was in vitro transcribed from the T7 promoter using MEGAscript T7 in vitro transcription kit (Ambion) and 0.75 μl of α-32P-UTP (40 μCi/μl, 800 mCi/mmmole, Perkin Elmer NEG007C001MC), followed by phenol/chloroform extraction. Precipitated RNA was resolved on a 6% acrylamide:urea gel, which was exposed to film. α-32P-UTP-labelled pri-miR-23a27a24-2 was cut from the gel and eluted in 0.3 M sodium acetate (pH 5.5), 2% SDS. In vitro processing was performed by incubation (37°C, 90 min) of α-32P-UTP-labelled pri-miR-23a27a24-2 with Flag-Drosha immunoprecipitated from HEK293T cells transfected with pCK-Flag-Drosha ± pMT23-HA-GSK3β-WT/S9A/K85R for 48 h. RNA was phenol/chloroform-extracted and separated on 6% and 12.5% acrylamide:urea gels alongside Decades RNA Markers (ThermoFisher), which were prepared according to the manufacturer’s protocol. The gel was exposed to film overnight at −80°C in a cassette with intensifying screen.

In vitro kinase assay

Twenty five amino acid peptides were synthesised corresponding to WT Drosha 289–313 (RERHRD-NRRPSLSERYKKEYKR) or S190A,S302A Drosha 289–313 (RERHRDNNRRAPALERSYKKEYKR), containing predicted GSK3β phosphorylation sites. Twenty five micrograms of the above Drosha peptides were incubated with 150 ng GSK3β, 10 μM cold ATP and 5 μl (γ-32P)-ATP (10 Ci/mmol, 2 mM/μl, Perkin Elmer) in 50 μl kinase assay buffer (60 mM HEPES-NaOH pH7.5, 3 mM MgCl2, 3 mM MnCl2, 3 μM sodium orthovanadate, 1.2 mM DTT, 0.05 μg/μl PEG20,000) for 1 h at 30°C. Reactions were terminated by incubation at 65°C for 20 min. A total of 10 μl of each reaction was dotted onto nitrocellulose membrane, left to dry for 2–3 min, washed x3 with TBST and exposed to X-ray film.

ACLY 3’UTR luciferase reporter assay

pMiRTarget-ACLY 3’UTR WT or pMiRTarget-ACLY 3’UTR miR-27a binding site (BS) mutant were transfected into HEK293T cells alongside the pdmLacZ β-galactosidase reporter plasmid using the calcium phosphate method (37). Twenty-fours hours post-transfection, cells were treated with 99021 (2 μM) and harvested after a further 24 h. Luciferase assays were performed using the Luclite assay (Packard, USA) and activity normalised for transfection efficiency using the Galacton kit (Tropix) as previously described (38).

Statistical analysis

Normally distributed continuous variables were assessed by Student’s t-test. Strength of correlation between two normally distributed continuous variables was assessed by Pearson’s correlation coefficient (r). P ≤ 0.05 was interpreted to denote statistical significance.

RESULTS

Inhibition of GSK3β reduces MiR biogenesis through inhibition of Drosha activity and repression of pri-MiR processing

As GSK3β interacts with and modulates the localisation of Drosha (34,41), we hypothesised that GSK3β expression and/or activity may alter pri-miR processing, and ultimately mature miR levels. To address this, a number of cell lines were treated with the highly potent and specific GSK3β inhibitor, 6-(2-(4-(2,4-Dichloro-phenyl)-5-(4-methyl-1H-imidazol-2-yl)-pyrimidin-2-ylamino)-ethylamino)-nicotinonitrile (known as CT 99021 - CHIR 99021 and referred to as 99021 throughout) (22), or transfected with a dominant-negative form of GSK3β, GSK3β-K85R (42,43) to control for possible off-target effects of 99021. GSK3β-K85R transfection reduced levels of mature miR-27a, −23a, −141 and −182 by up to 70% in HEK293T cells (Figure 1A). The same effect was observed in LNCaP prostate cancer cells following GSK3β-K85R transfection (Supplementary Figure S1A), and was corroborated by a 50% reduction in levels of the same mature miRs following 99021 treatment of LNCaP cells (Supplementary Figure S1B). Further, levels of corresponding pri-miRs were increased by up to 4-fold following GSK3β-K85R transfection or 99021 treatment of HEK293T cells (Figure 1B and C); significantly increased pri-miR levels were also observed following GSK3β-K85R transfection of HeLa cells (Supplementary Figure S1C).

As it has been previously demonstrated that GSK3β can phosphorylate Drosha and alter its subcellular localisation (34,41), it was possible that the effects of GSK3β inhibition on miR maturation may simply be attributable to altered Drosha localisation. However, 99021 treatment of LNCaP cells followed by cell fractionation showed no alteration in nuclear Drosha protein levels (Figure 1D), refuting this hypothesis. Additionally, decreased miR levels are not attributable to altered protein levels of MP or RISC components, since transfection of GSK3β-K85R did not alter protein levels of Drosha, DGC8, FUS, p72 or of the RISC component, AGO2 in HEK293T (Figure 1E) or HeLa cells (Supplementary Figure S1D). In addition, 99021 treatment of LNCaP prostate cancer cells did not alter Drosha protein levels (Supplementary Figure S1E). These data indicate that GSK3β can modulate miR biogenesis without altering abundance or localisation of key miR biogenesis pathway proteins.

Since GSK3β inhibition appears to inhibit pri-miR to mature miR processing, it was hypothesised that 99021-treatment may alter Drosha activity. To investigate this further, a reporter vector was generated in which the genomic miR-23a27a24-2 sequence is located 5’ of the luc2p gene, under the control of a CMV promoter (Figure 1F). Transcription yields the pri-miR-23a27a24-2 linked to the luc2p transcript and Drosha-mediated cleavage of the pri-miR disrupts the transcript, resulting in loss of luciferase activity, so Drosha activity is inversely correlated with luciferase activity. This approach has been shown to be a sensitive assay for Drosha-mediated cleavage of a specific pri-miR species in previous studies (44). Cos-1 cells were transfected with the Drosha reporter, followed by treatment with 99021. A stas-
Figure 1. Inhibition of GSK3β reduces MiR biogenesis through repression of pri-MiR processing. (A) qRT-PCR analysis of miR-27a, miR-23a, miR-24, miR-141 and miR-182 levels in HEK293T cells transfected with pMT23-HA-GSK3β(K85R) for 48 h. U18 was used as a normalisation gene. (B and C) qRT-PCR analysis of pri-miR-23a27a24-2 (i), pri-miR-141/200c (ii) and pri-miR-182 (iii) expression from HEK293T cells either (B) transfected with pMT23-HA-GSK3β(K85R), or (C) treated with 99021 (2 μM) for 48 h. β-Actin was used as normalisation gene. (A, B and C) Columns: mean ± SEM for three independent experiments performed in triplicate. (D) Western blot analysis of Drosha and MnSOD protein levels in cytoplasmic and nuclear fractions of LNCaP cells treated with 99021 for 48 h. MnSOD was used as a cytoplasmic control. A representative blot of three independent experiments is shown. (E) Western blot analysis of Drosha, AGO2, FUS, p72 and DGCR8 protein levels in HEK293T cells transfected with pMT23-HA-GSK3β(K85R) for 48 h. β-Tubulin was used as a loading control and for normalisation. A representative blot of three independent experiments is shown. (F) Luciferase activity in extracts of COS-1 cells transfected with 250 ng (i) CMV-GL4 or (ii,iii) CMV-Pri-miR-23a27a24-2-GL4, and treated with (i,ii) 99021 (0, 1, 2 μM), or co-transfected with 0, 100 or 400 ng (iii) pMT23-HA-GSK3β(K85R) for 48 h. Luciferase was normalised for transfection efficiency (β-galactosidase activity) and mean ± SEM of three independent experiments performed in duplicate is shown. *P ≤ 0.05, **P ≤ 0.005, ***P ≤ 0.0001. See also Supplementary Figure S1.
Figure 2. GSK3β activation enhances MiR biogenesis and GSK3β modulation alters pre-miR synthesis (A) qRT-PCR analysis of miR-27a, miR-23a, miR-24, miR-141 and miR-182 levels in HEK293T cells transfected with pMT23-HA-GSK3β (S9A) for 48 h. U18 was used as a normalisation gene. (B) qRT-PCR analysis of (i) pri-miR-23a27a24-2, (ii) pri-miR-141/200c and (iii) pri-miR-182 expression from HEK293T cells transfected with pMT23-HA-GSK3β (S9A) for 48 h. -Actin was used as normalisation gene. (A and B) Columns: mean ± SEM for three independent experiments performed in triplicate. (C) Western blot analysis of Drosha, AGO2, FUS, DGCR8 and p72 protein levels in HEK293T cells transfected with pMT23-HA-GSK3β (S9A) for 48 h. β-Tubulin was used as a loading control. (D) Luciferase activity in extracts of COS-1 cells transfected with 250 ng CMV-Pri-miR-23a27a24-2-GL4 and 0, 100 or 400 ng pMT23-HA-GSK3β (S9A) for 48 h. Luciferase was normalised for transfection efficiency (β-galactosidase activity) and mean
Constitutively active GSK3β S9A mutant increases Drosha cleavage activity and enhances miR biogenesis

To provide further evidence for the importance of GSK3β activity for miR maturation, converse experiments were performed using a vector expressing constitutively active GSK3β: GSK3β-S9A. Transfection of GSK3-S9A into HEK293T significantly increased levels of mature miR-27a, miR-23a, miR-24, miR-141 and miR-182 by up to 7.5-fold (Figure 2A), with similar effects also observed in HeLa cells (Supplementary Figure S2A). In contrast, levels of corresponding pri-miR precursors were significantly reduced by up to 80% in HEK293T cells following GSK3-S9A transfection (Figure 2B). As anticipated, GSK3-S9A did not alter protein levels of MP and RISC components, including Drosha (Figure 2C), and showed comparable localisation to both WT and dominant-negative GSK3β upon transfection into COS-1 and LNCaP cells (Supplementary Figure S4). However, expression of constitutively active GSK3β in COS-1 cells significantly reduced luciferase activity of the Drosha reporter vector by up to almost 40%, indicating enhanced Drosha activity in the presence of GSK3β-S9A (Figure 2D). These data support the hypothesis that active GSK3β promotes miR biogenesis by increasing Drosha-mediated cleavage of pri-miRs. If this is the case, GSK3β activity should increase, and its inhibition decrease, pre-miR levels. Thus, in vitro pri-miR processing assays were performed, in which in vitro transcribed and radio-labelled pri-miR-23a-27a-24-2 was incubated with Flag-Drosha MP complex immunoprecipitated from HEK293T cells post-transfection with WT, constitutively-active or dominant-negative GSK3β. As anticipated, no pre-miR was produced in the absence of Flag-Drosha (Figure 2E). Further, WT GSK3β enhanced production of pre-miRs in the presence of Drosha compared to Drosha alone (Figure 2E). S9A-GSK3β did not appear to increase pre-miR levels versus WT under these experimental conditions, however, addition of dominant-negative GSK3β-K85R significantly reduced pre-miR levels (Figure 2E). Pre-miR bands were observed in alignment with the 60 nt RNA marker (Figure 2E and Supplementary Figure S3), which is consistent with expected pre-miR-23a, −27a and −24 sizes of 57 nt, 62 nt and 59 nt, respectively. Additional experimental replicates are shown (Supplementary Figure S2B) and Western blotting was performed on input cell lysates to demonstrate equal expression of the GSK3β and Flag-Drosha constructs (Supplementary Figure S2C). Uncropped images of different exposure lengths from one biological replicate are shown (Supplementary Figure S3) to allow clear visualisation of both pre-miR bands and RNA size markers.

To confirm GSK3β-enhanced pre-miR production, small RNAs (<200 nt) were isolated from HEK293T cells treated with 99021 or transfected with S9A-GSK3β or K85R-GSK3β and qPCR performed using primers targeting the miR-27a stem-loop, without amplification of pri-miRs due to size selection. It was demonstrated that 99021 treatment significantly reduced levels of pre-miR-27a (Figure 2F), whilst constitutively-active S9A-GSK3β increased pre-miR-27a levels. Dominant-negative K85R-GSK3β decreased pre-miR levels (Figure 2F), in agreement with Figure 2E and effects observed upon 99021 treatment. As expected, GSK3β-WT and -S9A decreased levels of pri-miR-23a-27a-24-2, whilst 99021 and K85R-GSK3β increased pri-miR-23a-27a-24-2 levels under the same experimental conditions (Figure 2Fi).
Figure 3. GSK3β regulates pri-miR association with Drosha. (A and B) RNA-immunoprecipitation analysis of association of pri-miRs with Drosha in HEK293T cells ± (A) dominant-negative GSK3β-K85R or (B) constitutively-active GSK3β-S9A. Cells were transfected with Flag-Drosha ± (A) GSK3β-K85R or (B) GSK3β-S9A, then immunoprecipitated with anti-Flag antibody-bound beads and subjected to qRT-PCR analysis using (i) pri-miR-23a27a24-2, (ii) pri-miR-141/200c and (iii) pri-miR-182 primers. Columns: mean ± SEM for three independent experiments performed in duplicate, *P ≤ 0.05 compared to mock-transfected cells. (C) RNA-immunoprecipitation analysis of association of pri-miRs with wild-type GSK3β in HEK293T cells. Cells were transfected with HA-GSK3β, immunoprecipitated with anti-HA antibody-bound beads and subjected to qRT-PCR analysis using pri-miR-23a27a24-2, pri-miR-141/200c and pri-miR-182 primers. Columns: mean ± SEM for three independent experiments performed in duplicate, *P ≤ 0.05 compared to empty vector-transfected cells. (D) RNA-immunoprecipitation analysis of association of pri-miR-23a27a24-2 with constitutively-active and dominant-negative GSK3β mutants in HEK293T cells. Cells were transfected with HA-GSK3β-WT, HA-GSK3β-S9A or HA-GSK3β-K85R, immunoprecipitated with anti-HA antibody-bound beads and subjected to qRT-PCR analysis using pri-miR-23a27a24-2 primers. Columns: mean ± SEM for three independent experiments performed in duplicate, *P ≤ 0.05. See also Supplementary Figure S5.
whilst dominant-negative GSK3β down of pri-miR-141 type (Figure 3D). Similar effects were observed for pull-down of pri-miR-141 with GSK3β. It was also observed that constitutive activation of GSK3β/S-A significantly enhanced association of pri-miR-141/200c with Drosha by 5-fold (Figure 3Bi) and the same trend was observed for both pri-miR-23a27a24-2 and pri-miR-182 (Figure 3Bi and iii).

To investigate whether GSK3β itself can directly bind to pri-miRs, and its involvement in MP-mediated pri-miR cleavage, HEK293T cells were transfected with HA-tagged wild-type, dominant-negative or constitutively-active GSK3β mutant expression vectors, followed by RNA immunoprecipitation using anti-HA antibody. It was found that pull-down of pri-miR-23a27a24-2 and pri-miR-141/200c was significantly increased in the presence of HA-GSKβ compared to empty vector control (Figure 3C), suggesting that GSK3β is able to directly interact with pri-miRs, or that the presence of GSK3β in the MP (or MP-like complex) increases association of pri-miRs with this complex. Given the small fraction of pri-miR recovered in this assay, the latter may be the likelier explanation. It was also observed that constitutive activation of GSK3β increased its association with pri-miR-23a27a24-2, whilst dominant-negative GSK3β showed significantly reduced binding to pri-miR-23a27a24-4 compared to wild-type (Figure 3D). Similar effects were observed for pull-down of pri-miR-141/200c and pri-miR-182 (Supplementary Figure S5). These data support our hypothesis that GSK3β enhances miR biogenesis by increasing association of Drosha with pri-miR species in a global manner, and that the presence of GSK3β within the MP complex increases pri-miR binding to the complex, possibly through direct association of GSK3β with pri-miRs.

GSK3β alters Drosha association with MP components and interacts with DGCRC8 and p72 in a RNA-dependent manner to modulate Pri-MiR to Pre-MiR processing

To investigate if a direct interaction between GSK3β and MP components may facilitate the GSK3β-mediated increase in Drosha activity, immunofluorescent antibody staining was performed on PC3 prostate cancer cells to assess colocalisation of endogenous proteins. GSK3β staining (green) was observed in the cytoplasm, but discrete fluorescence was also observed in the nucleus, where it showed colocalisation with both Drosha and its cofactor, DGCRC8 (Figure 4A – colocalisation indicated by yellow staining). This supports the possibility of interaction between GSK3β and MP proteins. GSK3β was also found to colocalise with Drosha in the nuclei of Cos-1 and LNCaP cells following transient transfection of pMT23-HA-GSK3β and pCK-Flag-Drosha (Supplementary Figure S4). GSK3β/S-A and GSK3β-K85R demonstrated identical subcellular localisation to GSK3β-WT in both Cos-1 and LNCaP cells following transient transfection (Supplementary Figure S4).

In order to further address the possibility of interaction between Drosha and GSK3β, and to evaluate the effects of GSK3β activity on association of Drosha with other components of the MP complex, HEK293T cells were treated with 99021 for 48 h and immunoprecipitation performed using Drosha antibody-bound beads. An interaction between Drosha and GSK3β was not evident (data not shown), although it is possible that such interactions are so transient as to be undetectable using this approach. However, a significant 50% reduction of Drosha association with MP components, DGCRC8 and p72, was observed following 99021 treatment (Figure 4B, Supplementary Figure S6A), indicating that GSK3β may facilitate or enhance interactions between Drosha and its MP cofactors. To corroborate these findings, HEK293T cells were co-transfected with Flag-Drosha and constitutively-active/dominant-negative GSK3β mutants and immunoprecipitation experiments performed using anti-Flag antibody. Again, no evidence was found for a physical interaction between Drosha and either GSK3β mutant under these conditions (Figure 4C, Supplementary Figure S6B middle panel - bands in IP lanes are non-specific and are observed following addition of secondary antibody only). However, it was demonstrated that constitutively active GSK3β/S-A significantly increased (by 50%) the association of Drosha with DGCRC8, a MP cofactor that is responsible for correct orientation of Drosha on the pri-miR hairpin and is required for efficient Drosha RNAse activity (Figure 4C, Supplementary Figure S6B). Addition of GSK3β-K85R reduced Drosha:DGCRC8 binding compared to non-GSK3β mutant-transfected cells by 30%. Together these data suggest that GSK3β enhances interactions between Drosha and its vital MP cofactors DGCRC8 and p72 to accelerate pri-miR processing and promote miR biogenesis. Since no evidence of direct GSK3β:Drosha interaction was seen, it was hypothesised that GSK3β may directly interact with DGCRC8 and/or p72. To test this, HEK293T cells were treated with 99021 for 48 h and immunoprecipitation performed using GSK3β antibody-bound beads. Interaction of endogenous GSK3β with both DGCRC8 and p72 was confirmed, and reduced by ~40% upon treatment with GSK3β inhibitor (Figure 4D). In confirmation of this finding, DGCRC8 and p72 were also demonstrated to interact with HA-tagged exogenous GSK3β (Figure 4E). Addition of dominant-negative exogenous GSK3β-K85R reduced these associations by 60% compared to GSK3β-WT (Figure 4E). However, whilst GSK3β-S-A non-significantly increased Drosha association with DGCRC8 (Figure 4Ei), the presence of GSK3β-S-A significantly reduced interaction of Drosha with p72 (Figure 4Eii). These data confirm binding of GSK3β to MP components, DGCRC8 and p72, modulating Drosha activity and miR accumulation.

Immunoprecipitation cannot demonstrate whether interaction between GSK3β and DGCRC8 and p72 is direct or occurs via another factor, such as a pri-miR. To investigate the ability of WT GSK3β to interact with the above proteins in the absence of pri-miRs, GSK3β IP experiments were performed with or without RNAse A treatment. Association of GSK3β with both p72 and DGCRC8 was significantly reduced by over 50% following RNAse A treatment (Figure 4F, Supplementary Figure S6C). This suggests that an RNA species (mostly likely pri-miRs, given the known localisation of p72, DGCRC8 and GSK3β in the MP) is required
Figure 4. GSK3β modulates association of Drosha with DGCR8 and p72 and binds to microprocessor components to facilitate Drosha-mediated pri-miR production. (A) Analysis of GSK3β colocalisation with DGCR8 and Drosha in HEK293T cells by immunofluorescent antibody staining. Green, GSK3β; red, DGCR8 or Drosha; yellow, colocalisation (as indicated by arrows). Scale: as indicated by bars. Images are representative of two independent experiments, with five fields imaged per experiment. (B) Immunoprecipitation (IP) analysis of effects of 48 h 99021 (2 μM) treatment on interaction of endogenous Drosha with p72 and DGCR8 in HEK293T cells. 99021-treated HEK293T lysates were incubated with Drosha antibody- or rabbit IgG-bound
GSK3β nuclear localisation is required for its miR biogenesis-enhancing effects

In order to demonstrate the requirement of nuclear localisation of GSK3β for its biogenesis-promoting effects, nuclear localisation signal (NLS)-mutant HA-GSK3β constructs were generated. A putative NLS has been described between amino acids 85 and 123 of GSK3β (45). HEK293T cells were transfected with HA-GSK3β-K85A,K86A, HA-GSK3β-R96A and HA-GSK3β-R102G,K103A (as these have been previously shown nuclear exclusion (45)) and cellular fractionation performed. WT GSK3β was found in both cytoplasmic and nuclear compartments (Figure 5A). HA-GSK3β-R96A and HA-GSK3β-R102G,K103A did not demonstrate altered localisation compared to WT GSK3β (Figure 5A). HA-GSK3β-S9A and HA-GSK3β-K85R also demonstrated similar localisation profiles to WT (Supplementary Figure S7A). In contrast, HA-GSK3β-K85A,K86A was largely excluded from soluble nuclear and chromatin-bound fractions of HEK293T cells. Nuclear exclusion of HA-GSK3β-K85A,K86A was confirmed, and found to be decreased following GSK3β inhibition by 99021 (Figure 6A), indicating that Drosha is a substrate for GSK3β phosphorylation specifically at S300 and S302. To this end, HEK293T cells were treated with 99021 and lysates immunoprecipitated using anti-phosphoserine antibody and subjected to immunoblotting for Drosha. Phosphorylation of Drosha was confirmed, and found to be decreased following GSK3β inhibition by 99021 (Figure 6A), indicating that Drosha is a substrate for GSK3β kinase activity. However, some phospho-Drosha was still detectable following GSK3β inhibition (Figure 6A) implicating that Drosha is also a substrate for other kinases. To provide evidence for GSK3β phosphorylation of Drosha specifically at S300 and S302, HEK293T cells were transfected with WT Flag-Drosha or S300A,S302A phospho-mutant Flag-Drosha and treated with 99021, followed by anti-Flag immunoprecipitation and immunoblotting for phospho-serine. It was demonstrated that GSK3β phosphorylates Drosha at S300 and S302 to modulate Drosha association with DGCR8 and p72, alter Drosha cleavage activity and regulate mature MiR levels without altering Drosha localisation.
Figure 5. GSK3β nuclear localisation is required for its MiR biogenesis enhancing effects. (A) Western blot analysis of HA-GSK3β protein levels in cytoplasmic, soluble nuclear and chromatin fractions of HEK293T cells transfected with pMT23-HA-GSK3β-WT, pMT23-HA-GSK3β-K85A,K86A.
phosphorylation of WT Flag-Drosha is reduced following GSK3β inhibition (Figure 6Bi,ii), corroborating Drosha as a GSK3β substrate. In addition, phosphorylation of Flag-Drosha containing mutated S300 and S302 residues is reduced compared to Flag-Drosha-WT and is not lost following GSK3β inhibition (Figure 6Bi), supporting the hypothesis that GSK3β can phosphorylate Drosha at S300 and S302. Indeed, levels of phospho-Drosha are increased after 99021 treatment following ablation of S300 and S302 phosphorylation sites (Figure 6B). The differential effects of WT Flag-Drosha or S300A, S302A phospho-mutant Flag-Drosha are not attributable to altered Drosha localisation, since these constructs show comparable localisation profiles upon subcellular fractionation of transfected HEK293T cells (Supplementary Figure S8A).

To directly demonstrate phosphorylation of Drosha at S300 and/or S302 by GSK3β, *in vitro* kinase assays were performed, whereby recombinant GSK3β was incubated with 25aa peptides corresponding to WT or S300A, S302A mutant Drosha in the presence of (γ-32P)-ATP. Observed phosphorylation of WT Drosha peptide in the presence of GSK3β was lost upon mutation of S300 and S302 residues (Figure 6C, Supplementary Figure S8B), confirming that GSK3β phosphorylates Drosha at either one or both of these amino acids. We next sought to demonstrate the functional consequences of GSK3β phosphorylation on Drosha function. Cos-1 cells were transfected with the previously described Drosha reporter plasmid and either WT, or phospho-mimic S300E, S302D Drosha expression vector, which has been previously used to mimic phosphorylation of Drosha at S300 and S302 (41). It was demonstrated that Drosha S300E, S302D significantly reduced luciferase activity compared to WT Drosha, indicating increased pri-miR cleavage by phospho-mimic Drosha (Figure 6D), and suggesting that GSK3β phosphorylation of Drosha increases its RNase activity. Equal expression of both Drosha constructs was confirmed by Western blotting (Figure 6D, inset). To investigate the influence of Drosha phosphorylation at S300 and/or S302 on Drosha association with its MP cofactors, HEK293T cells were transfected with Flag-Drosha WT or S300E, S302D phospho-mimic and IP performed with Flag antibody-bound beads. Phospho-mimic Drosha demonstrated significantly increased association with both DGCR8 and p72 compared to WT Drosha (Figure 6E, Supplementary Figure S8C). To assess the functional effects of phospho-Drosha on mature and pri-miRs, HEK293T cells were transfected with Flag-Drosha WT or S300E, S302D phospho-mimic and qPCR performed for pri-miR-23a, 27a, 24-2 and −141/200c, and miR-23a, −27a, −141 and −182. No significant difference in pri-miRs levels was observed between Flag-Drosha WT and S300E, S302D transfected cells (Figure 6F), and whilst levels of miR-27a and −182 were not significantly altered in the presence of phospho-mimic Drosha (Figure 6Gi,iv), miR-23a and miR-141 levels were significantly increased in the presence of the S300E, S302D construct (Figure 6Gi,iii).

Together, these data suggest that GSK3β phosphorylates Drosha at S300 and/or S302, leading to enhanced association with p72 and DGCR8, increased Drosha RNase activity towards pri-miRs and increased levels of mature miRs.

**GSK3β-regulated MiR target proteins are increased following GSK3β inhibition**

Having established that GSK3β regulates miR biogenesis, we wished to determine whether this is likely to have downstream functional consequences. To this end, we studied the effects of GSK3β inhibition on expression of the miR targets, at the levels of target 3′UTR activity, mRNA levels and protein levels. Using a 3′UTR reporter construct for the miR-27a target *ACLY* (in which the *ACLY* 3′UTR was cloned 3′ of the luciferase gene in the pMiRTarget vector), it was found that luciferase activity was significantly increased following addition of 99021 (Figure 7A). This suggests that GSK3β inhibition relieves targeting of *ACLY* 3′UTR by miR-27a, presumably by preventing GSK3β-mediated up-regulation of miR-27a. However, when the miR-27a binding site was mutated in this reporter construct, which abrogated the miR-27a-mediated loss of *ACLY* 3′UTR activity observed for the WT construct (Supplementary Figure S9), we observed significantly higher 3′UTR activity compared to WT 3′UTR reporter (Figure 7A) – indicative of reduced miR-27a binding to *ACLY* 3′UTR, which was additionally not increased upon 99021 treatment. This supports the hypothesis that 99021 treatment reduces miR-27a levels, relieving repression of *ACLY* 3′UTR, and confirms that effects are specifically attributable to alterations in miR-27a, since no increase in 3′UTR activity upon GSK3β inhibition was observed when the miR-27a binding site was mutated. We next examined whether 99021 treatment affected
Figure 6. GSK3β phosphorylates Drosha at S^{300} and S^{302} to modulate Drosha association with DGCR8, alter Drosha cleavage activity and regulate mature MiR levels without altering Drosha localisation. (A) IP analysis of effects of 48 h 99021 (2 μM) treatment on endogenous Drosha phosphorylation. 99021-treated HEK293T lysates were incubated with anti-phosphoserine–agarose and Western blotting performed for Drosha. A representative image of three independent experiments is shown (i). Densitometry was performed using Image J software (ii,iii).

(B) IP analysis of effects of 48 h 99021 (2 μM) treatment on phosphorylation of phospho-Drosha peptides. A representative autoradiograph is shown (i). Densitometry was performed using Image J software (ii,iii).

(C) In vitro GSK3β phosphorylation of Drosha peptides. A representative autoradiograph is shown (i). Densitometry was performed using Image J software (ii,iii).

(D) Relative luciferase activity in Cos-1 cells expressing Drosha reporter. A representative blot is shown (i). Densitometry was performed using Image J software (ii,iii).

(E) IP analysis of effects of 99021 (2 μM) treatment on phos-Flag IPs. A representative blot is shown (i). Densitometry was performed using Image J software (ii,iii).

(F) Relative expression of miR-23a, miR-27a, miR-141, and miR-182. A representative blot is shown (i). Densitometry was performed using Image J software (ii,iii).
levels of the 3' UTR of ACYL, ZEB1, PTEN and FOXO1 (miR-23a, miR-141, miR-143 and miR-182/27a targets, respectively). It was demonstrated that for ZEB1 (Figure 7Bii) and PTEN (Figure 7Biii) 3' UTR levels were increased as anticipated. However, ACYL 3' UTR levels were unchanged (Figure 7Bi) and FOXO1 3' UTR levels decreased (Figure 7Biv) following addition of 99021. This may be due to the different mechanisms of repression of these targets by miR-27a. In cases where a miR acts via promoting translational repression, reduction of miR levels (here as a consequence of GSK3β inhibition) would result in derepression of translation leading to increased protein levels without necessarily affecting mRNA levels. However, in cases where the miR exerts its effects via promoting transcript degradation, we would expect GSK3β inhibition to result in increased mRNA expression with a corresponding increase in protein levels. In accordance with this, both ACYL and FOXO1 protein levels were increased following addition of 99021 (Figure 7C and F), as were protein levels of ZEB1 and PTEN (Figure 7D and E). These data demonstrate that GSK3β modulation of miR biogenesis has physiological consequences through altering protein levels of GSK3β-regulated miR targets.

**DISCUSSION**

miRs are dysregulated in many diseases, notably cancer, where they can act as tumour suppressors or oncogenes. Recent data have given tantalising clues as to the complex signalling pathways and cascades that impinge upon miR synthesis, and it is vital to fully understand regulatory processes governing miR biogenesis, and their perturbation in disease states, in order to exploit miRs as an ‘untapped’ repository of disease biomarkers and therapeutic targets.

GSK3β is a serine/threonine protein kinase that plays a key role in signal transduction during processes such as cell cycle progression, proliferation and inflammation through phosphorylation of target proteins and shows altered activity in a number of cancers. Interestingly, GSK3β has been shown to phosphorylate Drosha at residues S300 and S302, and it has been suggested that such modifications are required for Drosha nuclear localisation (34). In addition, small-molecule inhibition of GSK3β reduced mature levels of more than 90% of miRs in mESCs, purportedly due to loss of Drosha nuclear localisation (33).

We hypothesised that GSK3β could link pro-survival signalling pathways and miR biogenesis in human somatic cells, and sought to examine the effects of GSK3β on Drosha’s essential ribonuclease activity and to identify the mechanism by which GSK3β modulates miR biogenesis. We first transfected HEK293T cells with dominant-negative GSK3β-K23R. This decreased mature miR levels, increased pri-miR levels and reduced Drosha activity without altering Drosha protein levels or cellular localisation (Figure 1). These data suggested that inhibition of GSK3β reduces Drosha-mediated pri-miR cleavage, thereby decreasing levels of a number of different miRs. To corroborate this we used a specific small-molecule inhibitor of GSK3β, CHIR-99021 (99021), which demonstrates greater than 500-fold selectivity over closely-related kinases (22), and were able to fully replicate the results obtained with GSK3β-K23R: decreased mature miR levels and increased pri-miR levels, as attributable to reduced Drosha activity (Figure 1 and Supplementary Figure S1). These data further support the hypothesis that GSK3β inhibition reduces miR biogenesis. Our demonstration of similar extents of regulation for diverse miRs of different genomic contexts and subject to different modes of transcriptional regulation, when taken together with the finding that 90.4% of differentially-regulated miRs were downregulated following 99021 treatment of mESCs (33), are suggestive of widespread GSK3β modulation of miR biogenesis, although how widespread remains to be verified.

To provide additional evidence for the importance of GSK3β in global miR maturation, we employed constitutively-active GSK3βS9A and observed the converse effect: an increase in Drosha activity, leading to increased mature miR levels and a loss of pri-miRs (Figure 2A–D). Taken together, these data highlight an important role for GSK3β in facilitating miR biogenesis by increasing Drosha activity towards pri-miRs. To confirm our hypothesis that GSK3β regulates miR biogenesis at the level of MP activity, in vitro pri-miR processing assays were performed, whereby in vitro transcribed pri-miR-23a27a24-2 was in-

on phosphorylation of WT- or S300A,S302A phospho-mutant Flag-Drosha. HEK293T cells were transfected with WT- or S300A,S302A phospho-mutant Flag-Drosha, treated with 2 μM 99021 for 48 h, and subject to IP using anti-Flag antibody-bound beads, followed by Western blotting for phospho-serine and Flag. A representative image of three independent experiments is shown (i). (A and B) Columns: mean ± SEM for three independent experiments. (C) In vitro kinase assay analysis of GSK3β phosphorylation of WT or S300A,S302A-mutant Drosha. Recombinant GSK3β was incubated with 25aa peptides corresponding to WT or S300A,S302A-mutant Drosha for 60 min in the presence of (γ-32P)-ATP. Reaction products were spotted onto nitrocellulose membrane, washed and exposed to film. A representative image of three independent experiments is shown (i). Images of biological replicate experiments can be found in Supplementary Figure S8B. Densitometry was performed using Image J software (A and B). Columns: mean ± SEM for three independent experiments. (D) Luciferase activity in extracts of COS-1 cells transfected with 100 ng CMV-PrimIR-23a27a24-2-GL4 vector and 0, 100 or 250 ng pCK-Flag-Drosha(WT) or pCK-Flag-Drosha(S300E,S302D)(S300E,S302D) for 48 h. Luciferase was normalised for transfection efficiency (β-galactosidase activity) and mean ± SEM of three independent experiments performed in duplicate is shown. Western blotting analysis of Flag-Drosha protein levels was performed in HEK293T cells transfected with pCK-Flag-Drosha(WT) or pCK-Flag-Drosha(S300E,S302D) for 48 h to demonstrate equal transfection efficiency and expression of the two plasmids in luciferase reporter assays. (E) IP analysis of interactions between exogenous pCK-Flag-Drosha-WT/S300E,S302D and DGC85 or p72. HEK293T cells were transfected with pCK-Flag-Drosha(WT) or pCK-Flag-Drosha(S300E,S302D) for 48 h and subject to IP using anti-Flag antibody-bound beads. Western blotting for p72, DGC85 and Flag was performed. A representative blot of three independent experiments is shown (i). Images of biological replicate experiments can be found in Supplementary Figure S8C. Image J software was used for densitometry, and IP protein levels displayed relative to input (ii,iii). (F) qRT-PCR analysis of (i) pri-miR-23a27a24-2 or (ii) pri-miR-141/200c levels in HEK293T cells transfected with pCK-Flag-Drosha(WT) or pCK-Flag-Drosha(S300E,S302D) for 48 h. L19 was used as a normalisation gene. (G) qRT-PCR analysis of (i) miR-23a, (ii) miR-27a, (iii) miR-24, (iv) miR-141 and (v) miR-182 levels in HEK293T cells transfected with pCK-Flag-Drosha(WT) or pCK-Flag-Drosha(S300E,S302D) for 48 h. U18 was used as a normalisation gene. (F and G) Columns: mean ± SEM for three independent experiments performed in duplicate. *P ≤ 0.05, see also Supplementary Figure S8.
Figure 7. GSK3β-regulated miR target proteins are increased following GSK3β inhibition – mechanisms for enhanced MiR biogenesis by GSK3β. (A) Luciferase activity in lysates of HEK293T cells transfected with pMiRTarget-ACLY-WT or pMiRTarget-ACLY-miR27a binding site (BS) mutant (for details see Supplementary Figure S9) and treated with 99021 (2 μM, 24 h). Luciferase was normalised for transfection efficiency (β-galactosidase activity) and mean ± SEM of three independent experiments performed in duplicate is shown. (B) qRT-PCR analysis of (i) ACLY, (ii) ZEB1, (iii) PTEN and (iv) FOXO1 3′ UTR levels in HEK293T cells treated ± 99021 (2 μM) for 72 h. L19 was used as a normalisation gene. Columns: mean ± SEM for three independent experiments performed in triplicate. (C, D, E and F) Western blot analysis of (C) ACLY, (D) ZEB1, (E) PTEN and (F) FOXO1 protein levels in lysates of HEK293T cells treated ± 99021 (2 μM) for 72 h. β-Actin was used as a loading control and for normalisation. Representative blots of three independent experiments are shown (i). Densitometry was performed using Image J software (ii). Columns: mean ± SEM for three independent experiments. *P ≤ 0.05. (G) A mechanism for enhanced miR biogenesis by GSK3β. GSK3β phosphorylates Drosha at S100 and S102. This does not alter levels of miR biogenesis proteins or modulate Drosha localisation but increases Drosha association with DGCR8, p72 and pri-miRs and enhances Drosha pri-miR cleavage activity, reducing pri-miR and increasing mature miR levels. GSK3β achieves these effects as a component of the Microprocessor, binding to p72 and DGCR8 in a RNA-dependent manner.
cubated with Flag-Drosha-containing MP immunoprecipitated from cells transfected with GSK3β mutant. Should effects of GSK3β be at the level of pri-miR processing, we anticipated pri-miR levels to be increased by GSK3β-WT and -S9A, and reduced by GSK3β-K85R. Indeed, GSK3β increased Drosha-mediated pri-miR production, an effect entirely abrogated by GSK3β-K85R (Figure 2E). In support of this, size-selection qPCR showed increased pri-miR levels following GSK3β-S9A transfection, whilst 99021 treatment significantly reduced pri-miR-27a abundance (Figure 2Fi). This was corroborated by reduced pri-miR levels in the presence of GSK3β-WT and -S9A, and significantly increased pri-miR-27a following GSK3β-K85R transfection (Figure 2Fii), in addition to enhanced Drosha reporter activity in the presence of GSK3β-S9A (Figure 2D). Thus, we have provided substantial evidence that GSK3β acts at the level of the MP to increase Drosha RNase activity and enhance pri-miR to pre-miR processing.

To elucidate the mechanism(s) by which GSK3β influences Drosha activity, we first examined the effect of GSK3β inhibition or activation on Drosha: pri-miR binding using RNA-IP assays. It was found that dominant-negative GSK3β significantly reduced association of Drosha with several pri-miRs, whilst constitutively active GSK3β increased it (Figure 3A and B). These data indicate that GSK3β facilitates miR biogenesis by promoting interaction between Drosha and pri-miRs. We then investigated the ability of GSK3β itself to associate with pri-miRs, and found that the presence of HA:GSK3β resulted in significantly increased anti-HA immunoprecipitation of pri-miRs with respect to controls (Figure 3C). In corroboration of this, dominant-negative GSK3β was found to significantly reduce pri-miR-23a27a24-2 immunoprecipitation with reference to the wild-type protein, whilst the constitutively-active protein increased pri-miR-23a27a24-2 pull-down compared to WT-GSK3β (Figure 3D). There are two possible interpretations of these data: firstly, that GSK3β interacts directly with pri-miRs to enhance their processing, perhaps retaining pri-miRs in the correct orientation for efficient Drosha cleavage, since GSK3β lacks RNase function; secondly, that GSK3β forms an intrinsic and important stimulatory component of a pri-miR-binding complex (probably the MP), without direct pri-miR association. The latter scenario is most likely, since GSK3β has neither been demonstrated nor predicted to contain a DNA or RNA binding motif, and alters biogenesis of all miRs assayed, regardless of level of pri-miR structure branching, number of stem-loops in pri-miR or pri-miR length, factors which would be expected to determine extent of GSK3β regulation if it bound pri-miRs directly. Interestingly, GSK3β is known to phosphorylate proteins, for example, c-Jun, close to their DNA binding domain, altering affinity for DNA (46). Thus, it is a tempting hypothesis that GSK3β additionally phosphorylates DGCR8 or p72 near their RNA binding domains to increase affinity for pri-miR substrates. Further, functional assignment of GSK3β interactors in HepG2 cells identified 24 interactors as ‘nucleic acid binding proteins’ – the largest functional group described (47). This hints at previously unexplored but important functions of GSK3β in RNA processing pathways. It also appears that GSK3β may be prerequisite for Drosha: pri-miR interaction, since pri-miR pulldown by Drosha in the presence of dominant-negative GSK3β is reduced to background levels (Figure 3A).

To provide evidence for interactions between GSK3β and MP components in the nucleus, we first performed immunofluorescent staining and found evidence for nuclear colocalisation of GSK3β with both Drosha and its cofactor, DGCR8 (Figure 4A). Although GSK3β is often considered a cytoplasmic protein, our findings are supported by the observation that 48% of GSK3β interacting proteins are localised in the nucleus of cells (47), suggestive of an important nuclear role for GSK3β. In order to investigate potential interaction between GSK3β and Drosha, IP experiments were performed. No interaction was identified between GSK3β and Drosha under our experimental conditions, although it remains possible that such an association exists but is below the detection threshold for IP, particularly given the transient nature of kinase:substrate interactions and demonstration by ourselves (Figure 6A–C) and others (34) that Drosha is phosphorylated by GSK3β. Indeed, Drosha was not identified as a GSK3β-binding protein in a recent GSK3β interactome study (47). We did, however, demonstrate that GSK3β modulation alters interactions between Drosha and its MP cofactors: IP of endogenous Drosha demonstrated significant loss of association with both DGCR8 and p72 upon 99021 treatment of HEK293T cells (Figure 4B), and constitutively active GSK3β was shown to increase association of Drosha with DGCR8 (Figure 4C). Interestingly, DGCR8 has been identified as a phospho-protein, with phosphorylation at 23 sites demonstrated to increase its stability, resulting in a pro-growth miR profile (48). Although JNK and ERK proteins were identified as candidate kinases, the authors examined only a fraction of the kinome and additional potential DGCR8-phosphorylating proteins are not known. Given GSK3β’s primary function as a kinase, it is an attractive hypothesis that GSK3β could phosphorylate DGCR8 to increase its stability, promoting its increased interaction with Drosha and thus enhanced pri-miR cleavage. Indeed IP assays both with endogenous and HA-tagged GSK3β confirmed a previously undescribed interaction with DGCR8, which was significantly diminished in the presence of 99021 (Figure 4D) or the dominant-negative mutant (Figure 4E).

Interestingly, GSK3β also demonstrated a novel interaction with p72 (DDX17) (Figure 4D and E), a DEAD-box MP cofactor that acts as a specificity protein for processing distinct subsets of miRs, and is important for miR maturation (49). This provides additional evidence for the importance of GSK3β in miR biogenesis, and is of further interest since p72 has been demonstrated to alternatively splice GSK3β mRNA, increasing cellular levels of the shorter, more catalytically active GSK3β isoform-1 (50). This provides another level of complexity to regulation of miR maturation, whereby p72 not only regulates Drosha cleavage directly, but may also alter the equilibrium between the two GSK3β isoforms in order to regulate MP activity. Reciprocal regulation is also possible via GSK3β phosphorylation of p72, and/or the extent of the p72:GSK3β interaction itself. GSK3β has been demonstrated to interact with other miR-regulatory and RNA-binding proteins: for example, DDX1 and DDX21, which are closely-related to p72, and
the heterogeneous nuclear ribonucleoproteins hnRNPA2B1 and hnRNPK, whose family member, hnRNPA1, is a key MP component (47). Additionally, FUS and p68, further components of the MP complex, were identified as participants in a cluster with GSK3β (47), adding further weight to the argument that GSK3β facilitates miR biogenesis as a component of the MP complex, although modulation of Dicer activity by GSK3β has not been ruled out. Providing further evidence of the importance of GSK3β in pri-miR processing, its association with both DGCR8 and p72 was lost upon RNase treatment of HEK293T cells (Figure 4F), suggesting requirement of an RNA species, likely a pri-miR, for association of GSK3β with MP components and enhancement of Drosha activity.

Having demonstrated interaction of GSK3β with p72 and DGCR8 that is reduced in the absence of RNA, we sought to illustrate the requirement of GSK3β nuclear localisation for its miR biogenesis promoting activity. To this end, we tested a number of previously reported NLS mutants that had demonstrated nuclear exclusion in HEK293 and HeLa cells (45). HA-GSK3β mutants that had demonstrated nuclear exclusion in HEK293 and HeLa cells (45). HA-GSK3β-K85A,K86A, but not –R86A nor R106G,K103A, was found to be excluded from the nucleus of HEK293T cells, as demonstrated both by subcellular fractionation and immunofluorescent microscopy (Figure 5A and B). Subsequent analyses showed that interaction of HA-GSK3β-K85A,K86A with DGCR8 is reduced compared to WT-GSK3β (Figure 5Ci,ii). Interestingly, however, interaction with p72 was not altered by NLS mutation (Figure 5Ci,iii). This is likely to be because p72 localises to the cytoplasm in addition to the nucleus (Figure 5D), thus its interaction with NLS mutant GSK3β may be representative of cytoplasmic binding. Functionally, pri-miR levels were increased (Figure 5E) and mature miR levels reduced (Figure 5F) in the presence of HA-GSK3β-K85A,K86A compared to the WT protein, suggesting nuclear localisation of GSK3β is required for optimal Drosha cleavage of pri-miR substrates.

Given that GSK3β has been demonstrated to phosphorylate Drosha at S100 and S302 (34), we sought to clarify the impact of such post-translational modifications on Drosha RNase activity and to establish whether this is the mechanism by which GSK3β alters Drosha:DGCR8, Drosha:p72 and Drosha: pri-miR interactions. We first used IP methods to demonstrate that levels of phospho-Drosha are reduced upon 99021 treatment (Figure 6A), suggesting that GSK3β can phosphorylate Drosha in vitro. These data confirm previous reports describing Drosha phosphorylation by this kinase (34,41). To provide evidence that such phosphorylation occurs at S100 and S302, exogenous WT- or S100A,S302A-Flag-Drosha were immunoprecipitated and levels of serine-phosphorylated Drosha were examined. It was demonstrated that, in corroboration of the above discussed data, phosphorylation of WT Flag-Drosha is significantly decreased upon GSK3β inhibition, and that phosphorylation of S100A,S302A-Flag-Drosha is considerably reduced compared to WT Drosha (Figure 6B). Interestingly, phosphorylated WT-Drosha is still detectable upon GSK3β inhibition (Figure 6A and B), suggesting that Drosha is also serine-phosphorylated by other kinases. Further, levels of phospho-Drosha are increased following 99021 treatment of cells transfected with S100A,S302A-Flag-Drosha, raising the possibility that inhibition of GSK3β derepresses activity of other kinases that phosphorylate Drosha at other sites. Of note, our demonstration that abrogating GSK3β-mediated phosphorylation of Drosha at S100 and S302 (Supplementary Figure 5A) does not alter its cellular localisation contrasts with previous data illustrating loss of Drosha nuclear localisation upon 99021 treatment of mESCs (33), and loss of nuclear Drosha in GSK3β−/− MEFs compared to WT cells (34). This may be due to differences in mechanisms of GSK3β regulation of miR biogenesis between the mouse embryonic cell lines used in prior studies (33,34), and human HEK293T cells employed for our experiments, or effects of GSK3β manipulation independent of kinase activity at S100/S302.

Definitive evidence that GSK3β phosphorylates Drosha at S100 and/or S302 has been provided by in vitro kinase assays: GSK3β can phosphorylate WT, but not S100A,S302A mutant Drosha peptide in vitro (Figure 6C). In terms of the impact of GSK3β phosphorylation of S100/S302 on Drosha activity, luciferase activity of a pri-miR-23a27a24-specific Drosha reporter construct was reduced following addition of S100E,S302D phospho-mimic Drosha compared to WT-Drosha (Figure 6D), indicating enhanced Drosha cleavage. Further, phospho-mimic Drosha showed significantly enhanced interaction with its cofactors, DGCR8 and p72, as compared to WT-Drosha (Figure 6E), suggesting that GSK3β-mediated phosphorylation of Drosha at S100 and S302 increases miR biogenesis by increasing Drosha:cofactor interactions to promote pri-miR cleavage. This is further supported by significantly increased levels of miR-23a and −141 in HEK293T cells transfected with phospho-mimic Drosha compared to WT (Figure 6G). Minimal effects observed on pri-miR and mature miR-27a and −182 levels may be attributable to maximal Drosha activity in HEK293T cells transfected with WT Flag-Drosha, such that Drosha activity towards pri-miRs cannot be significantly elevated upon addition of Flag-Drosha:S100E,S302D. Together, these data support the hypothesis that GSK3β is the kinase responsible for Drosha phosphorylation at S100 and S302, leading to enhanced Drosha RNase activity and miR accumulation.

To demonstrate the physiological relevance of GSK3β regulation of miR biogenesis, we examined 3′UTR activity and protein levels of targets of miRs demonstrated above to be regulated by GSK3β. We demonstrated that 3′UTR activity of ACLY (a target of GSK3β-upregulated miR-27a) was increased upon 99021 treatment (Figure 7A), which reduces miR-27a levels (Supplementary Figure S1D). In addition, protein levels of ACLY (miR-27a target – Figure 7C), ZEB1 (miR-141 target – Figure 7D), PTEN (miR-141 target – Figure 7E) and FOXO1 (miR-182 and −27a target, Figure 7F) were significantly increased following addition of 99021, indicating derepression by their targeting miRs due to loss of GSK3β-enhanced miR biogenesis. These data demonstrates that GSK3β modulation of miR biogenesis has physiological consequences by altering protein levels of GSK3β-regulated miR targets.

It is clear that GSK3β constitutes an important link between multiple pro-survival signalling pathways and miR biogenesis. Perturbation of GSK3β activity may disrupt the highly-regulated process of miR maturation and may
play a role in disease pathology, carcinogenesis or maintenance of an oncogenic phenotype. In addition, alterations to the phosphorylation status of GSK3β in response to various stimuli may act as a rheostat that regulates miR biogenesis and the ratio of pri- to mature miRs by modulating Drosha activity. Further work is required to confirm whether GSK3β regulation of miR maturation is a global effect, and to establish whether GSK3β phosphorylates or interacts with other MP components in addition to Drosha, DGCR8 and p72. It is possible that additional auxiliary factors are required to confer specificity of GSK3β regulation of miR biogenesis, and that GSK3β may contribute to a pre-MP holoenzyme, associating with DGCR8, p72 and other factors to assemble pre-MP configuration concomitantly with Drosha binding. It will be very informative to further examine the GSK3β-regulated miRnome in human cells to see if the miR subset identified is enriched for particular cellular functions or processes, as this could have important implications for GSK3β as a drug target in cancer, where it displays contrasting and ambiguous roles.

In conclusion, these data are the first to identify an unexpected and entirely novel important role for GSK3β in post-transcriptional regulation of miR biogenesis as a component of the MP and RNase cofactor: GSK3β binds to p72 and DGCR8 in the nuclear MP in an RNA-dependent manner, leading to its phosphorylation Drosha at S308 and S302 (Figure 6A–C). This does not alter levels of miR biogenesis proteins or modulate Drosha localisation, but increases Drosha association with DGCR8, p72 and pri-miRs, and enhances Drosha RNase and pri-miR cleavage activity, reducing pri-miR and increasing mature miR levels, likely due to stabilisation of MP configuration. This has profound implications for GSK3β as a drug target in cancer and other pathologies, and for understanding miR biogenesis as a highly complex and stringently-controlled process on which a multitude of vital signalling cascades converge.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank Prof. V. Narry Kim, Seoul National University, for the kind gift of the pCK-Flag-Drosha construct, Dr Robert Kypta and Dr Hector Keun, Imperial College London, for advice and the kind gift of constructs and all members of the Bevan laboratory for insightful discussion.

FUNDING

The Wellcome Trust, The Rosetrees Trust, the Imperial Cancer Research UK Centre and Experimental Cancer Medicine Centre and a Movember/Prostate Cancer UK Centre of Excellence Program [CEO013-2-002]. Funding for open access charge: Imperial College Open Access Fund.

Conflict of interest statement. None declared.

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